ISSN 1948-0210 (online)

World Journal of *Stem Cells*

World J Stem Cells 2015 August 26; 7(7): 992-1077

Published by Baishideng Publishing Group Inc

WJSC

World Journal of Stem Cells

A peer-reviewed, online, open-access journal of stem cells

Editorial Board

2010-2015

The *World Journal of Stem Cells* Editorial Board consists of 719 members, representing a team of worldwide experts in infectious diseases. They are from 44 countries, including Argentina (2), Australia (10), Austria (6), Belgium (3), Brazil (10), Canada (16), China (74), Cyprus (1), Czech Republic (5), Denmark (7), Egypt (2), Finland (3), France (19), Germany (36), Greece (1), Hungary (3), India (10), Iran (9), Ireland (4), Israel (10), Italy (52), Japan (55), Jordan (1), Malaysia (1), Mexico (1), Morocco (1), Netherlands (8), Norway (3), Portugal (1), Romania (1), Russia (3), Singapore (19), Slovakia (1), South Korea (45), Spain (17), Sweden (4), Switzerland (4), Thailand (1), Tunisia (1), Turkey (5), United Arab Emirates (1), United Kingdom (29), United States (233), and Venezuela (1).

EDITOR-IN-CHIEF
Oscar Kuang-Sheng Lee, *Taipei*

STRATEGY ASSOCIATE EDITOR-IN-CHIEF
Philippe Bourin, *Toulouse*
Andras Dinnyes, *Godollo*
Umberto Galderisi, *Napoli*
Mikhail G Kolonin, *Houston*
Balazs Sarkadi, *Budapest*

GUEST EDITORIAL BOARD MEMBERS
Chia-Ning Chang, *Taichung*
Chuck CK Chao, *Taoyuan*
Chie-Pein Chen, *Taipei*
Fu-Chou Cheng, *Taichung*
Ing-Ming Chiu, *Miaoli*
Akon Higuchi, *Taoyuan*
Hossein Hosseinkhani, *Taipei*
Yu-Chen Hu, *Hsinchu*
Yen-Hua Huang, *Taipei*
Jyh-Cherng Ju, *Taichung*
Steven Shoei-Lung Li, *Kasohsiung*
Feng-Huei Lin, *Miaoli*
Shing-Hwa Liu, *Taipei*
Jui-Sheng Sun, *Taipei*
Tzu-Hao Wang, *Taoyuan*
Yau-Huei Wei, *Taipei*
Kuo-Liang Yang, *Hualien*
Chao-Ling Yao, *Chung-Li City*

MEMBERS OF THE EDITORIAL BOARD

Argentina

Foundation Benetti, *Santa Fe*
Luis E Politi, *Bahia Blanca*

Australia

Michael Kerry Atkinson, *Brisbane*
Peter Mark Bartold, *South Australia*
Jeremy M Crook, *Victoria*
Simon A Koblar, *South Australia*
Alice Pébay, *Parkville*
Kuldip S Sidhu, *Sydney*
Paul J Verma, *Clayton*
Ernst Jurgen Wolvetang, *Brisbane*
Cory J Xian, *Adelaide*
Xin-Fu Zhou, *Adelaide*

Austria

Ludwig Aigner, *Salzburg*
Ferdinand Frauscher, *Innsbruck*
Regina Grillari, *Vienna*
Mariann Gyöngyösi, *Vienna*
Günter Lepperdinger, *Innsbruck*
Peter Valent, *Vienna*

Belgium

Yves Beguin, *Liege*
Mieke Geens, *Brussels*
Najimi Mustapha, *Brussels*

Brazil

Niels Olsen Saraiva Camara, *São Paulo*
Ana Helena da Rosa Paz, *Porto Alegre*
KA Teixeira de Carvalho, *Curitiba-Paraná*
Armando de Mattos Carvalho, *Botucatu*
RC dos Santos Goldenberg, *Rio de Janeiro*
Irina Kerkis, *São Paulo*
LC De Moraes Sobrino Porto, *Rio de Janeiro*
Stevens Kastrup Rehen, *Rio de Janeiro*
Rodrigo Resende, *Divinópolis*
Naiara Zoccal Saraiva, *Jaboticabal*

Canada

Borhane Annabi, *Quebec*
Long-Jun Dai, *Vancouver*
Connie Jean Eaves, *Vancouver*
Santokh Gill, *Ottawa*
Jeffrey Henderson, *Toronto*
Rosario Isasi, *Quebec*
Xiao-Yan Jiang, *Vancouver*
Seung U Kim, *Vancouver*
William Allan King, *Guelph*
Ren-Ke Li, *Toronto*
Jeffrey A Medin, *Toronto*
Christopher Naugler, *Calgary*
Dominique Shum-Tim, *Quebec*
Jean-François Tanguay, *Quebec*
Kursad Turksen, *Ottawa*
Lisheng Wang, *Ottawa*

China

Xiu-Wu Bian, *Chongqing*
Andrew Burd, *Hong Kong*
Kai-Yong Cai, *Chongqing*
Chi-Kai Chen, *Shantou*
Ling-Yi Chen, *Tianjin*
Fu-Zhai Cui, *Beijing*
Yong Dai, *Shenzhen*
Yu-Cheng Dai, *Nanchang*

Li Deng, *Chengdu*
Jian Dong, *Shanghai*
Jun Dou, *Nanjing*
Jian-Xin Gao, *Shanghai*
Zhi-Liang Gao, *Guangzhou*
Zi-Kuan Guo, *Beijing*
Zhong-Chao Han, *Tianjin*
Ling-Ling Hou, *Beijing*
Jian-Guo Hu, *Bengbu*
Yi-Ping Hu, *Shanghai*
Dong-Sheng Huang, *Guangzhou*
Jian-Hua Huang, *Yinchuan*
Jiu-Hong Kang, *Shanghai*
Dong-Mei Lai, *Shanghai*
Anskar YH Leung, *Hong Kong*
Gang Li, *Hong Kong*
Gui-Rong Li, *Hong Kong*
Jin-Jun Li, *Shanghai*
Xiang-Yun Li, *Baoding*
Xiao-Rong Li, *Tianjin*
Zong-Jin Li, *Tianjin*
Qi-Zhou Lian, *Hong Kong*
Hong Liao, *Nanjing*
Kai-Yan Liu, *Beijing*
Lei Liu, *Chengdu*
Yi-Jia Lou, *Hangzhou*
Pauline Po Yee Lui, *Hong Kong*
Xue-Tao Pei, *Beijing*
Cai-Ping Ren, *Changsha*
Chun-Meng Shi, *Chongqing*
Guan-Bin Song, *Chongqing*
Jing-He Tan, *Tanan*
Kent Kam Sze Tsang, *Hong Kong*
Jin-Fu Wang, *Hangzhou*
Tie-Qiao Wen, *Shanghai*
Ji Wu, *Shanghai*
Ren-Hua Wu, *Shantou*
Rui-An Xu, *Xiamen*
Chuan Ye, *Guiyang*
Xi-Yong Yu, *Guangzhou*
Hao Zhang, *Beijing*
Shu-Ren Zhang, *Beijing*
Yun-Hai Zhang, *Hefei*
Lei Zhao, *Wuhan*
Xiao-Gang Zhong, *Nanning*
Bo Zhu, *Chongqing*
Zhong-Min Zou, *Chongqing*

Cyprus

Pantelis Georgiades, *Nicosia*

Czech Republic

Eva Bártová, *Brno*
Petr Dvorak, *Brno*
Vladimir Holan, *Prague*
Jaroslav Mokry, *Hradec Kralove*
Jakub Suchánek, *Hradec Kralove*

Denmark

Basem M Abdallah, *Odense*
Poul Hyttel, *Frederiksberg*
Lin Lin, *Tjele*
Morten Meyer, *Blommenslyst*
Nedime Serakinci, *Vejle*
Soren Paludan Sheikh, *Odense*
Vladimir Zachar, *Aalborg*

Egypt

Mohamed Ahmed Ghoneim, *Mansora*
Alaa Eldin Ismail, *Cairo*

Finland

Jukka Partanen, *Helsinki*
Petri Salven, *Helsinki*
Heli Teija Kristiina Skottman, *Tampere*

France

Ez-Zoubir Amri, *Nice*
Bernard Binetruy, *Marseille*
Alain Chapel, *Far*
Yong Chen, *Paris*
Dario Coleti, *Paris*
Christelle Coraux, *Reims*
Anne Claude Fernandez, *Montpellier*
Loïc Fouillard, *Cergy-Pontoise*
Norbert-Claude Gorin, *Paris*
Enzo Lalli, *Valbonne*
Gwendal Lazennec, *Montpellier*
Nathalie Lefort, *Evry*
Laurent Lescaudron, *Nantes*
David Magne, *Villeurbanne*
Muriel Perron, *Orsay*
Xavier Thomas, *Lyon*
Ali G Turhan, *Villejuif*
Didier Wion, *Grenoble*

Germany

Nasreddin Abolmaali, *Dresden*
James Adjaye, *Berlin*
Halvard Bonig, *Frankfurt*
Sven Brandau, *Essen*
Christian Buske, *Ulm*
Federico Calegari, *Dresden*
Denis Corbeil, *Dresden*
Hassan Dihazi, *Goettingen*
Juergen Dittmer, *Halle/Saale*
Thomas Dittmar, *Witten*
Frank Edenhofer, *Bonn*
José Tomás Egaña Erazo, *Munich*
Ursula Margarethe Gehling, *Langen*
Alexander Ghanem, *Bonn*
Eric Gottwald, *Eggenstein-Leopoldshafen*
Gerhard Gross, *Braunschweig*
Kaomei Guan, *Goettingen*
Christel Herold-Mende, *Heidelberg*
Jorg Kleeff, *Munich*
Gesine Kögler, *Düsseldorf*
Steffen Koschmieder, *Muenster*
Nan Ma, *Rostock*
Ulrich R Mahlknecht, *Homburg/Saar*
Ulrich Martin, *Hannover*
Glenn Mcgee, *Berlin*
Kurt Paul Pfannkuche, *Cologne*
Michael Platten, *Heidelberg*
Arjang Ruhparwar, *Heidelberg*
Heinrich Sauer, *Giessen*
Richard Schäfer, *Tübingen*
Nils Ole Schmidt, *Hamburg*
Sonja Schrepfer, *Hamburg*
Dimitry Spitkovsky, *Cologne*
Bodo-Eckehard Strauer, *Düsseldorf*
Sergey V Tokalov, *Dresden*
Wolfgang Wagner, *Aachen*

Greece

Nicholas P Anagnou, *Athens*

Hungary

Ferenc Uher, *Dioszegiut*

India

Anirban Basu, *Haryana*
Chiranjib Chakraborty, *Vellore*
Gugdutta U Gangenahalli, *New Delhi*
Minal Garg, *Lucknow*
Devendra K Gupta, *New Delhi*
Asok Mukhopadhyay, *New Delhi*
Riaz Ahmad Shah, *Kashmir*
Prathibha Harish Shetty, *Navi Mumbai*
Anjali Suhas Shiras, *Maharashtra*
Malancha Ta, *Bangalore*

Iran

Hossein Baharvand, *Tehran*
Mohamadreza B Eslaminejad, *Tehran*
Iraj Ragerdi Kashani, *Tehran*
Mansoureh Movahedin, *Tehran*
Seyed Javad Mowla, *Tehran*
Ghasem Hosseini Salekdeh, *Tehran*
Masoud Soleimani, *Tehran*
M Mehdi Yaghoobi, *Ostandari St. Kerman*
Arash Zaminy, *Tehran*

Ireland

Frank P Barry, *Galway*
Leo Quinlan, *Galway*
Philippe Taupin, *Dublin*
Ralf Michael Zwacka, *Galway*

Israel

Nadir Askenasy, *Petach Tikva*
Zeev Blumenfeld, *Haifa*
Benayahu Dafna, *Tel Aviv*
Benjamin Dekel, *Tel-Hashomer*
Dan Gazit, *Jerusalem*
Gal Goldstein, *Tel-Hashomer*
Eran Meshorer, *Jerusalem*
Rachel Sarig, *Rehovot*
Avichai Shimoni, *Tel-Hashomer*
Shimon Slavin, *Tel Aviv*

Italy

Andrea Barbuti, *Milan*

Carlo Alberto Beltrami, *Udine*
Bruno Bonetti, *Verona*
Paola Bruni, *Florence*
Laura Calzà, *Ozzano Emilia*
Giovanni Camussi, *Turin*
Domenico Capone, *Naples*
Francesco Carinci, *Ferrara*
Carmelo Carlo-Stella, *Milan*
Clotilde Castaldo, *Naples*
Angela Chambery, *Caserta*
Massimo De Felici, *Rome*
Enzo Di Iorio, *Zelarino*
Francesco Dieli, *Palermo*
Massimo Dominici, *Modena*
Stefania Filosa, *Naples*
Guido Frosina, *Genova*
Pompilio Giulio, *Milan*
Antonio Graziano, *Naples*
Brunella Grigolo, *Bologna*
Annalisa Grimaldi, *Varese*
Angela Gritti, *Milan*
Alessandro Isidori, *Pesaro*
Caterina AM La Porta, *Milan*
Giampiero La Rocca, *Palermo*
Giampiero Leanza, *Trieste*
Enrico Lucarelli, *Bologna*
Margherita Maioli, *Sassari*
Ferdinando Mannello, *Urbino*
Tullia Maraldi, *Modena*
Gianvito Martino, *Milan*
Monica Mattioli-Belmonte, *Ancona*
Fabrizio Michetti, *Rome*
Gabriella Minchiotti, *Naples*
Roberta Morosetti, *Rome*
Gianpaolo Papaccio, *Naples*
Felicita Pedata, *Florence*
Maurizio Pesce, *Milan*
Anna Chiara Piscaglia, *Rome*
Vito Pistoia, *Genova*
Francesca Pistollato, *Ispra*
Alessandro Poggi, *Genova*
Domenico Ribatti, *Bari*
Sergio Rutella, *Rome*
Sonia Scarfi', *Genova*
Arianna Scuteri, *Monza*
Luca Steardo, *Rome*
Gianluca Vadalà, *Rome*
Maria Teresa Valenti, *Verona*
Carlo Ventura, *Bologna*
Stefania Violini, *Lodi*

Japan

Manabu Akahane, *Nara*
Yasuto Akiyama, *Shizuoka*
Tomoki Aoyama, *Kyoto*
Sachiko Ezoe, *Osaka*
Yusuke Furukawa, *Tochigi*
Masayuki Hara, *Osaka*
Eiso Hiyama, *Hiroshima*
Kanya Honoki, *Kashihara*
Yuichi Hori, *Kobe*
Susumu Ikehara, *Osaka*
Ken-ichi Isobe, *Aichi*
Masamichi Kamihira, *Fukuoka*
Yonehiro Kanemura, *Osaka*
Hiroshi Kanno, *Yokohama*
Masaru Katoh, *Tokyo*
Eihachiro Kawase, *Kyoto*
Toru Kondo, *Sapporo*
Toshihiro Kushibiki, *Osaka*
Tao-Sheng Li, *Yamaguchi*
Yasuhisa Matsui, *Sendai*
Taro Matsumoto, *Tokyo*
Yuko Miyagoe-Suzuki, *Tokyo*
Hiroyuki Miyoshi, *Ibaraki*
Hiroyuki Mizuguchi, *Osaka*
Hiroshi Mizuno, *Sendai*
Takashi Nagasawa, *Kyoto*
Kohzo Nakayama, *Nagano*
Tetsuhiro Niidome, *Kyoto*
Toshio Nikaido, *Toyama*
Shoko Nishihara, *Tokyo*
Hirofumi Noguchi, *Okayama*
Tsukasa Ohmori, *Tochigi*
Katsutoshi Ozaki, *Tochigi*
Kumiko Saeki, *Tokyo*
Masanori Sasaki, *Hokkaido*
Kazunobu Sawamoto, *Aichi*
Goshi Shiota, *Yonago*
Mikiko C Siomi, *Tokyo*
Yoshiaki Sonoda, *Osaka*
Takashi Tada, *Kyoto*
Miyako Takaki, *Nara*
Kenzaburo Tani, *Fukuoka*
Shihori Tanabe, *Tokyo*
Shuji Toda, *Saga*
Atsunori Tsuchiya, *Niigata*
Kohichiro Tsuji, *Tokyo*
Shingo Tsuji, *Osaka*
Akihiro Umezawa, *Tokyo*
Hiroshi Wakao, *Sapporo*
Yoichi Yamada, *Nagoya*
Takashi Yokota, *Kanazawa*
Yukio Yoneda, *Ishikawa*
Kotaro Yoshimura, *Tokyo*
Katsutoshi Yoshizato, *Hiroshima*
Louis Yuge, *Hiroshima*

Jordan

Khitam Salem Odeh Alrefu, *Karak*

Malaysia

Rajesh Ramasamy, *Selangor*

Mexico

Marco A Velasco-Velazquez, *Mexico City*

Morocco

Radouane Yafia, *Ouarzazate*

Netherlands

Robert Paul Coppes, *Groningen*
Dirk Gijsbert de Rooij, *Amsterdam*
Christine Mummery, *Leiden*
Vered Raz, *Leiden*
Bernardus A Johannes Roelen, *Utrecht*
Marten Piet Smidt, *Utrecht*
Frank JT Staal, *Leiden*
Ruurd Torensma, *Nijmegen*

Norway

Brynjar Foss, *Stavanger*
Zhenhe Suo, *Oslo*
Berit Bølge Tysnes, *Bergen*

Portugal

Inês M Araújo, *Coimbra*

Romania

Mihaela Chivu Economescu, *Bucharest*

Russia

Igor A Grivennikov, *Moscow*
Sergey L Kiselev, *Moscow*
Serov Oleg, *Novosibirsk*

Singapore

Yu Cai, *Singapore*
Tong Cao, *Singapore*
Jerry Chan, *Singapore*
Ken Kwok-Keung Chan, *Singapore*
Woon Khiong Chan, *Singapore*
Gavin Stewart Dawe, *Singapore*
Peter Dröge, *Singapore*
Seet Li Fong, *Singapore*
Boon Chin Heng, *Singapore*
Yunhan Hong, *Singapore*
Yuin-Han Loh, *Singapore*
Koon Gee Neoh, *Singapore*
Steve Kah Weng Oh, *Singapore*
Kian Keong Poh, *Singapore*
Seeram Ramakrishna, *Singapore*
Herbert Schwarz, *Singapore*
Winston Shim, *Singapore*
Vivek M Tanavde, *Singapore*
Shu Wang, *Singapore*

Slovakia

Lubos Danisovic, *Bratislava*

South Korea

Kwang-Hee Bae, *Daejeon*
Hyuk-Jin Cha, *Seoul*
Jong Wook Chang, *Seoul*
Kyu-Tae Chang, *Chungcheongbuk-do*
Chong-Su Cho, *Seoul*
Myung Soo Cho, *Seoul*
Ssang-Goo Cho, *Seoul*
Kang-Yell Choi, *Seoul*
Ho Jae Han, *Gwangju*
Myung-Kwan Han, *Jeonju*
Chan Yeong Heo, *Gyeonggido*

Dong-Youn Hwang, *Seongnam*
Ki-Chul Hwang, *Seoul*
Sin-Soo Jeun, *Seoul*
Youngjoon Jun, *Gyeonggi-do*
Ji-Won Jung, *Chungbuk*
Jin Sup Jung, *Gyeongsangnam-do*
Kyung-Sun Kang, *Seoul*
Gilson Khang, *Jeonju*
Byung Soo Kim, *Seoul*
Haekwon Kim, *Seoul*
Hoeon Kim, *Daejeon*
Hyo-Soo Kim, *Seoul*
Jong-Hoon Kim, *Seoul*
Mee Kum Kim, *Seoul*
Moon Suk Kim, *Suwon*
Sang Gyung Kim, *Daegu*
Song Cheol Kim, *Seoul*
Yoon Jun Kim, *Seoul*
Dong Ryul Lee, *Seoul*
Jong Eun Lee, *Seoul*
Kwang-Bok Lee, *Chonbuk*
Soo-Hong Lee, *Gyunggi-do*
Younghee Lee, *Chungbuk*
Dae-Sik Lim, *Daejeon*
Kyu Lim, *Daejeon*
Do Sik Min, *Busan*
Byung Soon Park, *Seoul*
Jong-Beom Park, *Seoul*
Gyu-Jin Rho, *Jinju*
Chun Jeih Ryu, *Seoul*
Sun U Song, *Incheon*
Jong-Hyuk Sung, *Seoul*
Jong-Ho Won, *Seoul*
Seung Kwon You, *Seoul*

Spain

Luis M Antón Aparicio, *Coruña*
Angel Ayuso-Sacido, *Valencia*
Fernando Cobo, *Granada*
Maria P De Miguel, *Madrid*
Sabrina C Desbordes, *Barcelona*
Ramon Farre, *Barcelona*
Damian Garcia-Olmo, *Madrid*
Boulaiz Houria, *Granada*
Juan M Hurle, *Santander*
Antonia Aránega Jiménez, *Granada*
Marta Muñoz Llamosas, *España*
Juan Antonio Marchal, *Granada*
Pablo Menendez, *Granada*
María Dolores Miñana, *Valencia*
Eduardo Moreno, *Madrid*
Felipe Prosper, *Navarra*
Manuel Ramírez, *Madrid*

Sweden

M Quamrul Islam, *Linkoping*
Stefan Karlsson, *Lund*
Stephen C Strom, *Stockholm*
Rachael Victoria Sugars, *Huddinge*

Switzerland

Thomas Daikeler, *Basel*
Anis Feki, *Geneva*
Sabrina Mattoli, *Basel*
Arnaud Scherberich, *Basel*

Thailand

Rangsun Parnpai, *Nakhon Ratchasima*

Tunisia

Faouzi Jenhani, *Monastir*

Turkey

Kamil C Akcali, *Ankara*
Berna Arda, *Ankara*
Alp Can, *Ankara*
Y Murat Elcin, *Ankara*
Erdal Karaoz, *Kocaeli*

United Arab Emirates

Sherif M Karam, *Al-Ain*

United Kingdom

Malcolm Ronald Alison, *London*
Charles Archer, *Cardiff*
Dominique Bonnet, *London*
Kristin Mary Braun, *London*
Wei Cui, *London*
Nicholas R Forsyth, *Stoke-on-Trent*
Rasmus Freter, *Oxford*
Hassan T Hassan, *Paisley*
David C Hay, *Edinburgh*
Wael Kafienah, *Bristol*
Francis L Martin, *Lancaster*
Stuart McDonald, *London*
Pankaj Kumar Mishra, *Wolverhampton*
Ali Mobasheri, *Leicestershire*
Mike Modo, *London*
Donald Palmer, *London*
Stefano Pluchino, *Cambridge*
Julia Margaret Polak, *London*
Stefan Alexander Przyborski, *Durham*
James Alexander Ross, *Edinburgh*
Susan Gaynor Shawcross, *Manchester*
Alastair James Sloan, *Cardiff*
Virginie Sottile, *Nottingham*
Petros V Vlastarakos, *Stevenage*
Hong Wan, *London*
Christopher M Ward, *Manchester*
Heping Xu, *Foresterhill*
Lingfang Zeng, *London*
Rike Zietlow, *Cardiff*

United States

Gregor Barr Adams, *Los Angeles*
Arshak R Alexanian, *Milwaukee*
Ali Syed Arbab, *Charlestown*
Kinji Asahina, *Los Angeles*
Atsushi Asakura, *Minneapolis*
Prashanth Asuri, *Santa Clara*
Craig S Atwood, *Madison*
Debabrata Banerjee, *New Brunswick*
David W Barnes, *Lawrenceville*
Surinder Kumar Batra, *Omaha*
Aline M Betancourt, *New Orleans*
John J Bright, *Indianapolis*
Bruce Alan Bunnell, *New Orleans*
Matthew E Burow, *New Orleans*
Anthony Wing Sang Chan, *Atlanta*
Rebecca J Chan, *Indianapolis*
Joe Y Chang, *Houston*
G Rasul Chaudhry, *Rochester*
Caifu Chen, *Foster City*
Ke Cheng, *Los Angeles*
Jonathan Donald Chesnut, *Carlsbad*
Herman S Cheung, *Coral Gables*
Kent W Christopherson II, *Chicago*
David Wade Clapp, *Indianapolis*
Claudius Conrad, *Boston*
Charles Samuel Cox Jr, *Houston*
Ronald G Crystal, *New York*
Hiranmoy Das, *Columbus*
Ivana de la Serna, *Toledo*
Marcos de Lima, *Houston*
Douglas C Dean, *Lousiville*
Bridget M Deasy, *Pittsburgh*
Weiwen Deng, *Grand Rapids*
Goberdhan Dimri, *Evanston*
David Dingli, *Rochester*
Juan Domínguez-Bendala, *Miami*
Sergey V Doronin, *Stony Brook*
Fu-Liang Du, *Vernon*
Gary L Dunbar, *Mt. Pleasant*
Todd Evans, *New York*
Toshihiko Ezashi, *Columbia*
Vincent Falanga, *Providence*
Zhongling Fen, *Oceanside*
Markus Frank, *Boston*
Mohamed A Gaballa, *Sun City*
G Ian Gallicano, *Washington*
Yair Gazitt, *San Antonio*
Sanga Gehmert, *Houston*
Yong-Jian Geng, *Houston*
Jorge A Genovese, *Salt Lake City*
Mehrnaz Gharaee-Kermani, *Ann Arbor*
Ali Gholamrezanezhad, *Baltimore*
Joseph C Glorioso, *Pittsburgh*
W Scott Goebel, *Indianapolis*
Brigitte N Gomperts, *Los Angeles*
Joel S Greenberger, *Pittsburgh*
Kristbjorn Orri Gudmundsson, *Frederick*
Preethi H Gunaratne, *Houston*
Yan-Lin Guo, *Hattiesburg*
Robert G Hawley, *Washington*
Tong-Chuan He, *Chicago*
Mary JC Hendrix, *Chicago*
Charles C Hong, *Nashville*
Yiling Hong, *Dayton*
Courtney W Houchen, *Oklahoma City*
George TJ Huang, *Boston*
Jing Huang, *Bethesda*
Johnny Huard, *Pittsburgh*
Jaclyn Y Hung, *San Antonio*
Lorraine Iacovitti, *Philadelphia*
Tony Ip, *Worcester*
D Joseph Jerry, *Springfield*
Kun-Lin Jin, *Novato*
Lixin Kan, *Chicago*
Winston Whei-Yang Kao, *Cincinnati*
Sunil Kapila, *Ann Arbor*
Partow Kebriaei, *Houston*
Mary J Kelley, *Portland*
Sophia K Khaldoyanidi, *San Diego*

Mahesh Khatri, *Wooster*
Jaspal S Khillan, *Pittsburgh*
Michael R King, *Ithaca*
Katsuhiro Kita, *Galveston*
Prasanna Krishnamurthy, *Chicago*
Marlene Anastassova Kristeva, *Van Nuys*
John S Kuo, *Madison*
Mark A LaBarge, *Berkeley*
Uma Lakshmipathy, *Carlsbad*
Hillard Michael Lazarus, *Shaker Heights*
Techung Lee, *Buffalo*
Jianxue Li, *Boston*
Joshua Li, *Charlottesville*
Shaoguang Li, *Worcester*
Shengwen Calvin Li, *Orange*
Xiao-Nan Li, *Houston*
Ching-Shwun Lin, *San Francisco*
P Charles Lin, *Nashville*
Shuo Lin, *Los Angeles*
Ning Liu, *Madison*
Suling Liu, *Ann Arbor*
Zhenguo Liu, *Columbus*
Aurelio Lorico, *Las Vegas*
Jean-Pierre Louboutin, *Philadelphia*
Bingwei Lu, *Stanford*
Qing Richard Lu, *Dallas*
Nadya L Lumelsky, *Bethesda*
Hong-Bo R Luo, *Boston*
Hinh Ly, *Atlanta*
Teng Ma, *Tallahassee*
Kenneth Maiese, *Newark*
Zubin Master, *Albany*
Debra JH Mathews, *Baltimore*
Robert L Mauck, *Philadelphia*
Lucio Miele, *Jackson*
Robert H Miller, *Cleveland*
David K Mills, *Ruston*
Murielle Mimeault, *Omaha*
Prasun J Mishra, *Bethesda*
Kalpana Mujoo, *Houston*
Masato Nakafuku, *Cincinnati*
Mary B Newman, *Chicago*
Wenze Niu, *Dallas*
Christopher Niyibizi, *Hershey*
Jon M Oatley, *Pullman*
Seh-Hoon Oh, *Gainesville*
Shu-ichi Okamoto, *La Jolla*
Frank Pajonk, *Los Angeles*
Nishit Pancholi, *Chicago*
Deric M Park, *Charlottesville*
Gregory M Pastores, *New York*
Ming Pei, *Morgantown*
Derek A Persons, *Memphis*
Donald G Phinney, *Jupiter*
John S Pixley, *Reno*
Dimitris G Placantonakis, *New York*
George E Plopper, *Troy*
Mark EP Prince, *Ann Arbor*
April Pyle, *Los Angeles*
Derek Radisky, *Jacksonville*
Murugan Ramalingam, *Gaithersburg*
Pranela Rameshwar, *Newark*
Guangwen Ren, *Piscataway*
Brent A Reynolds, *Gainesville*
Jeremy N Rich, *Cleveland*
Angie Rizzino, *Omaha*
Fred Jerrold Roisen, *Louisville*
Rouel S Roque, *Henderson*
Carl Barth Rountree Jr, *Lititz*
Clinton T Rubin, *Stony Brook*
Donald Sakaguchi, *Ames*
Paul Ronald Sanberg, *Tampa*
Gerald Phillip Schatten, *Pittsburgh*
Stewart Sell, *Albany*
Arun Sharma, *Chicago*
Jinsong Shen, *Dallas*
Ashok Kumar Shetty, *Durham*
Songtao Shi, *Los Angeles*
Yanhong Shi, *Duarte*
Vassilios I Sikavitsas, *Norman*
Igor I Slukvin, *Madison*
Shay Soker, *Winston-Salem*
Hongjun Song, *Baltimore*
Edward F Srour, *Indianapolis*
Hua Su, *Francisco*
Jun Sun, *Rochester*
Tao Sun, *New York*
Siamak Tabibzadeh, *Stony Brook*
Kenichi Tamama, *Columbus*
Masaaki Tamura, *Manhattan*
Tetsuya S Tanaka, *Notre Dame*
Dean G Tang, *Smithville*
Hugh S Taylor, *New Haven*
Jonathan L Tilly, *Boston*
Jakub Tolar, *Minneapolis*
Deryl Troyer, *Manhattan*
Scheffer Chuei-Goong Tseng, *Miami*
Cho-Lea Tso, *Los Angeles*
Andre Van Wijnen, *Worcester*
Lyuba Varticovski, *Bethesda*
Tandis Vazin, *Berkeley*
Kent E Vrana, *Hershey*
Qi Wan, *Reno*
Charles Wang, *Los Angeles*
Guo-Shun Wang, *New Orleans*
Lianchun Wang, *Athens*
Limin Wang, *Ann Arbor*
Shu-Zhen Wang, *Birmingham*
Yigang Wang, *Cincinnati*
Zack Z Wang, *Scarborough*
Zhiqiang Wang, *Duarte*
David Warburton, *Los Angeles*
Li-Na Wei, *Minneapolis*
Christof Westenfelder, *Salt Lake City*
Marc Adrian Williams, *Rochester*
J Mario Wolosin, *New York*
Raymond Ching-Bong Wong, *Irvine*
Joseph C Wu, *Stanford*
Lizi Wu, *Gainesville*
Ping Wu, *Galveston*
Sean M Wu, *Boston*
Wen-Shu Wu, *Scarborough*
Meifeng Xu, *Cincinnati*
Xiaowei Xu, *Philadelphia*
Yan Xu, *Pittsburgh*
Dean Takao Yamaguchi, *Los Angeles*
Jun Yan, *Louisville*
Feng-Chun Yang, *Indianapolis*
Jing Yang, *Orange*
Phillip Chung-Ming Yang, *Stanford*
Shang-Tian Yang, *Columbus*
Xiaoming Yang, *Seattle*
Youxin Yang, *Boston*
Kaiming Ye, *Fayetteville*
Pampee Paul Young, *Nashville*
Hui Yu, *Pittsburgh*
John S Yu, *Los Angeles*
P Hong Yu, *Miami*
Seong-Woon Yu, *East Lansing*
Xian-Min Zeng, *Novato*
Ming Zhan, *Baltimore*
Baohong Zhang, *Greenville*
Chengcheng Zhang, *Dallas*
Qunzhou Zhang, *Los Angeles*
Yan Zhang, *Houston*
Ying Zhang, *Baltimore*
Pan Zheng, *Ann Arbor*
X Long Zheng, *Philadelphia*
Xuesheng Zheng, *Charlestown*
John F Zhong, *Los Angeles*
Bin Zhou, *Boston*
Feng C Zhou, *Indianapolis*
Xianzheng Zhou, *Minneapolis*

Venezuela

Pedro M Aponte, *Estado Aragua*

World Journal of
Stem Cells

Contents

Monthly Volume 7 Number 7 August 26, 2015

EDITORIAL

992 Signaling involved in stem cell reprogramming and differentiation
Tanabe S

REVIEW

999 Stem cell guidance through the mechanistic target of rapamycin
Maiese K

1010 Allogenic banking of dental pulp stem cells for innovative therapeutics
Collart-Dutilleul PY, Chaubron F, De Vos J, Cuisinier FJ

1022 Adoptive immunotherapy for acute leukemia: New insights in chimeric antigen receptors
Heiblig M, Elhamri M, Mauricette M, Thomas X

MINIREVIEWS

1039 Advancement in high dose therapy and autologous stem cell rescue in lymphoma
Isidori A, Clissa C, Loscocco F, Guiducci B, Barulli S, Malerba L, Gabucci E, Visani G

1047 Stem cells: Sources, and regenerative therapies in dental research and practice
Aly LAA

1054 Rat embryonic stem cells create new era in development of genetically manipulated rat models
Kawaharada K, Kawamata M, Ochiya T

ORIGINAL ARTICLE

Basic Study

1064 Simplified three-dimensional culture system for long-term expansion of embryonic stem cells
McKee C, Perez-Cruet M, Chavez F, Chaudhry GR

Contents

World Journal of Stem Cells
Volume 7 Number 7 August 26, 2015

ABOUT COVER

Editorial Board Member of *World Journal of Stem Cells*, Kumiko Saeki, MD, PhD, Director, Division of Stem Cell Therapy, Department of Disease Control, Research Institute, National Center for Global Health and Medicine, 1-21-1 Toyama Shinjuku-ku, Tokyo 162-8655, Japan

AIM AND SCOPE

World Journal of Stem Cells (*World J Stem Cells*, *WJSC*, online ISSN 1948-0210, DOI: 10.4252), is a peer-reviewed open access academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

WJSC covers topics concerning all aspects of stem cells: embryonic, neural, hematopoietic, mesenchymal, tissue-specific, and cancer stem cells; the stem cell niche, stem cell genomics and proteomics, and stem cell techniques and their application in clinical trials.

We encourage authors to submit their manuscripts to *WJSC*. We will give priority to manuscripts that are supported by major national and international foundations and those that are of great basic and clinical significance.

INDEXING/ABSTRACTING

World Journal of Stem Cells is now indexed in PubMed Central, PubMed, Digital Object Identifier, and Directory of Open Access Journals.

FLYLEAF

I-V Editorial Board

EDITORS FOR THIS ISSUE

Responsible Assistant Editor: *Xiang Li*
Responsible Electronic Editor: *Huan-Liang Wu*
Proofing Editor-in-Chief: *Lian-Sheng Ma*
Responsible Science Editor: *Shui Qiu*
Proofing Editorial Office Director: *Xiu-Xia Song*

NAME OF JOURNAL
World Journal of Stem Cells

ISSN
ISSN 1948-0210 (online)

LAUNCH DATE
December 31, 2009

FREQUENCY
Monthly

EDITOR-IN-CHIEF
Oscar Kuang-Sheng Lee, MD, PhD, Professor, Medical Research and Education of Veterans General Hospital-Taipei, No. 322, Sec. 2, Shih-pai Road, Shih-pai, Taipei 11217, Taiwan

EDITORIAL OFFICE
Jin-Lei Wang, Director
Xiu-Xia Song, Vice Director
World Journal of Stem Cells
Room 903, Building D, Ocean International Center, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China
Telephone: +86-10-85381891
Fax: +86-10-85381893
E-mail: editorialoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com

PUBLISHER
Baishideng Publishing Group Inc
8226 Regency Drive,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com

PUBLICATION DATE
August 26, 2015

COPYRIGHT
© 2015 Baishideng Publishing Group Inc. Articles published by this Open-Access journal are distributed under the terms of the Creative Commons Attribution Non-commercial License, which permits use, distribution, and reproduction in any medium, provided the original work is properly cited, the use is non-commercial and is otherwise in compliance with the license.

SPECIAL STATEMENT
All articles published in journals owned by the Baishideng Publishing Group (BPG) represent the views and opinions of their authors, and not the views, opinions or policies of the BPG, except where otherwise explicitly indicated.

INSTRUCTIONS TO AUTHORS
Full instructions are available online at http://www.wjgnet.com/1948-0210/g_info_20100313165700.htm

ONLINE SUBMISSION
http://www.wjgnet.com/esps/

World Journal of
Stem Cells

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4252/wjsc.v7.i7.992

World J Stem Cells 2015 August 26; 7(7): 992-998
ISSN 1948-0210 (online)
© 2015 Baishideng Publishing Group Inc. All rights reserved.

EDITORIAL

Signaling involved in stem cell reprogramming and differentiation

Shihori Tanabe

Shihori Tanabe, National Institute of Health Sciences, Tokyo 158-8501, Japan

Author contributions: Tanabe S is the sole contributor to this paper.

Conflict-of-interest statement: The author declares that no conflict of interest exists.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: http://creativecommons.org/licenses/by-nc/4.0/

Correspondence to: Shihori Tanabe, PhD, Senior Researcher at the National Institute of Health Sciences, 1-18-1, Kami-yoga, Setagaya-ku, Tokyo 158-8501, Japan. stanabe@nihs.go.jp
Telephone: +81-3-37001141
Fax: +81-3-37076950

Received: April 21, 2015
Peer-review started: April 24, 2015
First decision: May 13, 2015
Revised: May 29, 2015
Accepted: June 18, 2015
Article in press: June 19, 2015
Published online: August 26, 2015

Abstract

Stem cell differentiation is regulated by multiple signaling events. Recent technical advances have revealed that differentiated cells can be reprogrammed into stem cells. The signals involved in stem cell programming are of major interest in stem cell research. The signaling mechanisms involved in regulating stem cell reprogramming and differentiation are the subject of intense study in the field of life sciences. In this review, the molecular interactions and signaling pathways related to stem cell differentiation are discussed.

Key words: Stem cell; Signaling; Differentiation; Gene; Genome; Reprogramming

© The Author(s) 2015. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Signals in stem cell are regulated both genetically and epigenetically by many molecules. The programming of stem cell signaling is an important aspect of understanding stem cell phenotype transitions and functions. The differentiation process as well as intra- and inter-cellular signaling of stem cells is described in this article. The epigenetic regulation of these cells is also discussed.

Tanabe S. Signaling involved in stem cell reprogramming and differentiation. *World J Stem Cells* 2015; 7(7): 992-998 Available from: URL: http://www.wjgnet.com/1948-0210/full/v7/i7/992.htm DOI: http://dx.doi.org/10.4252/wjsc.v7.i7.992

INTRODUCTION

Stem cells are the source of all other cells, and they have the potential to differentiate into many types of cells that contribute to the various organs, such as the heart, lungs, liver, and blood. There are many kinds of stem cells: totipotent embryonic stem cells (ESCs) found in the embryo; mesenchymal stem cells (MSCs) that give rise primarily to bone marrow stroma, adipocytes, and cord blood; epithelial stem cells that reside in the intestine; induced pluripotent stem cells that are artificially reprogrammed from differentiated cells; and cancer stem cells (CSCs). In this article, signaling in stem cells and the role of signaling molecules in the differentiation and reprogramming of stem cells are discussed.

STEM CELL DIFFERENTIATION

Stem cell differentiation is tightly regulated. Among the various pathways related to cellular differentiation, epigenetic regulation is the key mechanism that controls midbrain dopaminergic neuron differentiation[1]. Urocortin, which is expressed in the developing ventral midbrain, mediates dopaminergic neuron differentiation *via* the up-regulation of acetylated histone H3 and the dopaminergic regulators *Nurr1*, *Foxa2* and *Pitx3*[1]. Dimethyl sulfoxide down-regulates the pluripotency genes *OCT4* (also known as *POU5F1*) and *NANOG* in human embryonic stem (ES) cells during definitive endoderm differentiation and controls hepatic differentiation[2].

A previous report indicated that N^6-methyladenosine (m^{6}A) transferase (Mettl3; methyl transferase-like 3) regulates murine naïve pluripotency[3]. mRNA methylation is a key RNA modification and an essential factor in epigenomic regulation and cell fate determination[3]. Pluripotent cells are affected by environmental factors such as culture conditions, and such factors can affect the spatial regulations of transplanted stem cells[4]. Several signaling pathways are involved in the transformation of stem cells, such as fibroblast growth factor 2 (FGF2)/Activin-A signaling and histone 3 lysine 4 trimethylation alterations[4]. Matrix-bound heparan sulfate (HS) proteoglycans are needed for the differentiation of stem cell-derived endodermal cells into airway epithelial cells[5]. The three-dimensional extracellular matrix scaffold is essential for effective functional expression of lung epithelial cells[5], and HS proteoglycans also appear to play an important role in this process. Human ES cells differentiate into osteogenic cells, which can be identified by *RUNX2* expression[6]. Monitoring the osteogenic differentiation of human ES cells is useful for the therapeutic strategies used in bone regeneration[6]. The three-dimensional poly(L-lactic acid) scaffold culture of human ES cells on a nano-fibrous matrix results in enhanced osteogenic differentiation[7]. *RUNX2* expression in nano-fibrous matrix culture is significantly higher than in control cultures[7]. Additionally, an efficient method for the osteogenic differentiation of human ES cells using primary bone-derived cells has been reported[8]. Some cellular factors from bone-derived cells may be involved in human ES cell differentiation[8]. Cell surface proteins play roles in stem cell culture differentiation[9]. The use of functionalized hyaluronic acid surfaces with either fibronectin or collagen enhances the attachment of human ES cells[9]. During the osteogenic differentiation of human ES cells, hyaluronic acid surfaces with collagen I and collagen IV have inductive effects on differentiation compared to fibronectin alone[9]. Transcription factors are regulated dynamically during human ES cell differentiation[10]. Transcription factor binding dynamics are classified into static, dynamic, enhanced, and suppressed states[10]. Transforming growth factor (TGF)-β and WNT signals are involved in human ES cell differentiation into mesendoderm and endoderm, whereas bone morphogenetic protein (BMP), vascular endothelial growth factor, and FGF2 are key regulators of the differentiation of ES cells into mesoderm. The inhibition of TGF-β, WNT or BMP signals result in ES cell differentiation into ectoderm[10]. *RUNX2* is down-regulated in late-stage cultures of MSCs[11,12]. The osteogenic differentiation capacity is decreased in late-stage cultures of MSCs compared to early-stage cultures; thus, gene expression levels can be used as an indicator of stem cell differentiation capacity[11]. Neural differentiation is regulated by epigenetic mechanisms[13]. Deep cortical layer neuronal markers such as *BCL11B* are expressed in differentiated neurons derived from neuroepithelial and early radial glia, and superficial layer neuronal markers such as *POU3F2*/*POU3F3* and *MEF2C* are expressed in differentiated progeny derived from mid radial glia[13]. Transcription factor activity is modulated dynamically during each stage of the neural progenitor population[13]. In addition to intracellular molecular network signaling, intercellular communication is also an important factor in stem cell signaling. Human neural stem/progenitor cells (hNSCs) and peripheral blood mononuclear cells (PBMCs) interact with each other when co-cultured under allogenic conditions. hNSCs increase the production of regulatory T cells, while PBMCs promote the differentiation of hNSCs[14]. The effect of intercellular communication on stem cell differentiation is an interesting topic for investigation. Cartilaginous tissue can be generated from human induced pluripotent stem (iPS) cells[15]. In severe combined immunodeficiency (SCID) mice transplanted with human iPS cell-derived differentiated cartilaginous cells, cartilage formation without tumor formation is observed[15].

STEM CELL SIGNALING

Tools for bioinformatics

A wealth of "big data" in gene and genome information, which has been collected *via* integrative profiling and is publically available on the internet, combined with recent advances in bioinformatics, have enabled us to analyze gene and signaling information. These advances include a useful web-accessible tool called the Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resource (http://david.abcc.ncifcrf.gov/home.jsp) and the cBioPortal for Cancer Genomics (http://www.cbioportal.org/index.do)[16,17]. The open-source tool Cytoscape is a molecular network analysis tool for integrating the networks with annotation and gene expression profiles (http://cytoscape.org/what_is_cytoscape.html).

Hedgehog pathway

Hedgehog (Hh) signaling is an important player in epithelial and mesenchymal regulation during embryonic limb and bone development and cell fate determination[18-20]. Sonic Hh signaling is involved in

vascular differentiation and regulates human CD34-positive cell function[20]. Hh signaling also has an important role in hematological CSCs[21]. Targets of the Hh signaling pathway include the Notch pathway, the epithelial-mesenchymal transition (EMT) pathway, the WNT signaling pathway, the TGF-β signaling pathway, and the regulation of cell cycle, adhesion, fate determination, and stem cell signaling[21].

Ephrin pathway

Ephrin signaling is involved in inflammatory bowel diseases[22]. Bone marrow stem cells and stem cell factors play important roles in mucosal regeneration. In the intestine of a rat model of inflammatory bowel disease, ephrin-B3 is up-regulated in bone marrow MSCs during mucosal regeneration[22]. Ephrin type-B receptor 3 (*EPHB3*), a tumor suppressor gene, is important in cancer cell metastasis, which is regulated by Notch signaling in colorectal cancers[23]. *EPHB3* down-regulation in tumorigenesis correlates with Wnt/β-catenin, Notch and mitogen-activated protein kinase (MAPK) signaling[23]. EphrinA1 expression decreases as β-catenin expression is inhibited in liver tumor cells[24]. The treatment of hepatocellular cancer with cell-permeable, gamma guanidine-based peptide nucleic acid antisense oligonucleotides resulted in the inhibition of β-catenin and Wnt target gene expression[23]. The EphrinA1 receptor has been reported to be an independent prognostic marker for different survival endpoints in clear cell renal cell carcinoma[25]. The use of Ephrin and ephrin receptor signaling as either a prognostic marker or for revealing cancer progression mechanisms is an interesting topic for future study.

WNT pathway

The WNT pathways regulate stem cell differentiation and proliferation. WNT3-WNT9B signaling is involved in the regulation of neural differentiation[26]. An increase in WNT9B is reported to control the switch between pluripotent and differentiated states *via* noncanonical Rho/JNK signaling, whereas canonical WNT3/β-catenin signaling promotes proliferation[26]. WNT5A is known to promote cancer cell invasion and proliferation[27]. WNT5A activation of the Wnt/β-catenin-independent pathway, including Src family kinases, induces the proliferation of certain cancer cell types, such as HeLa S3 cervical cancer cells and A549 lung cancer cells[27]. Interestingly, WNT5A is not involved in the proliferation of KKLS gastric cancer cells, although cancer invasion is dependent on the WNT5A pathway[27]. Wnt5A up-regulation is reported to induce EMT and metastasis by pancreatic cancer cells[28]. In this case, β-catenin-dependent canonical Wnt signaling is involved in promotion of EMT [28]. Graphene oxide is reported to target CSCs by inhibiting a number of signaling pathways including the WNT, Notch and STAT pathways[29]. Graphene oxide selectively inhibits CSC proliferation but does not affect normal fibroblast viability[29]. STAT3 signaling alters the tumor microenvironment and angiogenesis and promotes KRAS-induced lung tumorigenesis[30]. The Wnt signaling pathway is reported to be down-regulated in irradiated tumor cells[31]. Wnt signaling may be involved in tumor repopulation after radiotherapy[31]. Notch1 and Delta-like 4 signals are important factors for cell fate decisions[32]. The Notch pathway is a therapeutic target in cancer treatment[32]. Interleukin-27 (IL-27) down-regulates EMT- and stemness-related genes such as *SONIC HEDGEHOG* in adenocarcinoma and *OCT4A*, *SOX2*, *NOTCH1*, *KLF4*, *Nestin*, *SNAI1/SNAIL*, *SNAI2/SLUG* and *ZEB1* in squamous cell carcinoma[33]. *SNAI2*, an EMT-related gene, and stemness genes are known to be down-regulated by IL-27[33]. WNT signaling modulation also mediates the long-term expansion and chondrogenic differentiation of adult human MSCs[34]. WNT3A and FGF2 maintain the phenotype and multipotency of MSCs[34]. In ES cells, cyclin-dependent kinase 1 (CDK1) inhibition is reported to prevent teratoma formation[35]. CDK1 signaling and its association with other cancer signaling pathways such as the WNT pathway would be interesting topics for future study of the mechanisms of tumor formation by stem cells.

MicroRNA signaling

MicroRNAs (miRNAs) are involved in cancer cell signaling[36]. The tumor microenvironment that maintains cancer cells is regulated by microRNA-mediated gene expression[36]. Anti-miRNAs, antisense oligomers that inhibit onco-miRNAs, can be used as anti-cancer drugs[36]. The conserved, well-established promoter of the terminal differentiation *let-7a* miRNA induces mitochondrial reactive oxygen species production and up-regulates oxidative stress-responsive genes[37]. The regulation of energy metabolism in cancer cells *via let-7a* is a potential target of anti-cancer therapy because *let-7a* miRNA plays a tumor-suppressive role[37]. Human MSCs that support the tumor microenvironment deliver tumor regulatory miRNAs *via* extracellular vesicular trafficking[38]. The extracellular vesicles of hMSCs contain proteins and lipids on the vesicle membrane, and miRNAs and metabolites inside the vesicles[38]. The internalization of these vesicles by cancer cells may be one of the key mechanisms for cancer cell survival[38]. During the hypoxia-induced myogenic differentiation of ES cells, miRNA-26a is up-regulated and inhibits the mRNA expression of histone deacetylase 6 (*HDAC6*) and of stemness genes such as *Oct4* (also known as *Pou5f1*) and *Nanog*[39]. miRNA-26a-mediated signaling may be important for stem cell differentiation[39]. Hypoxia contributes to the development and phenotype of CSCs *via* miRNA- and cytokine-mediated microenvironmental regulation[40]. The vascular CSC niche is regulated by miRNAs, which may induce CSC generation and EMT *via* the NFkB, PI3K/Akt/mTOR, NOTCH, Wnt/β-catenin and Hedgehog signaling pathways[40].

In the murine cortex and hippocampus, subclasses of neurons and glial cells have been characterized by the expression of regulatory genes using large-scale

single-cell RNA sequencing[41]. These cells exhibit differential gene expression and epigenetic regulation and miRNAs have recently become a focus of these processes as selective cell markers[42]. Glioma cells expressing miR-302 are enriched by serum deprivation, and miR-302 may be a marker of CSCs[42]. miRNA-30c plays a role in sphere formation, self-renewal and neural differentiation in C6 glioma cells[43]. In these cells, the astrocyte marker glial fibrillary acidic protein (GFAP) is up-regulated during differentiation. However, GFAP expression decreases in miRNA-30c-overexpressing cells[43]. The fate of neural stem cells is also regulated by miRNAs[44]. The differentiation of neural stem cells into neurons is promoted by kuwanon V, a phytochemical isolated from mulberry tree (*Morus bombycis*) roots that may up-regulate the expression of miR-9, miR-29a and miR-181a[44]. Cancer stem cell self-renewal, differentiation and tumorigenesis are regulated by the Notch pathway, in which miRNAs such as the miR-34 family, miR-200 family, and miR-199-5p, miR-146a, miR-1 and miR-143 play important roles[45]. Notch signaling cross-talk with miRNAs may be involved in cancer cell proliferation, which suggests possible therapeutic approaches *via* miRNA re-expression in cancer[45]. Moreover, genome-wide analysis has revealed that long noncoding RNAs (lncRNAs) are regulated by Notch signals in acute leukemia[46]. lncRNAs are defined as transcripts of greater than 200 nucleotides that function by means other than coding for proteins, and regulate active and silent chromatin states[47]. Pluripotent states are epigenetically regulated by lncRNAs[47]. Epigenetic modifications in pluripotency are conducted *via* several steps such as histone modifications, DNA methylation and chromatin remodeling[48]. miRNA expression is also regulated in atherosclerosis[49]. Endothelial miRNA expression profiling *in silico* is one area for future study of miRNA signaling[49].

CELLULAR REPROGRAMMING

Stem cells can be reprogrammed from differentiated cells[50]. The abundant genes including *CDH1*, *RGS1*, and *NOTCH1* are related to the cell characteristics such as MSCs and gastric cancer cells[51]. A recent study revealed that neural stem cells could be reprogrammed from adult fibroblasts, senescent somatic cells, and blood cells by HMGA2/let-7[52]. These induced neural stem cells could be successfully differentiated into neurons, astrocytes, and oligodendrocytes *in vitro* and *in vivo*[52]. The clinical application of reprogrammed iPS cells has also been attempted[53]. The transplantation of reprogrammed cells efficiently overcame spinal cord injury in mice; however, the possibility of tumorigenesis is a concern during long-term therapy[53]. One of the mechanisms of tumor development in transplanted iPS cells seems to be the acquisition of mesenchymal features such as either snail up-regulation or epithelial-mesenchymal transition[53]. Careful investigation and gene expression analysis are needed before iPS cells can be utilized in clinical settings[53]. The selective targeting of human iPS cells to avoid tumorigenesis has been described using a stem cell-specific lectin probe and *Pseudomonas aeruginosa* exotoxin A fusion proteins[54]. iPS cells are reprogrammed from cancer cells that are used as a carcinogenesis model[55]. The expression of pluripotency associated genes such as either *Oct4* (also known as *Pou5f1*) or *Nanog* is regulated by the bromodomain and extraterminal domain family member BRD4[56]. BRD4 regulates transcription by binding to acetylated histones[56]. The induced differentiation of human iPS cells by interleukin-3/granulocyte colony-stimulating factor results in $CD45^+CD11b^+CD14^+CD163^+CD68^+$ monocyte/macrophage-type cell formation[57]. Mesenchymal-amoeboid transition is a suggested mechanism by which cancer cells might adopt a migration mode[58]. The cell migration mode is characterized by three qualities: adhesion, confinement, and contractility[58]. The change in cell phenotype that occurs during reprogramming may be related to migration parameters. *WNT5A* is reported to be up-regulated in MSCs compared to diffuse-type gastric cancer cells[59]. Genes related to EMT such as *WNT5A* or *NOTCH2* may have roles in maintaining mesenchymal features[59]. A genome-wide analysis of chromatin interactions in human ES cells, ES cell-derived mesendoderm, MSCs, neural progenitor cells and trophoblast-like cells revealed that 36% of active and inactive chromosomal compartment alterations occur during differentiation[60]. An integrative analysis of 111 reference human epigenomes has been performed by the NIH Roadmap Epigenomics Consortium; this analysis profiled histone modification patterns and gene regulation in several cell types and found that each identified mechanism of genetic regulation targets different tissues and cell types in human biology and disease[61].

The analysis of adipose-derived stem cell differentiation has revealed that keratinocyte progenitor cells are present in adipose tissue[62]. The reprogramming of fibroblasts into iPS cells requires WNT signaling[63]. The efficiency of cell reprogramming is regulated by WNT signaling in fibroblasts[63]. Single-cell mass cytometry has revealed that iPS cell reprogramming is related to mesenchymal-epithelial transition (MET)[64]. A time-resolved progression analysis of iPS cell reprogramming has revealed that the expression patterns are altered at each time point and that some similarities exist between Oct4-GFP, Nanog-Neo, and Nanog-GFP mouse embryonic fibroblast reprogramming systems[64]. NANOG, which regulates the pluripotency and reprogramming of iPS cells, binds to the OCT4 promoter, and its mutation enhances ES cell self-renewal[65].

In early-stage diabetic endothelial progenitor cells, the expression of anti-oxidative enzymes compensates for oxidative stress levels[66]. The relationship between oxidative stress and progenitor cell function may be an interesting future topic for future study of the reprogramming process. The expansion of human

ES cells can be successfully maintained using MSC feeder layers[67]. Considering that xenogeneic-free culture conditions are necessary for safe advances in regenerative medicine, the mechanisms that maintain pluripotent status and induce differentiation in the cellular microenvironment should be investigated.

CONCLUSION

In conclusion, stem cells have diverse internal signaling and communicate with surrounding cells. This communication among cells, such as immune cells and cancer cells, through signaling proteins and molecules leads to dramatic alterations in cell responses and pathological physiology. The intra- and inter-cellular signaling pathways in the cellular microenvironment must be elucidated to safely manipulate stem cells and program signaling cascades for therapeutic applications. Abundant data about cellular gene expression, genomics and epigenomics, which are "big-data", and database analyses are publically available *via* the internet and will contribute to our understanding of cell phenotype transition and the mechanisms that regulate various cell populations in the context of disease and therapy.

REFERENCES

1 **Huang HY**, Chiu TL, Chang HF, Hsu HR, Pang CY, Liew HK, Wang MJ. Epigenetic regulation contributes to urocortin-enhanced midbrain dopaminergic neuron differentiation. *Stem Cells* 2015; **33**: 1601-1617 [PMID: 25641682 DOI: 10.1002/stem.1949]

2 **Czysz K**, Minger S, Thomas N. DMSO efficiently down regulates pluripotency genes in human embryonic stem cells during definitive endoderm derivation and increases the proficiency of hepatic differentiation. *PLoS One* 2015; **10**: e0117689 [PMID: 25659159 DOI: 10.1371/journal.pone.0117689]

3 **Geula S**, Moshitch-Moshkovitz S, Dominissini D, Mansour AA, Kol N, Salmon-Divon M, Hershkovitz V, Peer E, Mor N, Manor YS, Ben-Haim MS, Eyal E, Yunger S, Pinto Y, Jaitin DA, Viukov S, Rais Y, Krupalnik V, Chomsky E, Zerbib M, Maza I, Rechavi Y, Massarwa R, Hanna S, Amit I, Levanon EY, Amariglio N, Stern-Ginossar N, Novershtern N, Rechavi G, Hanna JH. Stem cells. m6A mRNA methylation facilitates resolution of naïve pluripotency toward differentiation. *Science* 2015; **347**: 1002-1006 [PMID: 25569111 DOI: 10.1126/science.1261417]

4 **Wu J**, Okamura D, Li M, Suzuki K, Luo C, Ma L, He Y, Li Z, Benner C, Tamura I, Krause MN, Nery JR, Du T, Zhang Z, Hishida T, Takahashi Y, Aizawa E, Kim NY, Lajara J, Guillen P, Campistol JM, Esteban CR, Ross PJ, Saghatelian A, Ren B, Ecker JR, Izpisua Belmonte JC. An alternative pluripotent state confers interspecies chimaeric competency. *Nature* 2015; **521**: 316-321 [PMID: 25945737 DOI: 10.1038/nature14413]

5 **Shojaie S**, Ermini L, Ackerley C, Wang J, Chin S, Yeganeh B, Bilodeau M, Sambi M, Rogers I, Rossant J, Bear CE, Post M. Acellular lung scaffolds direct differentiation of endoderm to functional airway epithelial cells: requirement of matrix-bound HS proteoglycans. *Stem Cell Reports* 2015; **4**: 419-430 [PMID: 25660407 DOI: 10.1016/j.stemcr.2015.01.004]

6 **Zou L**, Kidwai FK, Kopher RA, Motl J, Kellum CA, Westendorf JJ, Kaufman DS. Use of RUNX2 expression to identify osteogenic progenitor cells derived from human embryonic stem cells. *Stem Cell Reports* 2015; **4**: 190-198 [PMID: 25680477 DOI: 10.1016/j.stemcr.2015.01.008]

7 **Smith LA**, Liu X, Hu J, Ma PX. The enhancement of human embryonic stem cell osteogenic differentiation with nano-fibrous scaffolding. *Biomaterials* 2010; **31**: 5526-5535 [PMID: 20430439 DOI: 10.1016/j.biomaterials.2010.03.065]

8 **Ahn SE**, Kim S, Park KH, Moon SH, Lee HJ, Kim GJ, Lee YJ, Park KH, Cha KY, Chung HM. Primary bone-derived cells induce osteogenic differentiation without exogenous factors in human embryonic stem cells. *Biochem Biophys Res Commun* 2006; **340**: 403-408 [PMID: 16389066 DOI: 10.1016/j.bbrc.2005.12.020]

9 **Doran MR**, Frith JE, Prowse AB, Fitzpatrick J, Wolvetang EJ, Munro TP, Gray PP, Cooper-White JJ. Defined high protein content surfaces for stem cell culture. *Biomaterials* 2010; **31**: 5137-5142 [PMID: 20378164 DOI: 10.1016/j.biomaterials.2010.03.015]

10 **Tsankov AM**, Gu H, Akopian V, Ziller MJ, Donaghey J, Amit I, Gnirke A, Meissner A. Transcription factor binding dynamics during human ES cell differentiation. *Nature* 2015; **518**: 344-349 [PMID: 25693565 DOI: 10.1038/nature14233]

11 **Tanabe S**, Sato Y, Suzuki T, Suzuki K, Nagao T, Yamaguchi T. Gene expression profiling of human mesenchymal stem cells for identification of novel markers in early- and late-stage cell culture. *J Biochem* 2008; **144**: 399-408 [PMID: 18550633 DOI: 10.1093/jb/mvn082]

12 **Wells CA**, Mosbergen R, Korn O, Choi J, Seidenman N, Matigian NA, Vitale AM, Shepherd J. Stemformatics: visualisation and sharing of stem cell gene expression. *Stem Cell Res* 2013; **10**: 387-395 [PMID: 23466562 DOI: 10.1016/j.scr.2012.12.003]

13 **Ziller MJ**, Edri R, Yaffe Y, Donaghey J, Pop R, Mallard W, Issner R, Gifford CA, Goren A, Xing J, Gu H, Cacchiarelli D, Tsankov AM, Epstein C, Rinn JL, Mikkelsen TS, Kohlbacher O, Gnirke A, Bernstein BE, Elkabetz Y, Meissner A. Dissecting neural differentiation regulatory networks through epigenetic footprinting. *Nature* 2015; **518**: 355-359 [PMID: 25533951 DOI: 10.1038/nature13990]

14 **Zhang H**, Shao B, Zhuge Q, Wang P, Zheng C, Huang W, Yang C, Wang B, Su DM, Jin K. Cross-talk between human neural stem/progenitor cells and peripheral blood mononuclear cells in an allogeneic co-culture model. *PLoS One* 2015; **10**: e0117432 [PMID: 25658950 DOI: 10.1371/journal.pone.0117432]

15 **Yamashita A**, Morioka M, Yahara Y, Okada M, Kobayashi T, Kuriyama S, Matsuda S, Tsumaki N. Generation of scaffoldless hyaline cartilaginous tissue from human iPSCs. *Stem Cell Reports* 2015; **4**: 404-418 [PMID: 25733017 DOI: 10.1016/j.stemcr.2015.01.016]

16 **Huang da W**, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 2009; **4**: 44-57 [PMID: 19131956 DOI: 10.1038/nprot.2008.211]

17 **Huang da W**, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res* 2009; **37**: 1-13 [PMID: 19033363 DOI: 10.1093/nar/gkn923]

18 **Johnson RL**, Tabin CJ. Molecular models for vertebrate limb development. *Cell* 1997; **90**: 979-990 [PMID: 9323126 DOI: 10.1016/S0092-8674(00)80364-5]

19 **St-Jacques B**, Hammerschmidt M, McMahon AP. Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. *Genes Dev* 1999; **13**: 2072-2086 [PMID: 10465785 DOI: 10.1101/gad.13.16.2072]

20 **Kanaya K**, Masaaki I, Okazaki T, Nakamura T, Horii-Komatsu M, Alev C, Akimaru H, Kawamoto A, Akashi H, Tanaka H, Asahi M, Asahara T. Sonic Hedgehog signaling regulates vascular differentiation and function in human CD34 positive cells: vasculogenic CD34(+) cells with Sonic Hedgehog. *Stem Cell Res* 2015; **14**: 165-176 [PMID: 25644021 DOI: 10.1016/j.scr.2015.01.003]

21 **Campbell V**, Copland M. Hedgehog signaling in cancer stem cells: a focus on hematological cancers. *Stem Cells Cloning* 2015; **8**: 27-38 [PMID: 25691811 DOI: 10.2147/SCCAA.S58613]

22 **Qu B**, Xin GR, Zhao LX, Xing H, Lian LY, Jiang HY, Tong JZ, Wang BB, Jin SZ. Testing stem cell therapy in a rat model of inflammatory bowel disease: role of bone marrow stem cells and stem cell factor in mucosal regeneration. *PLoS One* 2014; **9**: e107891 [PMID: 25309991 DOI: 10.1371/journal.pone.0107891]

23 **Jägle S**, Rönsch K, Timme S, Andrlová H, Bertrand M, Jäger M, Proske A, Schrempp M, Yousaf A, Michoel T, Zeiser R, Werner M, Lassmann S, Hecht A. Silencing of the EPHB3 tumor-suppressor gene in human colorectal cancer through decommissioning of a transcriptional enhancer. *Proc Natl Acad Sci USA* 2014; **111**: 4886-4891 [PMID: 24707046 DOI: 10.1073/pnas.1314523111]

24 **Delgado E**, Bahal R, Yang J, Lee JM, Ly DH, Monga SP. β-Catenin knockdown in liver tumor cells by a cell permeable gamma guanidine-based peptide nucleic acid. *Curr Cancer Drug Targets* 2013; **13**: 867-878 [PMID: 23822752 DOI: 10.2174/15680096113139990081]

25 **Toma MI**, Erdmann K, Diezel M, Meinhardt M, Zastrow S, Fuessel S, Wirth MP, Baretton GB. Lack of ephrin receptor A1 is a favorable independent prognostic factor in clear cell renal cell carcinoma. *PLoS One* 2014; **9**: e102262 [PMID: 25025847 DOI: 10.1371/journal.pone.0102262]

26 **Lee CT**, Bendriem RM, Kindberg AA, Worden LT, Williams MP, Drgon T, Mallon BS, Harvey BK, Richie CT, Hamilton RS, Chen J, Errico SL, Tsai SY, Uhl GR, Freed WJ. Functional consequences of 17q21.31/WNT3-WNT9B amplification in hPSCs with respect to neural differentiation. *Cell Rep* 2015; **10**: 616-632 [PMID: 25640183 DOI: 10.1016/j.celrep.2014.12.050]

27 **Shojima K**, Sato A, Hanaki H, Tsujimoto I, Nakamura M, Hattori K, Sato Y, Dohi K, Hirata M, Yamamoto H, Kikuchi A. Wnt5a promotes cancer cell invasion and proliferation by receptor-mediated endocytosis-dependent and -independent mechanisms, respectively. *Sci Rep* 2015; **5**: 8042 [PMID: 25622531 DOI: 10.1038/srep08042]

28 **Bo H**, Zhang S, Gao L, Chen Y, Zhang J, Chang X, Zhu M. Upregulation of Wnt5a promotes epithelial-to-mesenchymal transition and metastasis of pancreatic cancer cells. *BMC Cancer* 2013; **13**: 496 [PMID: 24156409 DOI: 10.1186/1471-2407-13-496]

29 **Fiorillo M**, Verre AF, Iliut M, Peiris-Pagés M, Ozsvari B, Gandara R, Cappello AR, Sotgia F, Vijayaraghavan A, Lisanti MP. Graphene oxide selectively targets cancer stem cells, across multiple tumor types: implications for non-toxic cancer treatment, via "differentiation-based nano-therapy". *Oncotarget* 2015; **6**: 3553-3562 [PMID: 25708684]

30 **Grabner B**, Schramek D, Mueller KM, Moll HP, Svinka J, Hoffmann T, Bauer E, Blaas L, Hruschka N, Zboray K, Stiedl P, Nivarthi H, Bogner E, Gruber W, Mohr T, Zwick RH, Kenner L, Poli V, Aberger F, Stoiber D, Egger G, Esterbauer H, Zuber J, Moriggl R, Eferl R, Győrffy B, Penninger JM, Popper H, Casanova E. Disruption of STAT3 signalling promotes KRAS-induced lung tumorigenesis. *Nat Commun* 2015; **6**: 6285 [PMID: 25734337 DOI: 10.1038/ncomms7285]

31 **Ma J**, Cheng J, Gong Y, Tian L, Huang Q. Downregulation of Wnt signaling by sonic hedgehog activation promotes repopulation of human tumor cell lines. *Dis Model Mech* 2015; **8**: 385-391 [PMID: 25713298 DOI: 10.1242/dmm.018887]

32 **Luca VC**, Jude KM, Pierce NW, Nachury MV, Fischer S, Garcia KC. Structural biology. Structural basis for Notch1 engagement of Delta-like 4. *Science* 2015; **347**: 847-853 [PMID: 25700513 DOI: 10.1126/science.1261093]

33 **Airoldi I**, Tupone MG, Esposito S, Russo MV, Barbarito G, Cipollone G, Di Carlo E. Interleukin-27 re-educates intratumoral myeloid cells and down-regulates stemness genes in non-small cell lung cancer. *Oncotarget* 2015; **6**: 3694-3708 [PMID: 25638163]

34 **Narcisi R**, Cleary MA, Brama PA, Hoogduijn MJ, Tüysüz N, ten Berge D, van Osch GJ. Long-term expansion, enhanced chondrogenic potential, and suppression of endochondral ossification of adult human MSCs via WNT signaling modulation. *Stem Cell Reports* 2015; **4**: 459-472 [PMID: 25733021 DOI: 10.1016/j.stemcr.2015.01.017]

35 **Huskey NE**, Guo T, Evason KJ, Momcilovic O, Pardo D, Creasman KJ, Judson RL, Blelloch R, Oakes SA, Hebrok M, Goga A. CDK1 inhibition targets the p53-NOXA-MCL1 axis, selectively kills embryonic stem cells, and prevents teratoma formation. *Stem Cell Reports* 2015; **4**: 374-389 [PMID: 25733019 DOI: 10.1016/j.stemcr.2015.01.019]

36 **Cheng CJ**, Bahal R, Babar IA, Pincus Z, Barrera F, Liu C, Svoronos A, Braddock DT, Glazer PM, Engelman DM, Saltzman WM, Slack FJ. MicroRNA silencing for cancer therapy targeted to the tumour microenvironment. *Nature* 2015; **518**: 107-110 [PMID: 25409146 DOI: 10.1038/nature13905]

37 **Serguienko A**, Grad I, Wennerstrøm AB, Meza-Zepeda LA, Thiede B, Stratford EW, Myklebost O, Munthe E. Metabolic reprogramming of metastatic breast cancer and melanoma by let-7a microRNA. *Oncotarget* 2015; **6**: 2451-2465 [PMID: 25669981]

38 **Vallabhaneni KC**, Penfornis P, Dhule S, Guillonneau F, Adams KV, Mo YY, Xu R, Liu Y, Watabe K, Vemuri MC, Pochampally R. Extracellular vesicles from bone marrow mesenchymal stem/stromal cells transport tumor regulatory microRNA, proteins, and metabolites. *Oncotarget* 2015; **6**: 4953-4967 [PMID: 25669974]

39 **Lee SW**, Yang J, Kim SY, Jeong HK, Lee J, Kim WJ, Lee EJ, Kim HS. MicroRNA-26a induced by hypoxia targets HDAC6 in myogenic differentiation of embryonic stem cells. *Nucleic Acids Res* 2015; **43**: 2057-2073 [PMID: 25662604 DOI: 10.1093/nar/gkv088]

40 **Collet G**, El Hafny-Rahbi B, Nadim M, Tejchman A, Klimkiewicz K, Kieda C. Hypoxia-shaped vascular niche for cancer stem cells. *Contemp Oncol* (Pozn) 2015; **19**: A39-A43 [PMID: 25691820 DOI: 10.5114/wo.2014.47130]

41 **Zeisel A**, Muñoz-Manchado AB, Codeluppi S, Lönnerberg P, La Manno G, Juréus A, Marques S, Munguba H, He L, Betsholtz C, Rolny C, Castelo-Branco G, Hjerling-Leffler J, Linnarsson S. Brain structure. Cell types in the mouse cortex and hippocampus revealed by single-cell RNA-seq. *Science* 2015; **347**: 1138-1142 [PMID: 25700174 DOI: 10.1126/science.aaa1934]

42 **Rafiee MR**, Malekzadeh Shafaroudi A, Rohban S, Khayatzadeh H, Kalhor HR, Mowla SJ. Enrichment of A Rare Subpopulation of miR-302-Expressing Glioma Cells by Serum Deprivation. *Cell J* 2015; **16**: 494-505 [PMID: 25685740]

43 **Chao CC**, Kan D, Lu KS, Chien CL. The role of microRNA-30c in the self-renewal and differentiation of C6 glioma cells. *Stem Cell Res* 2015; **14**: 211-223 [PMID: 25698399 DOI: 10.1016/j.scr.2015.01.008]

44 **Kong SY**, Park MH, Lee M, Kim JO, Lee HR, Han BW, Svendsen CN, Sung SH, Kim HJ. Kuwanon V inhibits proliferation, promotes cell survival and increases neurogenesis of neural stem cells. *PLoS One* 2015; **10**: e0118188 [PMID: 25706719 DOI: 10.1371/journal.pone.0118188]

45 **Prokopi M**, Kousparou CA, Epenetos AA. The Secret Role of microRNAs in Cancer Stem Cell Development and Potential Therapy: A Notch-Pathway Approach. *Front Oncol* 2015; **4**: 389 [PMID: 25717438 DOI: 10.3389/fonc.2014.00389]

46 **Trimarchi T**, Bilal E, Ntziachristos P, Fabbri G, Dalla-Favera R, Tsirigos A, Aifantis I. Genome-wide mapping and characterization of Notch-regulated long noncoding RNAs in acute leukemia. *Cell* 2014; **158**: 593-606 [PMID: 25083870 DOI: 10.1016/j.cell.2014.05.049]

47 **Flynn RA**, Chang HY. Long noncoding RNAs in cell-fate programming and reprogramming. *Cell Stem Cell* 2014; **14**: 752-761 [PMID: 24905165 DOI: 10.1016/j.stem.2014.05.014]

48 **El-Badawy A**, El-Badri N. Regulators of pluripotency and their implications in regenerative medicine. *Stem Cells Cloning* 2015; **8**: 67-80 [PMID: 25960670 DOI: 10.2147/SCCAA.S80157]

49 **Kumar S**, Kim CW, Son DJ, Ni CW, Jo H. Flow-dependent regulation of genome-wide mRNA and microRNA expression in endothelial cells in vivo. *Sci Data* 2014; **1**: 140039 [PMID: 25977794 DOI: 10.1038/sdata.2014.39]

50 **Tanabe S**. Origin of cells and network information. *World J Stem Cells* 2015; **7**: 535-540 [PMID: 25914760 DOI: 10.4252/wjsc.v7.i3.535]

51 **Tanabe S**, Aoyagi K, Yokozaki H, Sasaki H. Regulated genes in mesenchymal stem cells and gastric cancer. *World J Stem Cells* 2015; **7**: 208-222 [PMID: 25621121 DOI: 10.4252/wjsc.v7.i1.208]

52 **Yu KR**, Shin JH, Kim JJ, Koog MG, Lee JY, Choi SW, Kim HS, Seo Y, Lee S, Shin TH, Jee MK, Kim DW, Jung SJ, Shin S, Han DW, Kang KS. Rapid and Efficient Direct Conversion of Human Adult Somatic Cells into Neural Stem Cells by HMGA2/let-7b.

Cell Rep 2015 Jan 15; Epub ahead of print [PMID: 25600877 DOI: 10.1016/j.celrep.2014.12.038]

53 **Nori S**, Okada Y, Nishimura S, Sasaki T, Itakura G, Kobayashi Y, Renault-Mihara F, Shimizu A, Koya I, Yoshida R, Kudoh J, Koike M, Uchiyama Y, Ikeda E, Toyama Y, Nakamura M, Okano H. Long-term safety issues of iPSC-based cell therapy in a spinal cord injury model: oncogenic transformation with epithelial-mesenchymal transition. *Stem Cell Reports* 2015; **4**: 360-373 [PMID: 25684226 DOI: 10.1016/j.stemcr.2015.01.006]

54 **Tateno H**, Onuma Y, Ito Y, Minoshima F, Saito S, Shimizu M, Aiki Y, Asashima M, Hirabayashi J. Elimination of tumorigenic human pluripotent stem cells by a recombinant lectin-toxin fusion protein. *Stem Cell Reports* 2015; **4**: 811-820 [PMID: 25866158 DOI: 10.1016/j.stemcr.2015.02.016]

55 **Curry EL**, Moad M, Robson CN, Heer R. Using induced pluripotent stem cells as a tool for modelling carcinogenesis. *World J Stem Cells* 2015; **7**: 461-469 [PMID: 25815129 DOI: 10.4252/wjsc.v7.i2.461]

56 **Wu T**, Pinto HB, Kamikawa YF, Donohoe ME. The BET family member BRD4 interacts with OCT4 and regulates pluripotency gene expression. *Stem Cell Reports* 2015; **4**: 390-403 [PMID: 25684227 DOI: 10.1016/j.stemcr.2015.01.012]

57 **Lachmann N**, Ackermann M, Frenzel E, Liebhaber S, Brennig S, Happle C, Hoffmann D, Klimenkova O, Lüttge D, Buchegger T, Kühnel MP, Schambach A, Janciauskiene S, Figueiredo C, Hansen G, Skokowa J, Moritz T. Large-scale hematopoietic differentiation of human induced pluripotent stem cells provides granulocytes or macrophages for cell replacement therapies. *Stem Cell Reports* 2015; **4**: 282-296 [PMID: 25680479 DOI: 10.1016/j.stemcr.2015.01.005]

58 **Liu YJ**, Le Berre M, Lautenschlaeger F, Maiuri P, Callan-Jones A, Heuzé M, Takaki T, Voituriez R, Piel M. Confinement and low adhesion induce fast amoeboid migration of slow mesenchymal cells. *Cell* 2015; **160**: 659-672 [PMID: 25679760 DOI: 10.1016/j.cell.2015.01.007]

59 **Tanabe S**, Aoyagi K, Yokozaki H, Sasaki H. Gene expression signatures for identifying diffuse-type gastric cancer associated with epithelial-mesenchymal transition. *Int J Oncol* 2014; **44**: 1955-1970 [PMID: 24728500 DOI: 10.3892/ijo.2014.2387]

60 **Dixon JR**, Jung I, Selvaraj S, Shen Y, Antosiewicz-Bourget JE, Lee AY, Ye Z, Kim A, Rajagopal N, Xie W, Diao Y, Liang J, Zhao H, Lobanenkov VV, Ecker JR, Thomson JA, Ren B. Chromatin architecture reorganization during stem cell differentiation. *Nature* 2015; **518**: 331-336 [PMID: 25693564 DOI: 10.1038/nature14222]

61 **Kundaje A**, Meuleman W, Ernst J, Bilenky M, Yen A, Heravi-Moussavi A, Kheradpour P, Zhang Z, Wang J, Ziller MJ, Amin V, Whitaker JW, Schultz MD, Ward LD, Sarkar A, Quon G, Sandstrom RS, Eaton ML, Wu YC, Pfenning AR, Wang X, Claussnitzer M, Liu Y, Coarfa C, Harris RA, Shoresh N, Epstein CB, Gjoneska E, Leung D, Xie W, Hawkins RD, Lister R, Hong C, Gascard P, Mungall AJ, Moore R, Chuah E, Tam A, Canfield TK, Hansen RS, Kaul R, Sabo PJ, Bansal MS, Carles A, Dixon JR, Farh KH, Feizi S, Karlic R, Kim AR, Kulkarni A, Li D, Lowdon R, Elliott G, Mercer TR, Neph SJ, Onuchic V, Polak P, Rajagopal N, Ray P, Sallari RC, Siebenthall KT, Sinnott-Armstrong NA, Stevens M, Thurman RE, Wu J, Zhang B, Zhou X, Beaudet AE, Boyer LA, De Jager PL, Farnham PJ, Fisher SJ, Haussler D, Jones SJ, Li W, Marra MA, McManus MT, Sunyaev S, Thomson JA, Tlsty TD, Tsai LH, Wang W, Waterland RA, Zhang MQ, Chadwick LH, Bernstein BE, Costello JF, Ecker JR, Hirst M, Meissner A, Milosavljevic A, Ren B, Stamatoyannopoulos JA, Wang T, Kellis M. Integrative analysis of 111 reference human epigenomes. *Nature* 2015; **518**: 317-330 [PMID: 25693563 DOI: 10.1038/nature14248]

62 **Hasegawa T**, Sakamoto A, Wada A, Fukai T, Iida H, Ikeda S. Keratinocyte progenitor cells reside in human subcutaneous adipose tissue. *PLoS One* 2015; **10**: e0118402 [PMID: 25714344 DOI: 10.1371/journal.pone.0118402]

63 **Ross J**, Busch J, Mintz E, Ng D, Stanley A, Brafman D, Sutton VR, Van den Veyver I, Willert K. A rare human syndrome provides genetic evidence that WNT signaling is required for reprogramming of fibroblasts to induced pluripotent stem cells. *Cell Rep* 2014; **9**: 1770-1780 [PMID: 25464842 DOI: 10.1016/j.celrep.2014.10.049]

64 **Zunder ER**, Lujan E, Goltsev Y, Wernig M, Nolan GP. A continuous molecular roadmap to iPSC reprogramming through progression analysis of single-cell mass cytometry. *Cell Stem Cell* 2015; **16**: 323-337 [PMID: 25748935 DOI: 10.1016/j.stem.2015.01.015]

65 **Hayashi Y**, Caboni L, Das D, Yumoto F, Clayton T, Deller MC, Nguyen P, Farr CL, Chiu HJ, Miller MD, Elsliger MA, Deacon AM, Godzik A, Lesley SA, Tomoda K, Conklin BR, Wilson IA, Yamanaka S, Fletterick RJ. Structure-based discovery of NANOG variant with enhanced properties to promote self-renewal and reprogramming of pluripotent stem cells. *Proc Natl Acad Sci USA* 2015; **112**: 4666-4671 [PMID: 25825768 DOI: 10.1073/pnas.1502855112]

66 **Sukmawati D**, Fujimura S, Jitsukawa S, Ito-Hirano R, Ishii T, Sato T, Hayashi A, Itoh S, Mizuno H, Daida H, Tanaka R. Oxidative stress tolerance of early stage diabetic endothelial progenitor cell. *Regenerative Therapy* 2015; **1**: 38-44 [DOI: 10.1016/j.reth.2014.11.001]

67 **Akutsu H**, Machida M, Kanzaki S, Sugawara T, Ohkura T, Nakamura N, Yamazaki-Inoue M, Miura T, Vemuri MC, Rao MS, Miyado K, Umezawa A. Xenogeneic-free defined conditions for derivation and expansion of human embryonic stem cells with mesenchymal stem cells. *Regenerative Therapy* 2015; **1**: 18-29 [DOI: 10.1016/j.reth.2014.12.004]

P- Reviewer: Li SC, Maioli NA, Saeki K, Teresa Valenti M
S- Editor: Ji FF **L- Editor**: A **E- Editor**: Wu HL

WJSC
World Journal of
Stem Cells

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4252/wjsc.v7.i7.999

World J Stem Cells 2015 August 26; 7(7): 999-1009
ISSN 1948-0210 (online)
© 2015 Baishideng Publishing Group Inc. All rights reserved.

REVIEW

Stem cell guidance through the mechanistic target of rapamycin

Kenneth Maiese

Kenneth Maiese, Cellular and Molecular Signaling, Newark, NJ 07101, United States

Author contributions: Maiese K conceived, designed, and wrote this article.

Supported by The following grants to Kenneth Maiese: American Diabetes Association, American Heart Association, NIH NIEHS, NIH NIA, NIH NINDS, and NIH ARRA.

Conflict-of-interest statement: The author declares no conflicts of interest.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: http://creativecommons.org/licenses/by-nc/4.0/

Correspondence to: Kenneth Maiese, MD, Cellular and Molecular Signaling, 125 Main Street, Newark, NJ 07101, United States. wntin75@yahoo.com

Received: May 19, 2015
Peer-review started: May 20, 2015
First decision: June 18, 2015
Revised: June 29, 2015
Accepted: July 16, 2015
Article in press: July 18, 2015
Published online: August 26, 2015

Abstract

Stem cells offer great promise for the treatment of multiple disorders throughout the body. Critical to this premise is the ability to govern stem cell pluripotency, proliferation, and differentiation. The mechanistic target of rapamycin (mTOR), 289-kDa serine/threonine protein kinase, that is a vital component of mTOR Complex 1 and mTOR Complex 2 represents a critical pathway for the oversight of stem cell maintenance. mTOR can control the programmed cell death pathways of autophagy and apoptosis that can yield variable outcomes in stem cell survival and be reliant upon proliferative pathways that include Wnt signaling, Wnt1 inducible signaling pathway protein 1 (WISP1), silent mating type information regulation 2 homolog 1 (*Saccharomyces cerevisiae*) (SIRT1), and trophic factors. mTOR also is a necessary component for the early development and establishment of stem cells as well as having a significant impact in the regulation of the maturation of specific cell phenotypes. Yet, as a proliferative agent, mTOR can not only foster cancer stem cell development and tumorigenesis, but also mediate cell senescence under certain conditions to limit invasive cancer growth. mTOR offers an exciting target for the oversight of stem cell therapies but requires careful consideration of the diverse clinical outcomes that can be fueled by mTOR signaling pathways.

Key words: Apoptosis; Autophagy; Cancer; Cardiovascular; Erythropoietin; Mechanistic target of rapamycin; Neurodegeneration; Progenitor stem cells; Silent mating type information regulation 2 homolog; Wnt1 inducible signaling pathway; Wnt

© The Author(s) 2015. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Mechanistic target of rapamycin, the mechanistic target of rapamycin, can directly impact stem cell maintenance, proliferation, and differentiation to offer new therapeutic strategies for multiple disease entities.

Maiese K. Stem cell guidance through the mechanistic target of rapamycin. *World J Stem Cells* 2015; 7(7): 999-1009 Available from: URL: http://www.wjgnet.com/1948-0210/full/v7/i7/999.htm DOI: http://dx.doi.org/10.4252/wjsc.v7.i7.999

MECHANISTIC TARGET OF RAPAMYCIN SIGNALING

The mechanistic target of rapamycin (mTOR) is a

289-kDa serine/threonine protein kinase that is encoded by a single gene *FRAP1*[1,2]. mTOR, also known as the mammalian target of rapamycin and the FK506-binding protein 12-rapamycin complex-associated protein 1, oversees a complex array of cellular functions that involve gene transcription, cellular proliferation, senescence, metabolism, survival, and cellular death. The target of rapamycin (TOR) was initially identified in *Saccharomyces cerevisiae* with the genes *TOR1* and *TOR2* that encode two isoforms in yeast Tor1 and Tor2 through the use of rapamycin-resistant TOR mutants[3]. Rapamycin is a macrolide antibiotic derived from *Streptomyces hygroscopicus* that that can inhibit TOR as well as mTOR activity.

mTOR is a vital component for the function of the protein complexes mTOR Complex 1 (mTORC1) and mTOR Complex 2 (mTORC2) (Figure 1)[4-7]. Rapamycin primarily inhibits mTORC1 by blocking mTOR phosphorylation[8]. However, mTORC2 activity can be limited during chronic administration of rapamycin. mTORC1 is composed of Raptor (Regulatory-Associated Protein of mTOR), the proline rich Akt substrate 40 kDa (PRAS40), Deptor (DEP domain-containing mTOR interacting protein), and mLST8/GβL (mammalian lethal with Sec13 protein 8, termed mLST8). Phosphorylation of Raptor through the protein Ras homologue enriched in brain (Rheb) leads to mTORC1 activation. PRAS40 is inhibitory to mTOR activity and can prevent the binding of mTORC1 to Raptor[9]. Phosphorylation of PRAS40 by protein kinase B (Akt) frees PRAS40 from Raptor and allows PRAS40 to be sequestered by the cytoplasmic docking protein 14-3-3 to activate mTORC1[4-7]. Similar to PRAS40, Deptor inhibits mTORC1 activity through the binding of the FAT domain of mTOR (for FKBP associated protein, Ataxia-telangiectasia, and Transactivation/transformation domain-associated protein). In contrast to PRAS40 and Deptor, mLST8 fosters mTOR kinase activity through p70 ribosomal S6 kinase (p70S6K) and the eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4EBP1) that bind to Raptor[10]. PRAS40 can block mTORC1 activity by preventing p70S6K and 4EBP1 to associate with Raptor[9,11].

mTOR activity also is controlled by Akt and AMP activated protein kinase (AMPK) through the hamartin (tuberous sclerosis 1)/tuberin (tuberous sclerosis 2) (TSC1/TSC2) complex (Figure 1)[12,13]. TSC2 is considered to be a principal site to govern the activity of the TSC1/TSC2 complex that is an inhibitor of mTORC1. As a GTPase-activating protein (GAP) that can convert Ras homologue enriched in brain (Rheb-GTP) to the inactive GDP-bound form (Rheb-GDP), TSC2 prevents the activity of Rheb-GTP and blocks mTORC1 activity by limiting binding of 4EBP1 to mTORC1. Akt can phosphorylate TSC2 to disrupt the TSC1/TSC2 complex, force TSC2 to be sequestered by the cytoplasmic protein 14-3-3, and activate mTORC1[14]. It should be noted that under some cellular protection scenarios, a limited activity of TSC2 as well as AMPK appears necessary since complete knockdown of TSC2 can prevent cellular protection[15].

AMPK also provides a mechanism to control the activity of the TSC1/TSC2 complex, but in contrast to Akt serves to promote TSC2 activity and block mTORC1 function. AMPK phosphorylates TSC2 to enhance GAP activity to process Rheb-GTP into Rheb-GDP that can then block mTORC1 activity. Interestingly, AMPK can influence sirtuin (silent mating type information regulation 2 homolog) 1 (*S. cerevisiae*) (SIRT1) activity that can be critical for stem cell survival and proliferation[16]. AMPK increases the cellular NAD^+/NADH ratio that results in the deacetylation of the SIRT1 targets peroxisome proliferator-activated receptor-gamma coactivator 1 (PGC-1α) and forkhead transcription factors FoxO1[17] and FoxO3a[18]. AMPK also can increase nicotinamide phosphoribosyltransferase (NAMPT) activity that catalyzes the conversion of nicotinamide to nicotinamide mononucleotide[19], increases nicotinamide adenine dinucleotide (NAD^+) levels, decreases levels of the SIRT1 inhibitor nicotinamide, and promotes SIRT1 transcription[20-22]. SIRT1 up-regulation in combination with AMPK activation promotes the induction of autophagy that can protect endothelial cells exposed to oxidized low-density lipoproteins[23]. Similar to AMPK that is an inhibitor of mTOR, SIRT1 appears to exert its effects over cellular proliferation through blockade of mTOR[24]. SIRT1 inhibits mTOR activity to preserve the integrity of embryonic stem cells during oxidant stress[25]. SIRT1 also inhibits mTOR signaling to foster neuronal growth[26] and assist with mesangial cell proliferation during high glucose exposure[27].

In relation to mTORC2, this complex consists of Rictor (Rapamycin-Insensitive Companion of mTOR), mLST8, Deptor, the mammalian stress-activated protein kinase interacting protein (mSIN1), and the protein observed with Rictor-1 (Protor-1)[28-33]. Rictor and mSIN1 through mTORC2 can activate Akt to promote cell survival[11,29,34]. Protor-1 is a Rictor-binding subunit of mTORC2 and is believed to activate serum and glucocorticoid induced protein kinase 1 (SGK1), since loss of Protor-1 reduces the hydrophobic motif phosphorylation of SGK1 and the substrate N-Myc down-regulated gene 1 in the kidney (NRDG1)[35]. mTORC2 is a member of the protein kinase A/protein kinase G/protein kinase C (AGC) family of protein kinases and is activated by growth factors to control ion transport. mTORC2 also controls cytoskeleton remodeling through protein kinase C-α (PKCα) and oversees cell migration through the Rac guanine nucleotide exchange factors P-Rex1 and P-Rex2 and through Rho signaling. In contrast to mTORC1, mTORC2 is activated by the TSC1/TSC2 complex through the N-terminal region of TSC2 and the C-terminal region of Rictor[36].

MECHANISTIC TARGET OF RAPAMYCIN AND STEM CELL PROGRAMMED CELL DEATH

mTOR is an important component in the control of

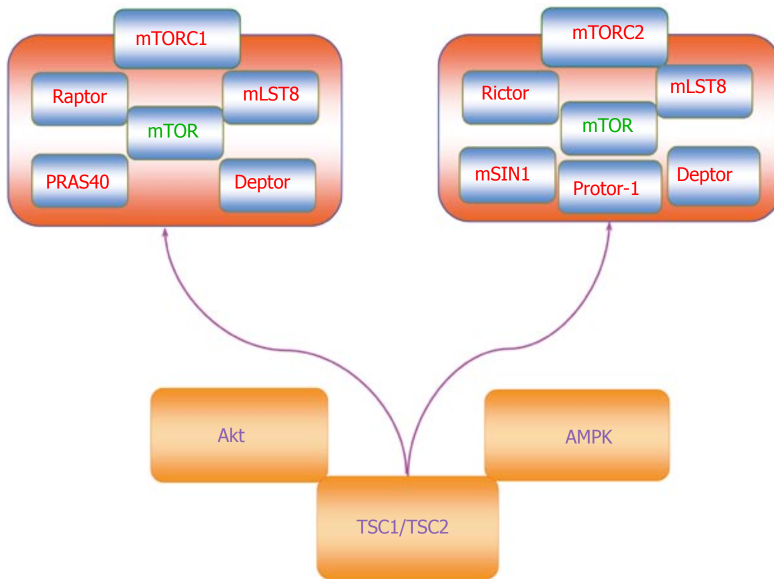


Figure 1 The components of the mechanistic target of rapamycin regulatory pathways. The mechanistic target of rapamycin (mTOR) is an important component of mTOR Complex 1 (mTORC1) and mTOR Complex 2 (mTORC2). The function and activity of mTOR is controlled by multiple pathways that include protein kinase B (Akt), AMP activated protein kinase (AMPK), and the hamartin (tuberous sclerosis 1)/tuberin (tuberous sclerosis 2) (TSC1/TSC2) complex. mTORC1 is consists of Raptor (Regulatory-Associated Protein of mTOR), the proline rich Akt substrate 40 kDa (PRAS40), Deptor (DEP domain-containing mTOR interacting protein), and mLST8/GβL (mammalian lethal with Sec13 protein 8, termed mLST8). mTORC2 is composed of Rictor (Rapamycin-Insensitive Companion of mTOR), mLST8, Deptor, the mammalian stress-activated protein kinase interacting protein (mSIN1), and the protein observed with Rictor-1 (Protor-1).

programmed cell death that involves autophagy and apoptosis for stem cell proliferation and survival (Figure 2). The process of autophagy recycles cytoplasmic components to remove defective organelles that can no longer be used by the cell[37]. Autophagy has three categories of chaperone-mediated autophagy, microautophagy, and macroautophagy[38]. Chaperone-mediated autophagy uses cytosolic chaperones that transport cytoplasmic components across lysosomal membranes[39]. Microautophagy sequesters components of the cytoplasm through invagination of the lysosomal membrane for digestion[40]. The most prevalent of the three categories is macroautophagy that sequesters cytoplasmic proteins and organelles into autophagosomes. These autophagosomes then fuse with lysosomes for degradation and are recycled for future use[24,41].

In yeast and mammalians, TOR and mTOR are associated with genes that control autophagy[4,6,42]. At least 33 autophagic related genes (*Atg*) have been identified in yeast that can affect multiple disorders including cancer, diabetes, vascular disease, and neurodegenerative disorders[37,38,40,43-48]. In this group, Atg1 and Atg13 (also known as Apg13) are associated with phosphoinositide 3-kinase (PI 3-K), Akt, and the TOR pathways. When Atg13 is dephosphorylated such as during starvation, Atg1 is active to promote autophagy. Phosphorylation of Atg13 through a TOR dependent pathway releases it from Atg1 and lessens Atg1 activity. In mammals, the homologues of Atg1 are UNC-51 like kinase 1 (ULK1) and ULK2[4]. Mammalian Atg13 binds to ULK1, ULK2, and FIP200 (focal adhesion kinase family interacting protein of 200 kDa) to activate ULKs, promote phosphorylation of FIP200 by ULKs, and lead to the induction of autophagy[49]. Activation of mTOR prevents the induction of autophagy by phosphorylating Atg13 and ULKs to inhibit the ULK-Atg13-FIP200 complex.

Autophagy can become a vital determinant for stem cell survival and proliferation. In some stem cell populations, activation of autophagy can lead to stem cell demise. Breast cancer stem cells have been shown to succumb to apoptosis during the activation of autophagy and the inhibition of Wnt signaling[46]. Wnt proteins are cysteine-rich glycosylated proteins that oversee stem cell proliferation and tumor cell growth[50-56]. Reduction in autophagy also may prevent the development of cellular senescence[57]. Endothelial progenitor cells that lead to the regeneration of vascular endothelium become dysfunctional during exposure to elevated glucose as a result of autophagy activity[58].

However, under other conditions, autophagy appears critical for stem cell survival. In endothelial progenitor cells, SIRT1 activity prevents apoptotic cell death during oxidative stress through the induction of autophagy[59]. In human embryonic stem cells challenged with oxidative stress, autophagy was found to be protective and required SIRT1 activity with the down-regulation of mTOR[25]. Furthermore, activation of SIRT1 is necessary to promote autophagy to maintain proteostasis, produce energy during nutrient deprivation, and maintain muscle stem cell activation[60]. In such cases, SIRT1 may have an inverse relationship with mTOR to foster stem cell survival[16,20].

The programmed cell death pathway of apoptosis also has an important role with mTOR signaling and

Figure 2 Mechanistic target of rapamycin governs stem cell development, pluripotency, survival, and differentiation. The mechanistic target of rapamycin (mTOR), mTOR Complex 1 (mTORC1), mTOR Complex 2 (mTORC2), protein kinase B (Akt), AMP activated protein kinase (AMPK), and the hamartin (tuberous sclerosis 1)/tuberin (tuberous sclerosis 2) (TSC1/TSC2) complex each play a role in stem cell development and proliferation with downstream pathways. Stem cell maintenance and survival is reliant upon mTOR signaling pathways that work in concert with Wnt signaling, Wnt1 inducible signaling pathway protein 1 (WISP1), silent mating type information regulation 2 homolog) 1 (*S. cerevisiae*) (SIRT1), and trophic factors such as erythropoietin (EPO). Stem cell development is under the regulation of Wnt and WISP1. Regulation of mTOR can control stem cell differentiation and stem cell pluripotency. As a result, mTOR signaling pathways have oversight of stem cell development, pluripotency, survival, and differentiation.

stem cell survival[61]. During the early stages of apoptotic cell injury, the loss of plasma membrane lipid phosphatidylserine (PS) asymmetry occurs[62-64]. If membrane PS externalization is not reversed and allowed to remain, inflammatory cells are activated that seek out membrane PS positive cells to engulf and remove these cells. Under such circumstances, these membrane PS positive cells may remain functional but their ultimate loss leads to tissue injury[65-71]. During the later phase of apoptotic cell injury, cellular DNA is destroyed which is usually not considered a reversible process[72-75]. During most conditions, activation of mTOR and its related pathways of PI 3-K and Akt can block apoptotic cell death in stem cells. Inhibition of mTOR, such as with rapamycin, leads to endothelial progenitor cell apoptotic death that may be related to inhibition of growth factor signaling[76]. Growth factors that include erythropoietin (EPO)[77,78] are cytoprotective against apoptosis through mTOR activity against sepsis-associated encephalopathy[79], oxidative stress[80], cerebral microglial injury[81], and beta-amyloid (Aβ) toxicity[82]. EPO has been shown to protect retinal progenitor cells from apoptotic cell death during oxidative stress through activation of the PI 3-K, Akt, and mTOR pathways (Figure 2)[83]. Interestingly, protection with EPO and mTOR may be lost with high exogenous EPO concentrations, since elevated concentrations of EPO result in decreased phosphorylation and activity of mTOR with subsequent increased apoptotic cell death[84]. Similar to EPO, other growth factors rely upon mTOR to maintain stem cell integrity (Figure 2). In murine experimental models, mTOR is used by the growth factors epidermal growth factor (EGF) and fibroblast growth factor (FGF) that are protective of stem cells and neurons[85-89] to maintain the proliferation of neural stem and progenitor cells[90]. EGF also uses the PI 3-K, Akt, and mTOR pathways to block cell injury such as during metabolic stress[91] and prevent memory impairment[92]. The growth factor brain derived neurotrophic factor (BDNF) relies upon mTOR activation for memory consolidation[93]. However, in some experimental models with growth factors, mTOR blockade with the induction of autophagy may take precedent over the inhibition of apoptosis to prevent cellular injury. During oxygen deprivation, cortical neurons are protected by BDNF through the induction of autophagy and the inhibition of mTOR[94].

MECHANISTIC TARGET OF RAPAMYCIN CONTROL OF STEM CELL PROLIFERATION AND MAINTENANCE

mTOR governs the proliferation and maintenance of stem cell in multiple systems of the body (Figure 2)[4]. The loss of the *mTOR* gene leads to limited trophoblast growth, faulty implantation, and inability to establish embryonic stem cells[95]. A decrease in proliferation of embryonic stem cells occurs during the deletion of the C-terminal six amino acids of mTOR that blocks the kinase activity of mTOR[96]. mTOR can maintain long-term undifferentiated growth of human embryonic

stem cells. Inhibition of mTOR promotes pluripotency, cell proliferation, and blocks mesoderm and endoderm activities in embryonic stem cells[97]. mTOR activity also leads to mesenchymal stem cell senescence[98]. Yet, under some conditions, activation of mTOR signaling components can lead to cell differentiation. In embryonic stem cells, mTOR signaling with p70S6K is limited, but once this signaling is increased, differentiation ensues[99].

In the nervous system, loss of mTORC1 activity in neural stem cells leads to reduced lineage expansion, prevention of differentiation, and blocked neuronal production[100]. Loss of mTOR activity during aging may influence decreased neurogenesis. In the aged brain, mTOR signaling is reduced which leads to a reduction in the proliferation of active neural stem cells[101]. mTOR activity appears important for the timing and control of neurogenesis. Inhibition of mTOR through the RTP801/REDD1 pathway delays neuronal differentiation. However, in newborn and mature neurons, levels of RTP801/REDD1 are diminished with increased mTOR activity to allow for the maturation of neurons[102]. Expression of mTOR is necessary for the neuronal phenotype of post mortem neuronal precursors[103]. Yet, the degree of mTOR activity may independently affect different populations of stem cells since in this model inhibition of mTOR activity leads to cell differentiation into astrocytic cells[90]. Akt and mTORC1 inhibition also has been shown to result in reduced neuronal stem cell self-renewal and earlier neuronal and astroglial differentiation[104]. Neighboring cells also may influence the growth of neuronal stem cells. Endothelial cells can promote mTOR activity and lead to the expansion of long-term glioblastoma stem-like cells[105].

In the cardiovascular system, mTOR is one of several components necessary for the proliferation of human embryonic stem cell-derived cardiomyocytes[106]. The activity of mTOR also controls the proliferation of hematopoietic stem and progenitor cells[107]. Maintenance of hematopoietic stem cells and inhibiting differentiation is tied to mTOR signaling and the reduction in phosphorylation of p70S6K[108]. Failure of endothelial progenitor cell development may be the result of decreased growth factor signaling and loss of mTOR activity[76]. Growth factors such as EPO have been shown to require mTOR activation to regulate bone homeostasis with osteoblastogenesis and osteoclastogenesis[109]. Differentiation of neural precursor cells that may be used for neurodegenerative disorders also is dependent upon EPO and mTOR[103]. mTOR may be necessary to increase angiogenesis from endothelial progenitor cells that may provide neuroprotection during cerebral ischemia[110]. The ability of human amniotic fluid stem cells to influence the differentiation of embryonic kidney cells is dependent upon mTOR activity[111].

During tumorigenesis, mTOR activation may affect neural precursor and oligodendroglial precursor cells to promote high-grade glioma proliferation[112]. Blockade of mTOR can prevent the conversion of astrocytoma cells to oligodendroglioma cells that can lead to glioblastoma multiforme[113]. Inhibition of mTOR signaling may reduce the population of cancer stem cells that can lead to disease recurrence and therapeutic resistance[114].

Under some conditions, mTOR may be protective against tumor cell growth by inhibiting proliferative pathways of Wnt. Wnt signaling can lead to rapid cell proliferation and cancerous growth, but in epithelial stem cells this process is blocked by mTOR that maintains cell senescence and prevents tumor growth[115]. Wnt may result in malignant melanoma[116], metastatic disease[117-120], and glioma proliferation[121,122]. It should be recognized that Wnt signaling also leads to beneficial and cytoprotective effects[52,54,123-125]. Loss of Wnt can result in human monocyte injury[126], impairment in bone repair[127], spinal cord injury[125], loss of neurogenesis[128], inhibition of wound healing[129], loss of stem cell differentiation[130], programmed cell death[38,66,131], and defects in placental development[132]. Wnt signaling activation can block inflammatory cell loss during neurodegenerative disorders[66,70,82,133], prevent cerebral ischemia[134,135], and protect cells during experimental diabetes[136-138]. Furthermore, trophic factors employ cytoprotective pathways of Wnt to prevent cerebral endothelial cell injury[137], preserve mesenchymal stem cells[139], block apoptosis during forkhead transcription factor activation[136,140], promote the maintenance of immune cells in the nervous system[81], and prevent Aβ toxicity in cerebral microglia[82]. However, prolonged exposure of growth factors such as EPO that rely upon Wnt signaling can have ill effects with the proliferation of cancer[141-143], inflammation, and blood-brain barrier injury[144].

In the Wnt signaling pathway, Wnt1 inducible signaling pathway protein 1 (WISP1), also known as CCN4, is a member of the six secreted extracellular matrix associated CCN family of proteins that are involved in cellular survival and stem cell proliferation[145]. WISP1 can activate the components of the mTOR pathway that determine stem cell survival (Figure 2)[21]. WISP1 increases mTOR activity by blocking the inhibitory actions of the mTOR component PRAS40[146]. WISP1 controls the post-translational phosphorylation of AMP activated protein kinase (AMPK), a pathway involved in stem cell proliferation and cellular metabolism[12,147]. WISP1 differentially decreases phosphorylation of TSC2 at Ser[1387], a target of AMPK, and increases phosphorylation of TSC2 at Thr[1462], a target of Akt[15].

As a tightly linked pathway to mTOR, WISP1 can significantly influence stem cell survival and proliferation. During stem cell migration, WISP1 expression is increased[148]. WISP1 is differentially regulated during human embryonic stem cell and adipose-derived stem cell differentiation. WISP1 expression is increased during human adipocyte differentiation[149] and is repressed in adipose-derived stem cells during hepatic differentiation[51]. WISP1 can modulate induced pluripotent stem cell reprogramming[150,151]. WISP1 is

one of several genes that are over-expressed during pancreatic regeneration[152]. WISP1 also may support vascular regeneration during saphenous vein crush injury[153]. WISP1 oversees cellular senescence[154] and does not appear to foster excessive cellular proliferation under circumstances involving aging vascular cells[155]. However, as a proliferative agent, WISP1 can lead to excessive cell growth. WISP1 may promote distant metastatic disease[156] and WISP1 expression is increased in neurofibromatosis type 1 tumorigenesis[157]. Variants of WISP1 can be extremely aggressive in promoting cell growth[158] in comparison to non-variant WISP1 expression that may be protective under specific scenarios and block tumor cell invasion, motility, and metastases[159].

FUTURE CONSIDERATIONS

Stem cells represent an important strategy as well as a vital experimental tool for the treatment of multiple disorders that can affect diverse systems of the body that include the brain, cardiovascular system, metabolism, and tumor cell growth. mTOR, a 289-kDa serine/threonine protein kinase and a critical component for the protein complexes of mTORC1 and mTORC2, oversees cellular development, proliferation, and senescence that can directly impact stem cell maintenance, proliferation, and differentiation.

Although mTOR is a highly attractive target to control stem cell maintenance and differentiation, the complexity of this system raises a number of considerations. How does mTOR interface with programmed cell death pathways that can directly affect stem cell populations? mTOR regulates the programmed cell death pathways of autophagy and apoptosis that have a complex outcome in stem cell survival. Through the modulation of Wnt signaling, activation of autophagy that necessitates inhibition of mTOR can block breast cancer stem cell growth. Yet, activation of autophagy that may work in concert with SIRT1 has been shown to play a vital role to maintain muscle stem cell activation and the protection of endothelial progenitor cells. Apoptosis that consists of both an early stage with membrane PS externalization and a late stage involving the destruction of genomic DNA usually relies upon activation of mTOR and its related pathways of PI 3-K, Akt, and growth factors such as EPO, EGF, FGF, and BDNF to block apoptotic cell death in stem cells. However, during some toxic environments, stem cells that become differentiated may require the induction of autophagy with mTOR inhibition to prevent apoptotic cell death.

Another consideration for mTOR is its variable role in the maintenance of stem cell populations and the eventual differentiation of cells into specific phenotypes. mTOR is necessary for trophoblast growth, implantation, the establishment of embryonic stem cells, and the maintenance of pluripotency. Loss of mTOR, such as in the aged brain, may be a factor that results in the reduction of the proliferation of active neural stem cells. Yet, mTOR signaling that can involve p70S6K also affects the modulation of stem cell genesis and cellular differentiation. The activation of mTOR rather that its inhibition can be necessary for stem cell differentiation as well as the ability to selectively promote the maturation of specific cell phenotypes. The control of stem cell development, migration, and proliferation by mTOR can be dependent upon both Wnt and WISP1 signaling.

Ultimately, consideration also must be given for the role mTOR plays to block tumorigenesis and the ability of mTOR signaling to at times accelerate tumor cell growth. Given its proliferative role, mTOR can foster cancer stem cell development and the conversion of differentiated cells into cells that have invasive growth. The degree of mTOR activity may be critical during tumorigenesis, since mTOR in some cell populations can either maintain cell senescence and prevent tumor growth or conversely promote cancer stem cell development that can lead to disease recurrence and therapeutic resistance. By clearly addressing the challenges that lie ahead, targeting mTOR and its signaling pathways offer an exciting approach to translate the development and utilization of stem cells into new therapeutic strategies for multiple disease entities.

REFERENCES

1 **Maiese K**, Chong ZZ, Shang YC, Wang S. mTOR: on target for novel therapeutic strategies in the nervous system. *Trends Mol Med* 2013; **19**: 51-60 [PMID: 23265840 DOI: 10.1016/j.molmed.2012.11.001]

2 **Neasta J**, Barak S, Hamida SB, Ron D. mTOR complex 1: a key player in neuroadaptations induced by drugs of abuse. *J Neurochem* 2014; **130**: 172-184 [PMID: 24666346 DOI: 10.1111/jnc.12725]

3 **Heitman J**, Movva NR, Hall MN. Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. *Science* 1991; **253**: 905-909 [PMID: 1715094]

4 **Chong ZZ**, Shang YC, Wang S, Maiese K. Shedding new light on neurodegenerative diseases through the mammalian target of rapamycin. *Prog Neurobiol* 2012; **99**: 128-148 [PMID: 22980037 DOI: 10.1016/j.pneurobio.2012.08.001]

5 **Gulhati P**, Bowen KA, Liu J, Stevens PD, Rychahou PG, Chen M, Lee EY, Weiss HL, O'Connor KL, Gao T, Evers BM. mTORC1 and mTORC2 regulate EMT, motility, and metastasis of colorectal cancer via RhoA and Rac1 signaling pathways. *Cancer Res* 2011; **71**: 3246-3256 [PMID: 21430067 DOI: 10.1158/0008-5472.can-10-4058]

6 **Maiese K**. Taking aim at Alzheimer's disease through the mammalian target of rapamycin. *Ann Med* 2014; **46**: 587-596 [PMID: 25105207 DOI: 10.3109/07853890.2014.941921]

7 **Zoncu R**, Efeyan A, Sabatini DM. mTOR: from growth signal integration to cancer, diabetes and ageing. *Nat Rev Mol Cell Biol* 2011; **12**: 21-35 [PMID: 21157483]

8 **Sarbassov DD**, Ali SM, Sengupta S, Sheen JH, Hsu PP, Bagley AF, Markhard AL, Sabatini DM. Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. *Mol Cell* 2006; **22**: 159-168 [PMID: 16603397]

9 **Wang H**, Zhang Q, Wen Q, Zheng Y, Lazarovici P, Jiang H, Lin J, Zheng W. Proline-rich Akt substrate of 40kDa (PRAS40): a novel downstream target of PI3k/Akt signaling pathway. *Cell Signal* 2012; **24**: 17-24 [PMID: 21906675 DOI: 10.1016/j.cellsig.2011.08.010]

10 **Kim DH**, Sarbassov DD, Ali SM, Latek RR, Guntur KV,

Erdjument-Bromage H, Tempst P, Sabatini DM. GbetaL, a positive regulator of the rapamycin-sensitive pathway required for the nutrient-sensitive interaction between raptor and mTOR. *Mol Cell* 2003; **11**: 895-904 [PMID: 12718876]
11 **Maiese K**. Driving neural regeneration through the mammalian target of rapamycin. *Neural Regen Res* 2014; **9**: 1413-1417 [PMID: 25317149 DOI: 10.4103/1673-5374.139453]
12 **Chong ZZ**, Maiese K. Mammalian target of rapamycin signaling in diabetic cardiovascular disease. *Cardiovasc Diabetol* 2012; **11**: 45 [PMID: 22545721 DOI: 10.1186/1475-2840-11-45]
13 **Martínez de Morentin PB**, Martinez-Sanchez N, Roa J, Ferno J, Nogueiras R, Tena-Sempere M, Dieguez C, Lopez M. Hypothalamic mTOR: the rookie energy sensor. *Curr Mol Med* 2014; **14**: 3-21 [PMID: 24236459]
14 **Hahn-Windgassen A**, Nogueira V, Chen CC, Skeen JE, Sonenberg N, Hay N. Akt activates the mammalian target of rapamycin by regulating cellular ATP level and AMPK activity. *J Biol Chem* 2005; **280**: 32081-32089 [PMID: 16027121]
15 **Shang YC**, Chong ZZ, Wang S, Maiese K. Tuberous sclerosis protein 2 (TSC2) modulates CCN4 cytoprotection during apoptotic amyloid toxicity in microglia. *Curr Neurovasc Res* 2013; **10**: 29-38 [PMID: 23244622]
16 **Maiese K**. SIRT1 and stem cells: In the forefront with cardiovascular disease, neurodegeneration and cancer. *World J Stem Cells* 2015; **7**: 235-242 [PMID: 25815111 DOI: 10.4252/wjsc.v7.i2.235]
17 **Maiese K**, Chong ZZ, Shang YC, Hou J. FoxO proteins: cunning concepts and considerations for the cardiovascular system. *Clin Sci* (Lond) 2009; **116**: 191-203 [PMID: 19118491]
18 **Cantó C**, Auwerx J. Caloric restriction, SIRT1 and longevity. *Trends Endocrinol Metab* 2009; **20**: 325-331 [PMID: 19713122]
19 **Maiese K**, Chong ZZ, Hou J, Shang YC. The vitamin nicotinamide: translating nutrition into clinical care. *Molecules* 2009; **14**: 3446-3485 [PMID: 19783937]
20 **Chong ZZ**, Shang YC, Wang S, Maiese K. SIRT1: new avenues of discovery for disorders of oxidative stress. *Expert Opin Ther Targets* 2012; **16**: 167-178 [PMID: 22233091 DOI: 10.1517/14728222.2012.648926]
21 **Maies K.** New Insights for Oxidative Stress and Diabetes Mellitus. *Oxid Med Cell Longev* 2015; **2015**: 875961 [PMID: 26064426 DOI: 10.1155/2015/875961]
22 **Wang Y**, Liang Y, Vanhoutte PM. SIRT1 and AMPK in regulating mammalian senescence: a critical review and a working model. *FEBS Lett* 2011; **585**: 986-994 [PMID: 21130086 DOI: 10.1016/j.febslet.2010.11.047]
23 **Jin X**, Chen M, Yi L, Chang H, Zhang T, Wang L, Ma W, Peng X, Zhou Y, Mi M. Delphinidin-3-glucoside protects human umbilical vein endothelial cells against oxidized low-density lipoprotein-induced injury by autophagy upregulation via the AMPK/SIRT1 signaling pathway. *Mol Nutr Food Res* 2014; **58**: 1941-1951 [PMID: 25047736 DOI: 10.1002/mnfr.201400161]
24 **Maiese K**, Chong ZZ, Shang YC, Wang S. Novel directions for diabetes mellitus drug discovery. *Expert Opin Drug Discov* 2013; **8**: 35-48 [PMID: 23092114 DOI: 10.1517/17460441.2013.736485]
25 **Ou X**, Lee MR, Huang X, Messina-Graham S, Broxmeyer HE. SIRT1 positively regulates autophagy and mitochondria function in embryonic stem cells under oxidative stress. *Stem Cells* 2014; **32**: 1183-1194 [PMID: 24449278 DOI: 10.1002/stem.1641]
26 **Guo W**, Qian L, Zhang J, Zhang W, Morrison A, Hayes P, Wilson S, Chen T, Zhao J. Sirt1 overexpression in neurons promotes neurite outgrowth and cell survival through inhibition of the mTOR signaling. *J Neurosci Res* 2011; **89**: 1723-1736 [PMID: 21826702 DOI: 10.1002/jnr.22725]
27 **Zhang S**, Cai G, Fu B, Feng Z, Ding R, Bai X, Liu W, Zhuo L, Sun L, Liu F, Chen X. SIRT1 is required for the effects of rapamycin on high glucose-inducing mesangial cells senescence. *Mech Ageing Dev* 2012; **133**: 387-400 [PMID: 22561310 DOI: 10.1016/j.mad.2012.04.005]
28 **Chong ZZ**, Shang YC, Zhang L, Wang S, Maiese K. Mammalian target of rapamycin: hitting the bull's-eye for neurological disorders. *Oxid Med Cell Longev* 2010; **3**: 374-391 [PMID: 21307646]
29 **Glidden EJ**, Gray LG, Vemuru S, Li D, Harris TE, Mayo MW. Multiple site acetylation of Rictor stimulates mammalian target of rapamycin complex 2 (mTORC2)-dependent phosphorylation of Akt protein. *J Biol Chem* 2012; **287**: 581-588 [PMID: 22084251]
30 **James MF**, Stivison E, Beauchamp R, Han S, Li H, Wallace MR, Gusella JF, Stemmer-Rachamimov AO, Ramesh V. Regulation of mTOR complex 2 signaling in neurofibromatosis 2-deficient target cell types. *Mol Cancer Res* 2012; **10**: 649-659 [PMID: 22426462 DOI: 10.1158/1541-7786.mcr-11-0425-t]
31 **Kamarudin MN**, Mohd Raflee NA, Hussein SS, Lo JY, Supriady H, Abdul Kadir H. (R)-(+)-α-lipoic acid protected NG108-15 cells against H_2O_2-induced cell death through PI3K-Akt/GSK-3β pathway and suppression of NF-κβ-cytokines. *Drug Des Devel Ther* 2014; **8**: 1765-1780 [PMID: 25336920 DOI: 10.2147/ddt.s67980]
32 **Maiese K**. Cutting through the complexities of mTOR for the treatment of stroke. *Curr Neurovasc Res* 2014; **11**: 177-186 [PMID: 24712647]
33 **Tang Z**, Baykal AT, Gao H, Quezada HC, Zhang H, Bereczki E, Serhatli M, Baykal B, Acioglu C, Wang S, Ioja E, Ji X, Zhang Y, Guan Z, Winblad B, Pei JJ. mTor is a signaling hub in cell survival: a mass-spectrometry-based proteomics investigation. *J Proteome Res* 2014; **13**: 2433-2444 [PMID: 24694195 DOI: 10.1021/pr500192g]
34 **Frias MA**, Thoreen CC, Jaffe JD, Schroder W, Sculley T, Carr SA, Sabatini DM. mSin1 is necessary for Akt/PKB phosphorylation, and its isoforms define three distinct mTORC2s. *Curr Biol* 2006; **16**: 1865-1870 [PMID: 16919458]
35 **Pearce LR**, Sommer EM, Sakamoto K, Wullschleger S, Alessi DR. Protor-1 is required for efficient mTORC2-mediated activation of SGK1 in the kidney. *Biochem J* 2011; **436**: 169-179 [PMID: 21413931]
36 **Huang J**, Dibble CC, Matsuzaki M, Manning BD. The TSC1-TSC2 complex is required for proper activation of mTOR complex 2. *Mol Cell Biol* 2008; **28**: 4104-4115 [PMID: 18411301]
37 **Vakifahmetoglu-Norberg H**, Xia HG, Yuan J. Pharmacologic agents targeting autophagy. *J Clin Invest* 2015; **125**: 5-13 [PMID: 25654545 DOI: 10.1172/jci73937]
38 **Maiese K**, Chong ZZ, Shang YC, Wang S. Targeting disease through novel pathways of apoptosis and autophagy. *Expert Opin Ther Targets* 2012; **16**: 1203-1214 [PMID: 22924465 DOI: 10.1517/14728222.2012.719499]
39 **Nakka VP**, Prakash-Babu P, Vemuganti R. Crosstalk Between Endoplasmic Reticulum Stress, Oxidative Stress, and Autophagy: Potential Therapeutic Targets for Acute CNS Injuries. *Mol Neurobiol* 2014 Dec 9; Epub ahead of print [PMID: 25482050 DOI: 10.1007/s12035-014-9029-6]
40 **Chen W**, Sun Y, Liu K, Sun X. Autophagy: a double-edged sword for neuronal survival after cerebral ischemia. *Neural Regen Res* 2014; **9**: 1210-1216 [PMID: 25206784 DOI: 10.4103/1673-5374.135329]
41 **Dello Russo C**, Lisi L, Feinstein DL, Navarra P. mTOR kinase, a key player in the regulation of glial functions: relevance for the therapy of multiple sclerosis. *Glia* 2013; **61**: 301-311 [PMID: 23044764 DOI: 10.1002/glia.22433]
42 **Peng N**, Meng N, Wang S, Zhao F, Zhao J, Su L, Zhang S, Zhang Y, Zhao B, Miao J. An activator of mTOR inhibits oxLDL-induced autophagy and apoptosis in vascular endothelial cells and restricts atherosclerosis in apolipoprotein $E^{-/-}$ mice. *Sci Rep* 2014; **4**: 5519 [PMID: 24980430 DOI: 10.1038/srep05519]
43 **Chen Y**, Liu XR, Yin YQ, Lee CJ, Wang FT, Liu HQ, Wu XT, Liu J. Unravelling the multifaceted roles of Atg proteins to improve cancer therapy. *Cell Prolif* 2014; **47**: 105-112 [PMID: 24661310 DOI: 10.1111/cpr.12095]
44 **François A**, Rioux Bilan A, Quellard N, Fernandez B, Janet T, Chassaing D, Paccalin M, Terro F, Page G. Longitudinal follow-up of autophagy and inflammation in brain of APPswePS1dE9 transgenic mice. *J Neuroinflammation* 2014; **11**: 139 [PMID: 25158693 DOI: 10.1186/s12974-014-0139-x]
45 **François A**, Terro F, Quellard N, Fernandez B, Chassaing D, Janet T, Rioux Bilan A, Paccalin M, Page G. Impairment of autophagy

in the central nervous system during lipopolysaccharide-induced inflammatory stress in mice. *Mol Brain* 2014; **7**: 56 [PMID: 25169902 DOI: 10.1186/s13041-014-0056-z]
46 **Fu Y**, Chang H, Peng X, Bai Q, Yi L, Zhou Y, Zhu J, Mi M. Resveratrol inhibits breast cancer stem-like cells and induces autophagy via suppressing Wnt/β-catenin signaling pathway. *PLoS One* 2014; **9**: e102535 [PMID: 25068516 DOI: 10.1371/journal.pone.0102535]
47 **Geng Y**, Ju Y, Ren F, Qiu Y, Tomita Y, Tomoeda M, Kishida M, Wang Y, Jin L, Su F, Wei C, Jia B, Li Y, Chang Z. Insulin receptor substrate 1/2 (IRS1/2) regulates Wnt/β-catenin signaling through blocking autophagic degradation of dishevelled2. *J Biol Chem* 2014; **289**: 11230-11241 [PMID: 24616100 DOI: 10.1074/jbc.M113.544999]
48 **Wu H**, Lu MH, Wang W, Zhang MY, Zhu QQ, Xia YY, Xu RX, Yang Y, Chen LH, Ma QH. Lamotrigine Reduces β-Site AβPP-Cleaving Enzyme 1 Protein Levels Through Induction of Autophagy. *J Alzheimers Dis* 2015 Apr 8; Epub ahead of print [PMID: 25854934 DOI: 10.3233/jad-143162]
49 **Jung CH**, Jun CB, Ro SH, Kim YM, Otto NM, Cao J, Kundu M, Kim DH. ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery. *Mol Biol Cell* 2009; **20**: 1992-2003 [PMID: 19225151]
50 **Berwick DC**, Harvey K. The regulation and deregulation of Wnt signaling by PARK genes in health and disease. *J Mol Cell Biol* 2014; **6**: 3-12 [PMID: 24115276 DOI: 10.1093/jmcb/mjt037]
51 **Heo J**, Ahn EK, Jeong HG, Kim YH, Leem SH, Lee SJ, Park EK, Yang M. Transcriptional characterization of Wnt pathway during sequential hepatic differentiation of human embryonic stem cells and adipose tissue-derived stem cells. *Biochem Biophys Res Commun* 2013; **434**: 235-240 [PMID: 23541944 DOI: 10.1016/j.bbrc.2013.02.109]
52 **Li F**, Chong ZZ, Maiese K. Winding through the WNT pathway during cellular development and demise. *Histol Histopathol* 2006; **21**: 103-124 [PMID: 16267791]
53 **Maiese K**, Li F, Chong ZZ, Shang YC. The Wnt signaling pathway: aging gracefully as a protectionist? *Pharmacol Ther* 2008; **118**: 58-81 [PMID: 18313758]
54 **Thorfve A**, Lindahl C, Xia W, Igawa K, Lindahl A, Thomsen P, Palmquist A, Tengvall P. Hydroxyapatite coating affects the Wnt signaling pathway during peri-implant healing in vivo. *Acta Biomater* 2014; **10**: 1451-1462 [PMID: 24342040 DOI: 10.1016/j.actbio.2013.12.012]
55 **Wexler EM**, Rosen E, Lu D, Osborn GE, Martin E, Raybould H, Geschwind DH. Genome-wide analysis of a Wnt1-regulated transcriptional network implicates neurodegenerative pathways. *Sci Signal* 2011; **4**: ra65 [PMID: 21971039 DOI: 10.1126/scisignal.2002282]
56 **Zeljko M**, Pecina-Slaus N, Martic TN, Kusec V, Beros V, Tomas D. Molecular alterations of E-cadherin and beta-catenin in brain metastases. *Front Biosci* (Elite Ed) 2011; **3**: 616-624 [PMID: 21196339]
57 **Chun P**. Role of sirtuins in chronic obstructive pulmonary disease. *Arch Pharm Res* 2015; **38**: 1-10 [PMID: 25304127 DOI: 10.1007/s12272-014-0494-2]
58 **Kim KA**, Shin YJ, Akram M, Kim ES, Choi KW, Suh H, Lee CH, Bae ON. High glucose condition induces autophagy in endothelial progenitor cells contributing to angiogenic impairment. *Biol Pharm Bull* 2014; **37**: 1248-1252 [PMID: 24989016]
59 **Chen J**, Xavier S, Moskowitz-Kassai E, Chen R, Lu CY, Sanduski K, Špes A, Turk B, Goligorsky MS. Cathepsin cleavage of sirtuin 1 in endothelial progenitor cells mediates stress-induced premature senescence. *Am J Pathol* 2012; **180**: 973-983 [PMID: 22234173 DOI: 10.1016/j.ajpath.2011.11.033]
60 **Tang AH**, Rando TA. Induction of autophagy supports the bioenergetic demands of quiescent muscle stem cell activation. *EMBO J* 2014; **33**: 2782-2797 [PMID: 25316028 DOI: 10.15252/embj.201488278]
61 **Maiese K**. mTOR: Driving apoptosis and autophagy for neurocardiac complications of diabetes mellitus. *World J Diabetes* 2015; **6**: 217-224 [PMID: 25789103 DOI: 10.4239/wjd.v6.i2.217]
62 **Maiese K**. Programming apoptosis and autophagy with novel approaches for diabetes mellitus. *Curr Neurovasc Res* 2015; **12**: 173-188 [PMID: 25742566]
63 **Weinberg E**, Maymon T, Weinreb M. AGEs induce caspase-mediated apoptosis of rat BMSCs via TNFα production and oxidative stress. *J Mol Endocrinol* 2014; **52**: 67-76 [PMID: 24198288 DOI: 10.1530/jme-13-0229]
64 **Yang Y**, Li H, Hou S, Hu B, Liu J, Wang J. The noncoding RNA expression profile and the effect of lncRNA AK126698 on cisplatin resistance in non-small-cell lung cancer cell. *PLoS One* 2013; **8**: e65309 [PMID: 23741487 DOI: 10.1371/journal.pone.0065309]
65 **Bailey TJ**, Fossum SL, Fimbel SM, Montgomery JE, Hyde DR. The inhibitor of phagocytosis, O-phospho-L-serine, suppresses Müller glia proliferation and cone cell regeneration in the light-damaged zebrafish retina. *Exp Eye Res* 2010; **91**: 601-612 [PMID: 20696157 DOI: 10.1016/j.exer.2010.07.017]
66 **Chong ZZ**, Li F, Maiese K. Cellular demise and inflammatory microglial activation during beta-amyloid toxicity are governed by Wnt1 and canonical signaling pathways. *Cell Signal* 2007; **19**: 1150-1162 [PMID: 17289346]
67 **Hou J**, Chong ZZ, Shang YC, Maiese K. Early apoptotic vascular signaling is determined by Sirt1 through nuclear shuttling, forkhead trafficking, bad, and mitochondrial caspase activation. *Curr Neurovasc Res* 2010; 7: 95-112 [PMID: 20370652]
68 **Lin SH**, Maiese K. The metabotropic glutamate receptor system protects against ischemic free radical programmed cell death in rat brain endothelial cells. *J Cereb Blood Flow Metab* 2001; **21**: 262-275 [PMID: 11295881]
69 **Popescu NI**, Lupu C, Lupu F. Extracellular protein disulfide isomerase regulates coagulation on endothelial cells through modulation of phosphatidylserine exposure. *Blood* 2010; **116**: 993-1001 [PMID: 20448108]
70 **Shang YC**, Chong ZZ, Hou J, Maiese K. Wnt1, FoxO3a, and NF-kappaB oversee microglial integrity and activation during oxidant stress. *Cell Signal* 2010; **22**: 1317-1329 [PMID: 20462515]
71 **Viola G**, Bortolozzi R, Hamel E, Moro S, Brun P, Castagliuolo I, Ferlin MG, Basso G. MG-2477, a new tubulin inhibitor, induces autophagy through inhibition of the Akt/mTOR pathway and delayed apoptosis in A549 cells. *Biochem Pharmacol* 2012; **83**: 16-26 [PMID: 21964343]
72 **Kim S**, Kang IH, Nam JB, Cho Y, Chung DY, Kim SH, Kim JS, Cho YD, Hong EK, Sohn NW, Shin JW. Ameliorating the effect of astragaloside IV on learning and memory deficit after chronic cerebral hypoperfusion in rats. *Molecules* 2015; **20**: 1904-1921 [PMID: 25625683 DOI: 10.3390/molecules20021904]
73 **Maiese K**. Novel applications of trophic factors, wnt and wisp for neuronal repair and regeneration in metabolic disease. *Neural Regenn Res* 2015; **10**: 518-528 [DOI: 10.4103/1673-5374.155427]
74 **Xin YJ**, Yuan B, Yu B, Wang YQ, Wu JJ, Zhou WH, Qiu Z. Tet1-mediated DNA demethylation regulates neuronal cell death induced by oxidative stress. *Sci Rep* 2015; **5**: 7645 [PMID: 25561289 DOI: 10.1038/srep07645]
75 **Yu T**, Li L, Chen T, Liu Z, Liu H, Li Z. Erythropoietin attenuates advanced glycation endproducts-induced toxicity of Schwann cells in vitro. *Neurochem Res* 2015; **40**: 698-712 [PMID: 25585642 DOI: 10.1007/s11064-015-1516-2]
76 **Miriuka SG**, Rao V, Peterson M, Tumiati L, Delgado DH, Mohan R, Ramzy D, Stewart D, Ross HJ, Waddell TK. mTOR inhibition induces endothelial progenitor cell death. *Am J Transplant* 2006; **6**: 2069-2079 [PMID: 16796720]
77 **Maiese K**, Chong ZZ, Hou J, Shang YC. Oxidative stress: Biomarkers and novel therapeutic pathways. *Exp Gerontol* 2010; **45**: 217-234 [PMID: 20064603]
78 **Maiese K**, Li F, Chong ZZ. New avenues of exploration for erythropoietin. *JAMA* 2005; **293**: 90-95 [PMID: 15632341]
79 **Wang GB**, Ni YL, Zhou XP, Zhang WF. The AKT/mTOR pathway mediates neuronal protective effects of erythropoietin in sepsis. *Mol Cell Biochem* 2014; **385**: 125-132 [PMID: 24057122 DOI: 10.1007/s11010-013-1821-5]

80 **Chong ZZ**, Shang YC, Wang S, Maiese K. PRAS40 is an integral regulatory component of erythropoietin mTOR signaling and cytoprotection. *PLoS One* 2012; **7**: e45456 [PMID: 23029019 DOI: 10.1371/journal.pone.0045456]
81 **Shang YC**, Chong ZZ, Wang S, Maiese K. Erythropoietin and Wnt1 govern pathways of mTOR, Apaf-1, and XIAP in inflammatory microglia. *Curr Neurovasc Res* 2011; **8**: 270-285 [PMID: 22023617]
82 **Shang YC**, Chong ZZ, Wang S, Maiese K. Prevention of β-amyloid degeneration of microglia by erythropoietin depends on Wnt1, the PI 3-K/mTOR pathway, Bad, and Bcl-xL. *Aging* (Albany NY) 2012; **4**: 187-201 [PMID: 22388478]
83 **Sanghera KP**, Mathalone N, Baigi R, Panov E, Wang D, Zhao X, Hsu H, Wang H, Tropepe V, Ward M, Boyd SR. The PI3K/Akt/mTOR pathway mediates retinal progenitor cell survival under hypoxic and superoxide stress. *Mol Cell Neurosci* 2011; **47**: 145-153 [PMID: 21463685 DOI: 10.1016/j.mcn.2011.03.010]
84 **Andreucci M**, Fuiano G, Presta P, Lucisano G, Leone F, Fuiano L, Bisesti V, Esposito P, Russo D, Memoli B, Faga T, Michael A. Downregulation of cell survival signalling pathways and increased cell damage in hydrogen peroxide-treated human renal proximal tubular cells by alpha-erythropoietin. *Cell Prolif* 2009; **42**: 554-561 [PMID: 19508320]
85 **Chang ZY**, Yeh MK, Chiang CH, Chen YH, Lu DW. Erythropoietin protects adult retinal ganglion cells against NMDA-, trophic factor withdrawal-, and TNF-α-induced damage. *PLoS One* 2013; **8**: e55291 [PMID: 23383140 DOI: 10.1371/journal.pone.0055291]
86 **Czubak P**, Bojarska-Junak A, Tabarkiewicz J, Putowski L. A modified method of insulin producing cells' generation from bone marrow-derived mesenchymal stem cells. *J Diabetes Res* 2014; **2014**: 628591 [PMID: 25405207 DOI: 10.1155/2014/628591]
87 **Maiese K**. Protein kinase C modulates the protective ability of peptide growth factors during anoxia. *J Auton Nerv Syst* 1994; **49** Suppl: S187-S193 [PMID: 7836678]
88 **Maiese K**, Boccone L. Neuroprotection by peptide growth factors against anoxia and nitric oxide toxicity requires modulation of protein kinase C. *J Cereb Blood Flow Metab* 1995; **15**: 440-449 [PMID: 7714002 DOI: 10.1038/jcbfm.1995.55]
89 **Maiese K**, Boniece I, DeMeo D, Wagner JA. Peptide growth factors protect against ischemia in culture by preventing nitric oxide toxicity. *J Neurosci* 1993; **13**: 3034-3040 [PMID: 7687284]
90 **Sato A**, Sunayama J, Matsuda K, Tachibana K, Sakurada K, Tomiyama A, Kayama T, Kitanaka C. Regulation of neural stem/progenitor cell maintenance by PI3K and mTOR. *Neurosci Lett* 2010; **470**: 115-120 [PMID: 20045038]
91 **Kimura R**, Okouchi M, Kato T, Imaeda K, Okayama N, Asai K, Joh T. Epidermal growth factor receptor transactivation is necessary for glucagon-like peptide-1 to protect PC12 cells from apoptosis. *Neuroendocrinology* 2013; **97**: 300-308 [PMID: 23147408 DOI: 10.1159/000345529]
92 **Ramanan VK**, Nho K, Shen L, Risacher SL, Kim S, McDonald BC, Farlow MR, Foroud TM, Gao S, Soininen H, Kloszewska I, Mecocci P, Tsolaki M, Vellas B, Lovestone S, Aisen PS, Petersen RC, Jack CR, Jr., Shaw LM, Trojanowski JQ, Weiner MW, Green RC, Toga AW, De Jager PL, Yu L, Bennett DA, Saykin AJ. FASTKD2 is associated with memory and hippocampal structure in older adults. *Mol Psychiatry* 2014 Nov 11; Epub ahead of print [PMID: 25385369 DOI: 10.1038/mp.2014.142]
93 **Slipczuk L**, Bekinschtein P, Katche C, Cammarota M, Izquierdo I, Medina JH. BDNF activates mTOR to regulate GluR1 expression required for memory formation. *PLoS One* 2009; **4**: e6007 [PMID: 19547753]
94 **Chen A**, Xiong LJ, Tong Y, Mao M. Neuroprotective effect of brain-derived neurotrophic factor mediated by autophagy through the PI3K/Akt/mTOR pathway. *Mol Med Rep* 2013; **8**: 1011-1016 [PMID: 23942837 DOI: 10.3892/mmr.2013.1628]
95 **Gangloff YG**, Mueller M, Dann SG, Svoboda P, Sticker M, Spetz JF, Um SH, Brown EJ, Cereghini S, Thomas G, Kozma SC. Disruption of the mouse mTOR gene leads to early postimplantation lethality and prohibits embryonic stem cell development. *Mol Cell Biol* 2004; **24**: 9508-9516 [PMID: 15485918]
96 **Murakami M**, Ichisaka T, Maeda M, Oshiro N, Hara K, Edenhofer F, Kiyama H, Yonezawa K, Yamanaka S. mTOR is essential for growth and proliferation in early mouse embryos and embryonic stem cells. *Mol Cell Biol* 2004; **24**: 6710-6718 [PMID: 15254238]
97 **Zhou J**, Su P, Wang L, Chen J, Zimmermann M, Genbacev O, Afonja O, Horne MC, Tanaka T, Duan E, Fisher SJ, Liao J, Chen J, Wang F. mTOR supports long-term self-renewal and suppresses mesoderm and endoderm activities of human embryonic stem cells. *Proc Natl Acad Sci USA* 2009; **106**: 7840-7845 [PMID: 19416884]
98 **Zhang D**, Yan B, Yu S, Zhang C, Wang B, Wang Y, Wang J, Yuan Z, Zhang L, Pan J. Coenzyme Q10 inhibits the aging of mesenchymal stem cells induced by D-galactose through Akt/mTOR signaling. *Oxid Med Cell Longev* 2015; **2015**: 867293 [PMID: 25789082 DOI: 10.1155/2015/867293]
99 **Easley CA**, Ben-Yehudah A, Redinger CJ, Oliver SL, Varum ST, Eisinger VM, Carlisle DL, Donovan PJ, Schatten GP. mTOR-mediated activation of p70 S6K induces differentiation of pluripotent human embryonic stem cells. *Cell Reprogram* 2010; **12**: 263-273 [PMID: 20698768]
100 **Hartman NW**, Lin TV, Zhang L, Paquelet GE, Feliciano DM, Bordey A. mTORC1 targets the translational repressor 4E-BP2, but not S6 kinase 1/2, to regulate neural stem cell self-renewal in vivo. *Cell Rep* 2013; **5**: 433-444 [PMID: 24139800 DOI: 10.1016/j.celrep.2013.09.017]
101 **Romine J**, Gao X, Xu XM, So KF, Chen J. The proliferation of amplifying neural progenitor cells is impaired in the aging brain and restored by the mTOR pathway activation. *Neurobiol Aging* 2015; **36**: 1716-1726 [PMID: 25655438 DOI: 10.1016/j.neurobiolaging.2015.01.003]
102 **Malagelada C**, López-Toledano MA, Willett RT, Jin ZH, Shelanski ML, Greene LA. RTP801/REDD1 regulates the timing of cortical neurogenesis and neuron migration. *J Neurosci* 2011; **31**: 3186-3196 [PMID: 21368030 DOI: 10.1523/jneurosci.4011-10.2011]
103 **Marfia G**, Madaschi L, Marra F, Menarini M, Bottai D, Formenti A, Bellardita C, Di Giulio AM, Carelli S, Gorio A. Adult neural precursors isolated from post mortem brain yield mostly neurons: an erythropoietin-dependent process. *Neurobiol Dis* 2011; **43**: 86-98 [PMID: 21324364 DOI: 10.1016/j.nbd.2011.02.004]
104 **Magri L**, Cambiaghi M, Cominelli M, Alfaro-Cervello C, Cursi M, Pala M, Bulfone A, Garcìa-Verdugo JM, Leocani L, Minicucci F, Poliani PL, Galli R. Sustained activation of mTOR pathway in embryonic neural stem cells leads to development of tuberous sclerosis complex-associated lesions. *Cell Stem Cell* 2011; **9**: 447-462 [PMID: 22056141]
105 **Galan-Moya EM**, Le Guelte A, Lima Fernandes E, Thirant C, Dwyer J, Bidere N, Couraud PO, Scott MG, Junier MP, Chneiweiss H, Gavard J. Secreted factors from brain endothelial cells maintain glioblastoma stem-like cell expansion through the mTOR pathway. *EMBO Rep* 2011; **12**: 470-476 [PMID: 21460795]
106 **Földes G**, Mioulane M, Wright JS, Liu AQ, Novak P, Merkely B, Gorelik J, Schneider MD, Ali NN, Harding SE. Modulation of human embryonic stem cell-derived cardiomyocyte growth: a testbed for studying human cardiac hypertrophy? *J Mol Cell Cardiol* 2011; **50**: 367-376 [PMID: 21047517 DOI: 10.1016/j.yjmcc.2010.10.029]
107 **Benyoucef A**, Calvo J, Renou L, Arcangeli ML, van den Heuvel A, Amsellem S, Mehrpour M, Larghero J, Soler E, Naguibneva I, Pflumio F. The SCL/TAL1 Transcription Factor Represses the Stress Protein DDiT4/REDD1 in Human Hematopoietic Stem/Progenitor Cells. *Stem Cells* 2015; **33**: 2268-2279 [PMID: 25858676 DOI: 10.1002/stem.2028]
108 **Iriuchishima H**, Takubo K, Matsuoka S, Onoyama I, Nakayama KI, Nojima Y, Suda T. Ex vivo maintenance of hematopoietic stem cells by quiescence induction through Fbxw7& amp; alpha; overexpression. *Blood* 2011; **117**: 2373-2377 [PMID: 21190997 DOI: 10.1182/blood-2010-07-294801]
109 **Kim J**, Jung Y, Sun H, Joseph J, Mishra A, Shiozawa Y, Wang J, Krebsbach PH, Taichman RS. Erythropoietin mediated bone formation is regulated by mTOR signaling. *J Cell Biochem* 2012; **113**: 220-228 [PMID: 21898543 DOI: 10.1002/jcb.23347]

110 **Moon HE**, Byun K, Park HW, Kim JH, Hur J, Park JS, Jun JK, Kim HS, Paek SL, Kim IK, Hwang JH, Kim JW, Kim DG, Sung YC, Koh GY, Song CW, Lee B, Paek SH. COMP-Ang1 Potentiates EPC Treatment of Ischemic Brain Injury by Enhancing Angiogenesis Through Activating AKT-mTOR Pathway and Promoting Vascular Migration Through Activating Tie2-FAK Pathway. *Exp Neurobiol* 2015; **24**: 55-70 [PMID: 25792870 DOI: 10.5607/en.2015.24.1.55]

111 **Siegel N**, Rosner M, Unbekandt M, Fuchs C, Slabina N, Dolznig H, Davies JA, Lubec G, Hengstschläger M. Contribution of human amniotic fluid stem cells to renal tissue formation depends on mTOR. *Hum Mol Genet* 2010; **19**: 3320-3331 [PMID: 20542987]

112 **Venkatesh HS**, Johung TB, Caretti V, Noll A, Tang Y, Nagaraja S, Gibson EM, Mount CW, Polepalli J, Mitra SS, Woo PJ, Malenka RC, Vogel H, Bredel M, Mallick P, Monje M. Neuronal Activity Promotes Glioma Growth through Neuroligin-3 Secretion. *Cell* 2015; **161**: 803-816 [PMID: 25913192 DOI: 10.1016/j.cell.2015.04.012]

113 **Hu X**, Pandolfi PP, Li Y, Koutcher JA, Rosenblum M, Holland EC. mTOR promotes survival and astrocytic characteristics induced by Pten/AKT signaling in glioblastoma. *Neoplasia* 2005; **7**: 356-368 [PMID: 15967113]

114 **Kolev VN**, Wright QG, Vidal CM, Ring JE, Shapiro IM, Ricono J, Weaver DT, Padval MV, Pachter JA, Xu Q. PI3K/mTOR dual inhibitor VS-5584 preferentially targets cancer stem cells. *Cancer Res* 2015; **75**: 446-455 [PMID: 25432176 DOI: 10.1158/0008-5472.can-14-1223]

115 **Castilho RM**, Squarize CH, Chodosh LA, Williams BO, Gutkind JS. mTOR mediates Wnt-induced epidermal stem cell exhaustion and aging. *Cell Stem Cell* 2009; **5**: 279-289 [PMID: 19733540 DOI: 10.1016/j.stem.2009.06.017]

116 **Uzdensky AB**, Demyanenko SV, Bibov MY. Signal transduction in human cutaneous melanoma and target drugs. *Curr Cancer Drug Targets* 2013; **13**: 843-866 [PMID: 23675881]

117 **James RG**, Davidson KC, Bosch KA, Biechele TL, Robin NC, Taylor RJ, Major MB, Camp ND, Fowler K, Martins TJ, Moon RT. WIKI4, a novel inhibitor of tankyrase and Wnt/ß-catenin signaling. *PLoS One* 2012; **7**: e50457 [PMID: 23227175 DOI: 10.1371/journal.pone.0050457]

118 **Kafka A**, Bašić-Kinda S, Pećina-Šlaus N. The cellular story of dishevelleds. *Croat Med J* 2014; **55**: 459-467 [PMID: 25358879]

119 **Klinke DJ**. Induction of Wnt-inducible signaling protein-1 correlates with invasive breast cancer oncogenesis and reduced type 1 cell-mediated cytotoxic immunity: a retrospective study. *PLoS Comput Biol* 2014; **10**: e1003409 [PMID: 24426833 DOI: 10.1371/journal.pcbi.1003409]

120 **Knoblich K**, Wang HX, Sharma C, Fletcher AL, Turley SJ, Hemler ME. Tetraspanin TSPAN12 regulates tumor growth and metastasis and inhibits β-catenin degradation. *Cell Mol Life Sci* 2014; **71**: 1305-1314 [PMID: 23955570 DOI: 10.1007/s00018-013-1444-8]

121 **Liu Y**, Yan W, Zhang W, Chen L, You G, Bao Z, Wang Y, Wang H, Kang C, Jiang T. MiR-218 reverses high invasiveness of glioblastoma cells by targeting the oncogenic transcription factor LEF1. *Oncol Rep* 2012; **28**: 1013-1021 [PMID: 22766851 DOI: 10.3892/or.2012.1902]

122 **Tu Y**, Gao X, Li G, Fu H, Cui D, Liu H, Jin W, Zhang Y. MicroRNA-218 inhibits glioma invasion, migration, proliferation, and cancer stem-like cell self-renewal by targeting the polycomb group gene Bmi1. *Cancer Res* 2013; **73**: 6046-6055 [PMID: 23950210 DOI: 10.1158/0008-5472.can-13-0358]

123 **Maiese K**, Chong ZZ, Shang YC, Hou J. Rogue proliferation versus restorative protection: where do we draw the line for Wnt and forkhead signaling? *Expert Opin Ther Targets* 2008; **12**: 905-916 [PMID: 18554157 DOI: 10.1517/14728222.12.7.905]

124 **Wang S**, Sun Z, Zhang X, Li Z, Wu M, Zhao W, Wang H, Chen T, Yan H, Zhu J. Wnt1 positively regulates CD36 expression via TCF4 and PPAR-γ in macrophages. *Cell Physiol Biochem* 2015; **35**: 1289-1302 [PMID: 25721714 DOI: 10.1159/000373951]

125 **Xu D**, Zhao W, Pan G, Qian M, Zhu X, Liu W, Cai G, Cui Z. Expression of Nemo-like kinase after spinal cord injury in rats. *J Mol Neurosci* 2014; **52**: 410-418 [PMID: 24395089 DOI: 10.1007/s12031-013-0191-5]

126 **Borrell-Pagès M**, Romero JC, Badimon L. LRP5 negatively regulates differentiation of monocytes through abrogation of Wnt signalling. *J Cell Mol Med* 2014; **18**: 314-325 [PMID: 24266894 DOI: 10.1111/jcmm.12190]

127 **Macsai CE**, Georgiou KR, Foster BK, Zannettino AC, Xian CJ. Microarray expression analysis of genes and pathways involved in growth plate cartilage injury responses and bony repair. *Bone* 2012; **50**: 1081-1091 [PMID: 22387305 DOI: 10.1016/j.bone.2012.02.013]

128 **L'Episcopo F**, Tirolo C, Testa N, Caniglia S, Morale MC, Deleidi M, Serapide MF, Pluchino S, Marchetti B. Plasticity of subventricular zone neuroprogenitors in MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) mouse model of Parkinson's disease involves cross talk between inflammatory and Wnt/β-catenin signaling pathways: functional consequences for neuroprotection and repair. *J Neurosci* 2012; **32**: 2062-2085 [PMID: 22323720 DOI: 10.1523/jneurosci.5259-11.2012]

129 **Liu J**, Wang Y, Pan Q, Su Y, Zhang Z, Han J, Zhu X, Tang C, Hu D. Wnt/β-catenin pathway forms a negative feedback loop during TGF-β1 induced human normal skin fibroblast-to-myofibroblast transition. *J Dermatol Sci* 2012; **65**: 38-49 [PMID: 22041457 DOI: 10.1016/j.jdermsci.2011.09.012]

130 **Shah N**, Morsi Y, Manasseh R. From mechanical stimulation to biological pathways in the regulation of stem cell fate. *Cell Biochem Funct* 2014; **32**: 309-325 [PMID: 24574137 DOI: 10.1002/cbf.3027]

131 **He B**, Reguart N, You L, Mazieres J, Xu Z, Lee AY, Mikami I, McCormick F, Jablons DM. Blockade of Wnt-1 signaling induces apoptosis in human colorectal cancer cells containing downstream mutations. *Oncogene* 2005; **24**: 3054-3058 [PMID: 15735684]

132 **Kohan-Ghadr HR**, Smith LC, Arnold DR, Murphy BD, Lefebvre RC. Aberrant expression of E-cadherin and β-catenin proteins in placenta of bovine embryos derived from somatic cell nuclear transfer. *Reprod Fertil Dev* 2012; **24**: 588-598 [PMID: 22541547 DOI: 10.1071/rd11162]

133 **Marchetti B**, Pluchino S. Wnt your brain be inflamed? Yes, it Wnt! *Trends Mol Med* 2013; **19**: 144-156 [PMID: 23312954 DOI: 10.1016/j.molmed.2012.12.001]

134 **Chong ZZ**, Shang YC, Hou J, Maiese K. Wnt1 neuroprotection translates into improved neurological function during oxidant stress and cerebral ischemia through AKT1 and mitochondrial apoptotic pathways. *Oxid Med Cell Longev* 2010; **3**: 153-165 [PMID: 20716939]

135 **Xing Y**, Zhang X, Zhao K, Cui L, Wang L, Dong L, Li Y, Liu Z, Wang C, Zhang X, Zhu C, Qiao H, Ji Y, Cao X. Beneficial effects of sulindac in focal cerebral ischemia: a positive role in Wnt/β-catenin pathway. *Brain Res* 2012; **1482**: 71-80 [PMID: 22981403 DOI: 10.1016/j.brainres.2012.08.057]

136 **Chong ZZ**, Hou J, Shang YC, Wang S, Maiese K. EPO relies upon novel signaling of Wnt1 that requires Akt1, FoxO3a, GSK-3β, and β-catenin to foster vascular integrity during experimental diabetes. *Curr Neurovasc Res* 2011; **8**: 103-120 [PMID: 21443457]

137 **Chong ZZ**, Shang YC, Maiese K. Vascular injury during elevated glucose can be mitigated by erythropoietin and Wnt signaling. *Curr Neurovasc Res* 2007; **4**: 194-204 [PMID: 17691973]

138 **Pandey S**. Targeting Wnt-Frizzled signaling in cardiovascular diseases. *Mol Biol Rep* 2013; **40**: 6011-6018 [PMID: 24057182 DOI: 10.1007/s11033-013-2710-4]

139 **Danielyan L**, Schäfer R, Schulz A, Ladewig T, Lourhmati A, Buadze M, Schmitt AL, Verleysdonk S, Kabisch D, Koeppen K, Siegel G, Proksch B, Kluba T, Eckert A, Köhle C, Schöneberg T, Northoff H, Schwab M, Gleiter CH. Survival, neuron-like differentiation and functionality of mesenchymal stem cells in neurotoxic environment: the critical role of erythropoietin. *Cell Death Differ* 2009; **16**: 1599-1614 [PMID: 19609278 DOI: 10.1038/cdd.2009.95]

140 **Maiese K**. Foxo proteins in the nervous system. *Anal Cell Pathol* (Amst) 2015; **2015:** Article ID 569392 [DOI: 10.1155/2015/569392]

141 **Hedley BD**, Allan AL, Xenocostas A. The role of erythropoietin and erythropoiesis-stimulating agents in tumor progression.

Clin Cancer Res 2011; **17**: 6373-6380 [PMID: 21750199 DOI: 10.1158/1078-0432.ccr-10-2577]
142 **Sadoff L**. Erythropoietin and cancer. *JAMA* 2005; **293**: 1858; author reply 1858-1859 [PMID: 15840858]
143 **Zhang C**, Li Z, Cao Q, Qin C, Cai H, Zhou H, Qian J, Tao L, Ju X, Yin C. Association of erythropoietin gene rs576236 polymorphism and risk of adrenal tumors in a Chinese population. *J Biomed Res* 2014; **28**: 456-461 [PMID: 25469114 DOI: 10.7555/jbr.28.20130126]
144 **Ogunshola OO**, Moransard M, Gassmann M. Constitutive excessive erythrocytosis causes inflammation and increased vascular permeability in aged mouse brain. *Brain Res* 2013; **1531**: 48-57 [PMID: 23892106 DOI: 10.1016/j.brainres.2013.07.033]
145 **Maiese K**. WISP1: Clinical insights for a proliferative and restorative member of the CCN family. *Curr Neurovasc Res* 2014; **11**: 378-389 [PMID: 25219658]
146 **Shang YC**, Chong ZZ, Wang S, Maiese K. Wnt1 inducible signaling pathway protein 1 (WISP1) targets PRAS40 to govern β-amyloid apoptotic injury of microglia. *Curr Neurovasc Res* 2012; **9**: 239-249 [PMID: 22873724]
147 **Xia W**, Zhang F, Xie C, Jiang M, Hou M. Macrophage migration inhibitory factor confers resistance to senescence through CD74-dependent AMPK-FOXO3a signaling in mesenchymal stem cells. *Stem Cell Res Ther* 2015; **6**: 82 [PMID: 25896286 DOI: 10.1186/s13287-015-0076-3]
148 **Lough D**, Dai H, Yang M, Reichensperger J, Cox L, Harrison C, Neumeister MW. Stimulation of the follicular bulge LGR5+ and LGR6+ stem cells with the gut-derived human alpha defensin 5 results in decreased bacterial presence, enhanced wound healing, and hair growth from tissues devoid of adnexal structures. *Plast Reconstr Surg* 2013; **132**: 1159-1171 [PMID: 24165598 DOI: 10.1097/PRS.0b013e3182a48af6]
149 **Murahovschi V**, Pivovarova O, Ilkavets I, Dmitrieva RM, Döcke S, Keyhani-Nejad F, Gögebakan Ö, Osterhoff M, Kemper M, Hornemann S, Markova M, Klöting N, Stockmann M, Weickert MO, Lamounier-Zepter V, Neuhaus P, Konradi A, Dooley S, von Loeffelholz C, Blüher M, Pfeiffer AF, Rudovich N. WISP1 is a novel adipokine linked to inflammation in obesity. *Diabetes* 2015; **64**: 856-866 [PMID: 25281430 DOI: 10.2337/db14-0444]
150 **Jung DW**, Kim WH, Williams DR. Reprogram or reboot: small molecule approaches for the production of induced pluripotent stem cells and direct cell reprogramming. *ACS Chem Biol* 2014; **9**: 80-95 [PMID: 24245936 DOI: 10.1021/cb400754f]
151 **Yang CS**, Lopez CG, Rana TM. Discovery of nonsteroidal anti-inflammatory drug and anticancer drug enhancing reprogramming and induced pluripotent stem cell generation. *Stem Cells* 2011; **29**: 1528-1536 [PMID: 21898684 DOI: 10.1002/stem.717]
152 **Lim HW**, Lee JE, Shin SJ, Lee YE, Oh SH, Park JY, Seong JK, Park JS. Identification of differentially expressed mRNA during pancreas regeneration of rat by mRNA differential display. *Biochem Biophys Res Commun* 2002; **299**: 806-812 [PMID: 12470650]
153 **Price RM**, Tulsyan N, Dermody JJ, Schwalb M, Soteropoulos P, Castronuovo JJ. Gene expression after crush injury of human saphenous vein: using microarrays to define the transcriptional profile. *J Am Coll Surg* 2004; **199**: 411-418 [PMID: 15325611]
154 **Du J**, Klein JD, Hassounah F, Zhang J, Zhang C, Wang XH. Aging increases CCN1 expression leading to muscle senescence. *Am J Physiol Cell Physiol* 2014; **306**: C28-C36 [PMID: 24196529 DOI: 10.1152/ajpcell.00066.2013]
155 **Marchand A**, Atassi F, Gaaya A, Leprince P, Le Feuvre C, Soubrier F, Lompré AM, Nadaud S. The Wnt/beta-catenin pathway is activated during advanced arterial aging in humans. *Aging Cell* 2011; **10**: 220-232 [PMID: 21108734 DOI: 10.1111/j.1474-9726.2010.00661.x]
156 **Ono M**, Inkson CA, Sonn R, Kilts TM, de Castro LF, Maeda A, Fisher LW, Robey PG, Berendsen AD, Li L, McCartney-Francis N, Brown AC, Crawford NP, Molinolo A, Jain A, Fedarko NS, Young MF. WISP1/CCN4: a potential target for inhibiting prostate cancer growth and spread to bone. *PLoS One* 2013; **8**: e71709 [PMID: 23977121 DOI: 10.1371/journal.pone.0071709]
157 **Pasmant E**, Ortonne N, Rittié L, Laurendeau I, Lévy P, Lazar V, Parfait B, Leroy K, Dessen P, Valeyrie-Allanore L, Perbal B, Wolkenstein P, Vidaud M, Vidaud D, Bièche I. Differential expression of CCN1/CYR61, CCN3/NOV, CCN4/WISP1, and CCN5/WISP2 in neurofibromatosis type 1 tumorigenesis. *J Neuropathol Exp Neurol* 2010; **69**: 60-69 [PMID: 20010302 DOI: 10.1097/NEN.0b013e3181c79bff]
158 **Tanaka S**, Sugimachi K, Kameyama T, Maehara S, Shirabe K, Shimada M, Wands JR, Maehara Y. Human WISP1v, a member of the CCN family, is associated with invasive cholangiocarcinoma. *Hepatology* 2003; **37**: 1122-1129 [PMID: 12717393 DOI: 10.1053/jhep.2003.50187]
159 **Soon LL**, Yie TA, Shvarts A, Levine AJ, Su F, Tchou-Wong KM. Overexpression of WISP-1 down-regulated motility and invasion of lung cancer cells through inhibition of Rac activation. *J Biol Chem* 2003; **278**: 11465-11470 [PMID: 12529380 DOI: 10.1074/jbc.M210945200]

P- Reviewer: Hopfner M, Hung LY **S- Editor**: Ji FF
L- Editor: A **E- Editor**: Wu HL

World Journal of Stem Cells

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4252/wjsc.v7.i7.1010

World J Stem Cells 2015 August 26; 7(7): 1010-1021
ISSN 1948-0210 (online)
© 2015 Baishideng Publishing Group Inc. All rights reserved.

REVIEW

Allogenic banking of dental pulp stem cells for innovative therapeutics

Pierre-Yves Collart-Dutilleul, Franck Chaubron, John De Vos, Frédéric J Cuisinier

Pierre-Yves Collart-Dutilleul, Frédéric J Cuisinier, BioNano Laboratory EA 4203, Montpellier 1 University, 34193 Montpellier Cedex5, France

Franck Chaubron, Institut Clinident BioPharma, Biopôle Clermont-Limagne, 63360 Saint Beauzire, France

John De Vos, INSERM U1040, Genetic Instability of Human Pluripotent Stem Cells, 34197 Montpellier, France

John De Vos, CHRU Montpellier, Hospital Saint-Eloi, Institut de Recherche en Biothérapie, 34197 Montpellier, France

Author contributions: Collart-Dutilleul PY, Chaubron F, De Vos J and Cuisinier FJ solely contributed to this paper.

Conflict-of-interest statement: The authors declare that they have no conflict of interests.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: http://creativecommons.org/licenses/by-nc/4.0/

Correspondence to: Frédéric J Cuisinier, DDS, PhD, BioNano Laboratory EA 4203, Montpellier 1 University, 545 Av Pr JL Viala, 34193 Montpellier Cedex5,
France. frederic.cuisinier@univ-montp1.fr
Telephone: +33-4-11759225
Fax: +33-4-11759201

Received: October 13, 2014
Peer-review started: October 14, 2014
First decision: January 8, 2015
Revised: June 8, 2015
Accepted: June 18, 2015
Article in press: June 19, 2015
Published online: August 26, 2015

Abstract

Medical research in regenerative medicine and cell-based therapy has brought encouraging perspectives for the use of stem cells in clinical trials. Multiple types of stem cells, from progenitors to pluripotent stem cells, have been investigated. Among these, dental pulp stem cells (DPSCs) are mesenchymal multipotent cells coming from the dental pulp, which is the soft tissue within teeth. They represent an interesting adult stem cell source because they are recovered in large amount in dental pulps with non-invasive techniques compared to other adult stem cell sources. DPSCs can be obtained from discarded teeth, especially wisdom teeth extracted for orthodontic reasons. To shift from promising pre-clinical results to therapeutic applications to human, DPSCs must be prepared in clinical grade lots and transformed into advanced therapy medicinal products (ATMP). As the production of patient-specific stem cells is costly and time-consuming, allogenic biobanking of clinical grade human leukocyte antigen (HLA)-typed DPSC lines provides efficient innovative therapeutic products. DPSC biobanks represent industrial and therapeutic innovations by using discarded biological tissues (dental pulps) as a source of mesenchymal stem cells to produce and store, in good manufacturing practice (GMP) conditions, DPSC therapeutic batches. In this review, we discuss about the challenges to transfer biological samples from a donor to HLA-typed DPSC therapeutic lots, following regulations, GMP guidelines and ethical principles. We also present some clinical applications, for which there is no efficient therapeutics so far, but that DPSCs-based ATMP could potentially treat.

Key words: Adult stem cells; Multipotent stem cells; Cell-based therapy; Cell tissue bank

© The Author(s) 2015. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: To achieve clinical applications, stem cell-based therapy must shift from lab experimentation to clinical grade stem cells. We present here the development of advanced therapy medicinal products (ATMP) by the banking of dental pulp stem cells (DPSCs) for allogenic use. The dental pulp represents an efficient tool for industrial applications due to its accessibility after wisdom teeth extraction for orthodontic purpose. DPSC therapeutic batches can be produced in good manufacturing practice condition after human leukocyte antigen typing and stored in allogenic biobanks. We propose some clinical applications, for which there is no efficient therapeutics so far, but that DPSCs-based ATMP could potentially treat.

Collart-Dutilleul PY, Chaubron F, De Vos J, Cuisinier FJ. Allogenic banking of dental pulp stem cells for innovative therapeutics. *World J Stem Cells* 2015; 7(7): 1010-1021 Available from: URL: http://www.wjgnet.com/1948-0210/full/v7/i7/1010.htm DOI: http://dx.doi.org/10.4252/wjsc.v7.i7.1010

INTRODUCTION

Adult organisms contain postnatal somatic stem cells that are involved in symmetrical and asymmetrical cell divisions, allowing stem cell compartment maintenance and cell differentiation[1]. Thus, adult stem cells provide replacement and repair cells for normal turnover or injured tissues[2]. As these stem cells are able to renew particular tissues, they have motivated research on how to apply them in the clinic. Because of their self-renewal and ability to regenerate tissue, stem cells could provide long-lasting clinical benefits to recipients. Among these potentially beneficial cells, mesenchymal stromal cells are spindle-shaped, plastic-adherent cells isolated from bone marrow, adipose tissue, dental pulp and many other tissue sources[3,4]. They are also called mesenchymal stem cells (MSCs) in reference to their significant self-renewing properties and ability to form skeletal and connective tissue, and are suggested to be responsible for the normal turnover and maintenance of adult mesenchymal tissues[2,5]. MSCs are now the focus of intensive efforts in order to elucidate their nature and properties, and to develop cell-based therapies with real clinical applications[6]. Moreover, MSCs provide promising therapeutic benefits as they primarily mediate positive effects through paracrine mechanisms independent of cell differentiation[7]. Many preclinical and clinical trials have been completed and the major hurdles are now cell engraftment and survival, stem cell fate control, and donor-patient compatibility for allogenic applications. Several current efforts are directed at promoting the registration and banking of stem cell lines and providing associated data[8,9]. Banking MSCs, with shared materials and data, is an important step for the efficient progress of stem cell research and clinical translation. Emphasis on clinical applications is increasing, with an aim of establishing clinical grade, human leukocyte antigen (HLA)-matched banks for clinical translation[10].

DENTAL PULP STEM CELLS

Teeth are formed of two main parts, the crown and the root, that can be defined by anatomic criteria. They are linked by the periodontal ligament to the supporting alveolar bone, which is composed of both compact and trabecular bone. The dental crown consists of enamel, dentin, and dental pulp tissue. During tooth growth and development, ameloblasts form enamel and odontoblasts generate primary dentin. After tooth eruption, ameloblasts disappear from the surface of the enamel; consequently, enamel formation ceases to occur naturally *in vivo*. In contrast, odontoblasts, along the inner surface of the dentin inside the pulp chamber, continue to deposit dentin matrix to form secondary dentin throughout life. In addition to secondary dentin, odontoblasts can form tertiary (reparative) dentin in response to several stimuli, such as mechanical, chemical, and/or bacterial stimulation. Even when odontoblasts have been damaged, the reparative dentin can be formed in the dental pulp to protect against further disruption of the pulp tissue. This reparative dentinogenesis has been thought to be mediated by newly generated odontoblasts arising from dental pulp tissue. These findings led to the speculation that odontogenic progenitor cells or stem cells may exist in dental pulp tissue[11]. The first type of dental stem cell was subsequently isolated from the human pulp tissue and given the name dental pulp stem cells (DPSCs)[12]. Dental pulp is a soft connective tissue entrapped within the dental crown, and divided into four layers. The external layer is made up of odontoblasts producing dentin; the second layer is poor in cells and rich in collagen fibers; and the third layer contains progenitor cells and undifferentiated cells, some of which are considered stem cells. From this layer, undifferentiated cells migrate to various districts where they can differentiate under different stimuli and make new differentiated cells and tissues. The innermost layer is the core of the pulp and comprises the vascular area and nerves[13]. Dental pulp is an interesting source of adult stem cells because of the large amount of cells present and the non-invasiveness of the isolation methods compared to other adult tissue sources[13-15]. MSCs defined as dental stem cells can be obtained from human permanent and primary teeth, human wisdom teeth[12], human exfoliated deciduous teeth[16], apical papilla[17], the periodontal ligament[18,19] and the dental follicle[20,21].

Dental pulp tissue from human third molar, exfoliated deciduous or supernumerary teeth represent an easily accessible source for harvesting MSCs as these teeth are often discarded.

Stem cells that reside in dental pulp (DPSCs) have been described as a population of MSCs, as they match the definition given by the Mesenchymal and Tissue

Stem Cell Committee of the International Society for Cellular Therapy[3]: DPSC are plastic-adherent when maintained in standard culture conditions; they express some specific surface molecules such as CD105, CD73, CD90 and lack expression of CD45, CD34, CD14, CD19 and HLA-DR surface molecules; and they have the ability to differentiate into osteoblasts, adipocytes and chondroblasts *in vitro*[11,12,18,22-24]. Moreover, DPSCs can differentiate into a large array of cells and tissues[25-28] and a comparison of their multipotency with Bone Marrow Stem Cells has demonstrated that proliferation, availability, and cell number of DPSCs were greater than for bone marrow MSCs[24,29].

In addition, DPSCs were also found to undergo myogenic and neurogenic differentiation capacities *in vitro*, expressing respective gene markers and exhibiting neuron-like cell morphologies. The plasticity and multipotential capability of DPSCs can be explained by the fact that dental pulp is made of both ectodermic and mesenchymal components, and contains neural crest-derived cells[13]. Concerning cell surface molecules, the persistence of negative results for CD45 demonstrates that these cells are not derived from a hematopoietic source, although they are of mesenchymal origin[25]. Like all MSCs, DPSCs are also heterogeneous and the various markers may be expressed differently by subpopulations of these stem cells[24]. A selected subpopulation of $CD34^+/CD45^-$ DPSCs, which represented roughly 10% of dental pulp cells, has also been described. These cells displayed an increased capacity of self-expanding and differentiating in pre-osteoblasts, and were able to self-maintain and renew for long time[17]. Although MSCs were originally described as CD34 negative, it seems that this subpopulation of DPSCs expresses the CD34 cell surface antigen in the manner reserved for the most primitive stromal stem cells (other than hematopoietic) that was gradually lost after the differentiation of lineage committed progenitors[30].

DPSCS BIOBANKING

The term "biobank" describes various facilities that store biological samples, from small tissue collections to wide repositories featuring a variety of tissues and biological sample types[31,32].

Storage and collection of biological material might be accompanied by various medical and epidemiological data that are used for current research and for potential future works[33]. Biobanking has been defined as a structured resource for genetic and medical research and their therapeutic applications. It includes human biological material and extensive associated information[34,35].

Several types of biobanks can be distinguished, according to the purpose and target: case control, clinical trial, tissue, biomolecular resource center, stem cells[36]. Stem cell biobanks have received much attention as a new biologic resource for both research and clinical applications, leading to the development of stem cell banks around the world[37]. Stem cells of dental origin represent a promising source of new stem cells as, in western countries, 80% of teenagers and/or young adults have their wisdom teeth extracted. Furthermore, dental pulp is naturally protected within the pulp chamber, the inner cavity of the tooth, in a sterile environment. Pulp from one wisdom tooth generally contains between 200000 and 300000 DPSCs[38]. Studies have indicated that DPSC isolation was feasible for 5 d after tooth extraction[39]. Efficient results were obtained by cryopreserving second-passage DPSC cultures, but could also be achieved by isolating and cryopreserving entire pulp tissues, with digestion and culture performed post-thaw[40]. Such minimal processing may be of interest for the banking of samples for which there are no immediate plans for expansion and use. Furthermore, cell recovery could be achieved by mechanical disruption with a single-use device, in accordance with the GMP (European Good Manufacturing Practices) standard. Dental pulp and DPSC recovery is represented in Figure 1.

Immunologic considerations: Allogenic use and immunomodulation

Immune mechanisms confer immediate protection against foreign organisms (innate immunity) and specific immune responses to neutralize pathogens (adaptive immunity). The immune system recognizes tissue compatibility and can raise an effective immune response against pathogens or incompatible allogenic tissues. Tissue compatibility or incompatibility is determined from allelic similarities or disparities at genetic loci that encode the major histocompatibility complex (MHC) antigens, also called the HLA system. The HLA system encodes two major classes of highly polymorphic cell surface glycoproteins: HLA class I molecules are expressed on all nucleated cells and HLA class II molecules are expressed on antigen-presenting cells, thymic epithelial cells, and B lymphocytes. These immunological principles, which apply to organ or tissue transplantation, can be extended to transplantation of DPSCs or DPSC-derived tissue, especially for HLA class I molecules (HLA-A, HLA-B and HLA-C)[41]. Thus, a major clinical challenge to DPSC banking will be to overcome the immunological barriers to the transplantation of DPSC-derived tissues in order to prevent rejection[42,43]. Ensuring HLA compatibility is certainly the most interesting method of minimizing the risk of rejection. Embryonic stem cells have been shown to express very low levels of HLA class I proteins, with a moderate increase during differentiation[44]. Regarding MSCs, HLA expression remains unclear, but they have been extensively studied for their immunomodulatory properties[45-49]. Indeed, MSCs exert a profound inhibitory effect on T cell proliferation *in vitro* and *in vivo*, with similar effects on B cells, dendritic cells and natural killer cells[50]. Moreover, T-cell inhibition is not restricted by HLA type, and immunosuppressive effects are

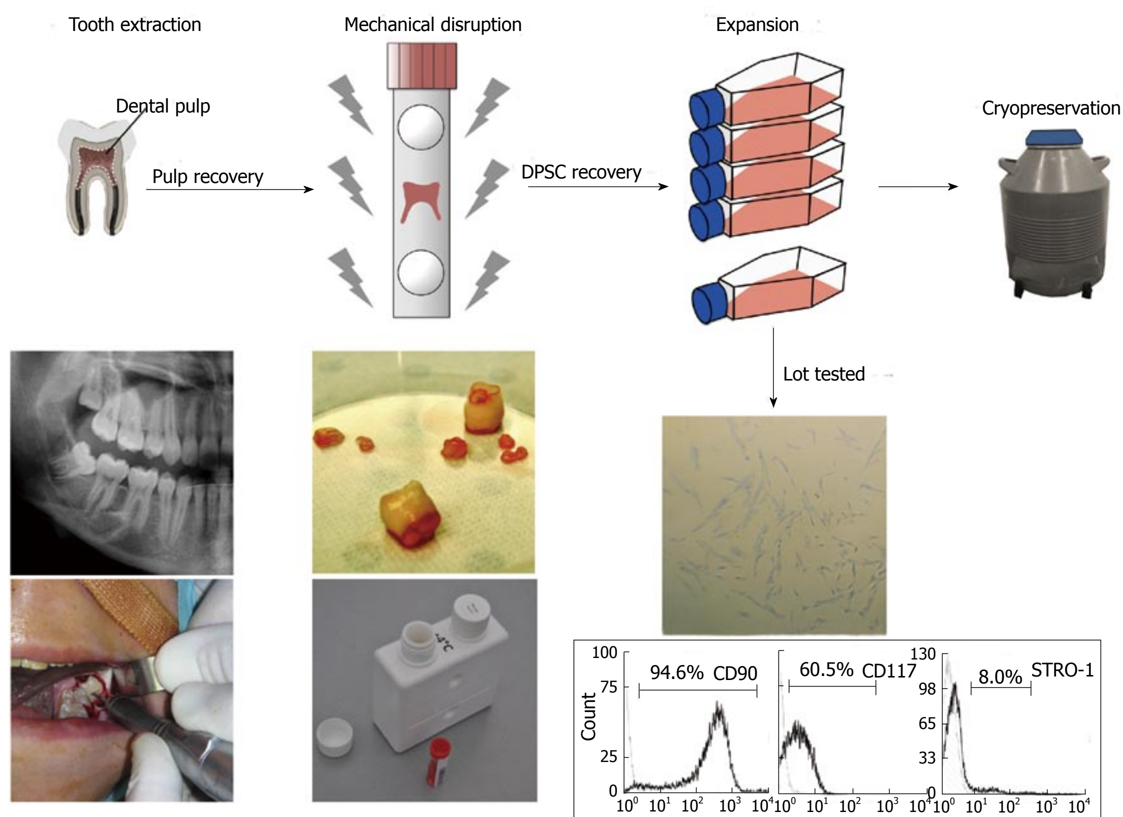


Figure 1 Dental Pulp tissue and dental pulp stem cell recovery. Wisdom teeth are extracted in aseptic conditions and transferred to the cell bank in a sterile transport tube. The teeth are then cracked opened and the pulps are mechanically disrupted in a tissue grinder/homogenizer. The cell suspensions obtained are screened for expression of stemness markers by flow cytometry, before storage in liquid nitrogen. DPSC: Dental pulp stem cell.

mediated through soluble factors and the generation of regulatory cells[47,51]. These findings suggest that MSCs can induce peripheral tolerance, enhancing their potential for therapeutic applications[45,48]. A higher immunosuppression of T-cell alloreactivity has also been demonstrated in DPSCs in comparison with bone marrow stem cells[52]. These properties distinguish DPSCs as one of the most accessible cell sources for cell-based therapy in regenerative medicine and inflammation-related diseases[46].

To overcome rejection and use DPSCs in transplantation medicine, the formation of a histocompatibility bank is an attractive option, where DPSCs are stored after HLA isotyping. Ideally, considering the large numbers of wisdom teeth extracted from genetically diverse populations, adequate levels of isotype matching with patients may be achieved. The establishment of an HLA-organized DPSC allogenic bank could be sufficient to provide stem cells for a large number of patients. The concept of haplobanking with HLA homozygous cell lines would also limit the number of HLA mismatches[53]. Several studies have been conducted to determine the number of donors needed to cover the population of a country. These findings are dependent on ethnic disparities of the population and the type of stem cells considered. The creation of a bank containing highly selected homozygous lines is an attractive approach to HLA matching. Selected homozygous lines can provide HLA matches for a wide percentage of populations. Estimates of the number of homozygous cell lines needed have mainly been established considering embryonic stem cells, for the main proteins HLA-A, HLA-B and HLA-DR. Data from cadaveric organ donors, cord blood bank or *in vitro* fertilization-derived embryos led to the estimate that approximately 150-190 human embryonic stem cell lines with various HLA genotypes, or a collection of 10-30 homozygous lines for the common HLA types, would be sufficient to provide HLA-matches for a wide part of the population in the United Kingdom[54], Japan[55,56], the United States[57] or China[58]. Because of the low incidence (1.5%) of HLA-homozygous individuals in the normal population[54], a systematic collection of discarded wisdom teeth would be of prime interest. The determination of the HLA types of 100 DPSC lines from teeth collected in Japan revealed 2 homozygous lines for all the 3 considered HLA loci. These 2 homozygous lines therefore have the potential to cover approximately 20% of the Japanese population with a perfect match[59].

Methods and good manufacturing practices

The production and marketing of stem cell-based therapy faces imperative steps, including product characterization, safety testing and clinical trials design. At both national and international levels, numerous standards and regulations must be followed in order

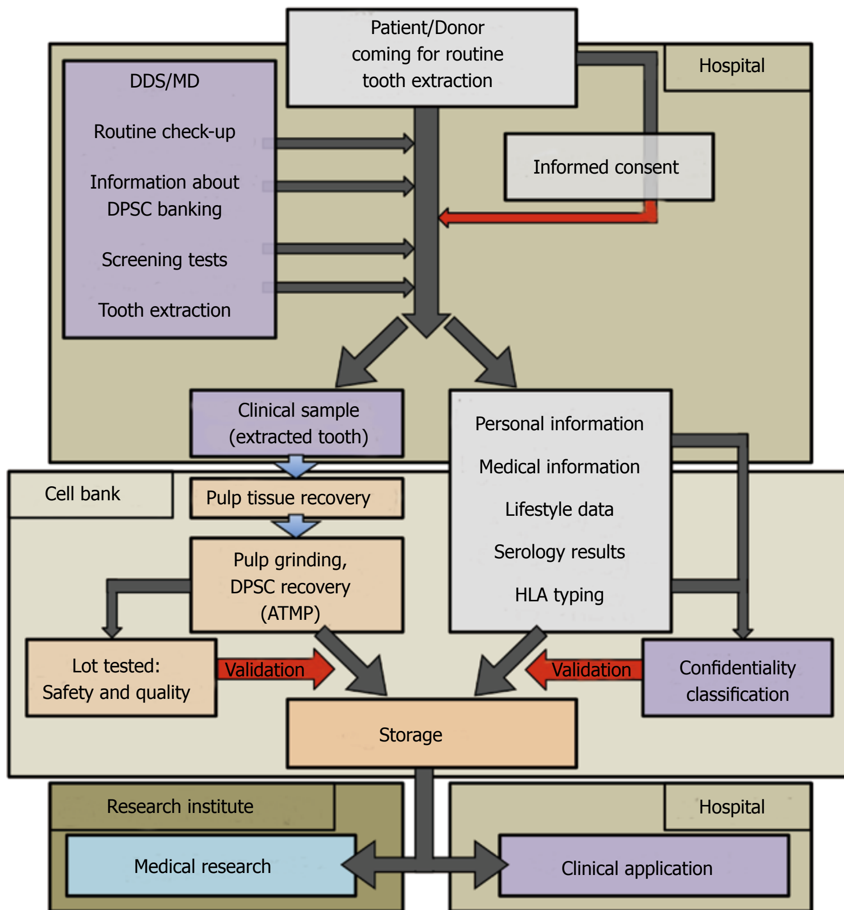


Figure 2 Process flowchart for current Good Manufacturing Practices manufacturing of dental pulp stem cell lots/therapeutic products. The chart is divided into 4 areas: Hospital for tooth recovery, Cell Bank, Research Institute and Hospital for clinical applications: (1) Hospital: direct contact between patients and authorized medical staff, such as Medical Doctor (MD) or Doctor of Dental Surgery (DDS). Clinical sample and donor's personal data are recovered; (2) Cell Bank: current Good Manufacturing Practices manufacturing of Advanced Therapy Medicinal Products (ATMP) from the biological samples (dental pulps). All data concerning the donors are anonymous; (3) Research Institute: dental pulp stem cell (DPSC) lots are used for animal experiments to develop new therapeutics; and (4) Hospital: clinical grade DPSC lots are used for therapeutics. Red arrows represent critical parameters related to each step of processing (informed consent, quality, safety, confidentiality). HLA: Human leukocyte antigen.

to translate DPSCs into clinical products. There are variations in these international and national guidelines, and in the regulations that are applied to the collection and storage of human tissue, personal data and medical records[32]. The Food and Drug Administration, in the United States, and the European Medicines Agency (EMA), in Europe, are responsible for creating and enforcing these regulations. In Europe, stem cells for clinical therapies are classified under advanced therapy medicinal products (ATMP) unless they are minimally manipulated and intended for homologous use[60]. A Committee for advanced therapies (CAT) has even been created to evaluate cell production marketing by assessing the quality, safety and efficacy of ATMPs, in accordance with the regulatory framework. EMA regulation defines the current Good Manufacturing Practices (cGMP) guidelines to manufacture ATMPs[61]. Even though clinical grade production of DPSCs needs to be implemented, DPSCs can be isolated, stored, and eventually expanded by applying rational modifications to the commonly used methods[15,62], in order to continue complying with good manufacturing practices[63] from the donor (patient having his/her tooth extracted, in aseptic condition) to the storage tank. The critical step of enzymatic pulp tissue digestion can be replaced by mechanical disruption in single use devices, such as a tissue grinder/homogenizer. Fetal bovine serum usually required for *in vitro* expansion can be replaced by human serum supplements derived from peripheral blood serum, peripheral blood plasma, or platelet lysate[64]. Moreover, genetic stability has been demonstrated for DPSCs for up to 9 cell passages[65,66].

Legal and practical issues (consent, confidentiality, commercialization)

Translation of DPSC research into clinical applications relies on abundant *in vitro* and *in vivo* preclinical data. However, when it comes to potential therapeutic applications, some barriers can appear, due to restrictions specified in the consent document used for the collection of biological materials, questions about ownership of the collected DPSCs, and the confidentiality of the information associated with the cell lines[10]. The constitution of an allogenic DPSC bank contains

procedures to ensure anonymity, although authorized parties can access some clinically relevant information.

The rights of donors and the interests of researchers are protected by incorporating relevant government legislation (ethical committee review) and procedures (*e.g.*, anonymity and consent). It is crucial to explain the use and transfer of cells and data at the time of informed consent, especially highlighting features that distinguish collection for research from collection for a biobank[67]. The whole process, from the patient coming for tooth extraction to storage of DPSC lots, is presented in Figure 2.

Allogenic DPSC biobanking brings together a multitude of data on individuals, including health and lifestyle. Thus, the way the informed consent is obtained should reflect the personal information used for medical research, taking into account that the patient was originally coming for a routine tooth extraction. As informed consent is derived from the standard that every donor has the right to self-determination, the patient must be informed about the nature of biobanking, the procedures in which the tooth he has donated might be involved, and the expected outcome of the research[36,68]. The physical and intellectual property of biological samples collected must be clearly established and explained[69].

INNOVATIVE THERAPEUTICS

Upon discovery of stem cells in the dental pulp, DPSCs demonstrated their ability to regenerate a complex consisting of a mineralized matrix of odontoblasts and connective tissue containing blood vessels similar to that observed in normal human tooth[12]. Since then, the use range of potential medical applications based on DPSCs include the repair and regeneration of bone[13,70], the central nervous system[27,71,72], liver tissue[73], heart tissue[74], eyes[75], muscles[76,77], and salivary gland cells[78,79]. Overall, it holds great potential in the field of regenerative medicine and tissue engineering[13,23] alone or combined with various biomaterials[80-84]. Some have proposed that DPSCs may have greater potential than the current MSC gold standard, the bone marrow-derived MSC[29].

Allogenic banking of DPSCs could boost industrial and therapeutic innovations by providing tools for unsolved medical problems, including the production of advanced therapy medicinal products (ATMP). We present here some clinical applications, for which there is no efficient therapeutics so far, but that DPSCs-based ATMP could potentially treat.

Spinal cord injuries

Chronic medullary lesion, a result of spinal cord trauma, is characterized by neurologic deficiency without evolution. Six months after trauma, these lesions are considered chronic, with no chance of improvement. According to the Christopher Reeves foundation, these spinal cord lesions affect 1.2 million persons in the United States and 300000 persons in Europe. Numerous preclinical studies have been conducted to graft stem cells of various origins into injured spinal cords, such as neural stem cells[85], embryonic stem cells[86-88], with encouraging results[89]. DPSCs, due to their embryologic origin, express some markers of both mesenchymal and neuroectodermic origin[12,16]. Indeed, DPSCs originate from migrating cranial neural crest cells. During embryonic development, these neural crest cells differentiate into a wide variety of cell types, including neurons of the peripheral nervous system[27]. MSCs have been thought to be usable in the treatment of spinal cord injuries, and adult human DPSCs could provide an ideal source of stem cells for therapeutic applications in such neurological pathologies[90]. The efficiency of DPSCs in improving neural regeneration has been shown *in vitro*[72,73,91] and *in vivo* after spinal cord injury[92-94]. These preclinical data enhance the therapeutic potential of intrathecally administrated HLA-typed DPSC lots in treatment of nerve tissue injuries.

Sjögren's syndrome

Sjögren's syndrome is an autoimmune pathology affecting 0.2% to 3% of the general population[95]. It is a chronic inflammation of the salivary and lacrimal glands, characterized by lymphocytic infiltration of the exocrine glands with a polyclonal B cell activation[96]. Although the pathogenesis of primary Sjögren's syndrome remains unclear, T cells and B cells have been shown to be involved. Pharmacological treatments have limited efficiency, with only the capacity to temporarily ameliorate symptoms, and with no modification of the overall course of the disease[97]. Given the lack of disease-modifying drugs, treatment options are now focused on biotherapies[98]. As detailed above, immunomodulatory properties of MSCs have been demonstrated *in vitro* and *in vivo*, suggesting a therapeutic potential for autoimmune disease treatments[47], especially through anti-inflammatory cytokines production and T regulatory cells promotion[99]. These immunomodulatory properties have also been demonstrated for DPSCs, identifying them as a cell source for cell-based therapy of immune and inflammation-related diseases[47]. Intravenous injection and local injection of DPSC lots into salivary glands represents a potential novel immunotherapeutic tool for autoimmune Sjögren's syndrome.

Irradiated salivary glands

Cancers that originate from the aerodigestive epithelium, including carcinomas of the head and neck, are the leading causes of cancer-related mortality worldwide, accounting for about 2 million deaths and 500000 new cancers diagnosed annually[100,101]. Treatment involves chemotherapy, radiotherapy, and surgery. Radiation-induced salivary hypofunction is one of the major developments that affect survivors. Even though radiotherapy is focused on the cancerous area, radiations often affect salivary glands, causing severe hyposialia and oral dryness after orofacial cancer

treatment; hyposalivation underlying xerostomia after radiotherapy is still a major problem in the treatment of head and neck cancer. To date, the only treatment for this oral dryness is the use of artificial saliva to supply salivary glands, a treatment with limited efficiency. Salivary stem cell (salisphere) transplantation has been shown to functionally restore salivary gland efficiency after radiation-induced impairment of salivary gland function and consequential xerostomia[79]. Furthermore, it was demonstrated that DPSCs used as a cell source for the treatment of salivary gland hypofunction could partially revert this hypofunction[80]. Thus, stem cell-based therapy has great potential in prevention or treatment of radiation-induced hyposalivation[102]. New therapeutic strategies are now being considered using stem cells injected intravenously or directly into salivary glands to allow salivary gland cell reactivation[103-106].

Acute periodontitis

Periodontal diseases are highly prevalent diseases that can affect up to 90% of the population worldwide. They have various forms, from gingivitis, the mildest form caused by dental plaque, to periodontitis, which induces the loss of connective tissue and bone support, and causes tooth loss in adults[107]. Acute periodontitis is an inflammatory disease of the periodontium triggered by the host's immune response and resulting in the progressive loss of gingival tissue, periodontal ligament and supporting alveolar bone[108]. Actual therapeutics consist of the control of bacterial infection and the stabilization of tissue loss. Regenerative treatment using bone grafts, gingiva grafts, and growth factors offer interesting possibilities, but only in specific indications[109-111], and with unpredictable results[112]. In this context, topical application of stem cells in periodontal lesions appeared to be a promising strategy to regenerate periodontium[113]. *In vitro* studies demonstrated the ability of DPSCs to differentiate into osteoblast and cementoblast lineage, and to participate in periodontal ligament and cementum regeneration[114,115]. *In vivo* experiments enhanced the therapeutic potential of dental stem cell grafting to regenerate periodontal tissues[116-118]. Allogenic transplantation could enhance periodontal tissue repair and limit local inflammation through MSC immunomodulation[108,119].

Endodontic regeneration

Dental pulp, the soft connective tissue described above, is the tissue entrapped within the teeth, in which are recovered DPSCs. In case of dental decay, this tissue can be infected and become necrotic, because it is encased in a thick dentin wall, and consists of a microcirculatory system originating from a very small opening at the apex of the root. This anatomical configuration limits the development vascular supply during pulp regeneration. Endodontic treatment, when needed, includes the removal of vital and necrotic tissues from the root canal system, along with infected root dentine. It aims to prepare the canal space to facilitate disinfection by irrigants and medicaments. Prevention of reinfection is then achieved through the provision of a fluid-tight root canal filling and a coronal restoration[120]. The potential possibility of regeneration of pulp tissue by cell therapy is a promising approach for the future treatment of pulpitis or peri-apical disease assuring longevity of teeth and improved quality of life. It has been demonstrated that transplantation of DPSC was capable of inducing complete pulp regeneration in a root canal after pulpectomy[121]. Thus, the use of DPSC, combined with a supporting scaffold, could be used to treat and heal infected root canals, providing an interesting alternative to the actual inert fillings used in endodontics. Root canals anatomy limits the use of rigid scaffold systems in pulp regeneration: scaffolds for pulp regeneration should be injectable, with fibrous structures that ideally mimic the extracellular matrix of the pulp tissue and support stem cells growth.

Induced pluripotent stem generation from DPSC

Pluripotent stem cells can be induced from fibroblasts by retroviral introduction of Oct3/4, Sox2, c-Myc and Klf4[122]. These induced pluripotent stem (iPS) cells are similar to embryonic stem cells in morphology, proliferation and differentiation capacities[123]. They proliferate extensively and differentiate into virtually any desired cell type, providing an unlimited source of replacement cells for human therapy[124]. It has been shown that DPSCs could be also reprogrammed into iPS cells, with a higher efficiency rate than dermal fibroblasts. DPSCs-derived iPS cells were indistinguishable from human embryonic stem cells, highlighting the potential of DPSCs as an alternative source for generating iPS cells[125,126]. Many reprogrammed cell lines could easily be established from DPSCs obtained from young patients with a low risk of bacterial contamination and genetic modification, as extracted wisdom teeth are generally aseptically obtained from the mandible and are protected from ultraviolet and other damage by surrounding hard tissues. It was shown that iPS cells could be efficiently generated from DPSCs using the conventional 4 reprogramming factors (Oct3/4, Sox2, c-Myc and Klf4)[59,125,126], as well as using only 3 factors (Oct3/4, Sox2 and Klf4)[59], or even using only 2 non-oncogenic factors (Oct4 and Sox2)[30]. Interestingly, the efficiency rate of reprogramming was related to the donor's age, with higher rate for younger patients with wisdom teeth still under maturation[59].

With respect to safety, it would be ideal not to use retrovirus vectors for transient expression of the reprogramming genes. DPSCs are assumed to offer high efficiency of iPS cell generation even with the use of non-integrating vectors such as Sendai viruses or modified mRNA. Clinical use of iPS in regenerative medicine is very promising. However, time-efficiency and financial considerations argue in favor of the use of allogenic rather than autologous iPS lines. Similarly to DPSCs, biobanking of iPS lines would be a reasonable

strategy. In this setting, DPSC banking could be of great help to establish iPS cell banks with a sufficient repertoire of HLA types, since the establishment of clinical-grade iPS cell lines from individual patients would require much time and incur a high cost[59].

From DPSC to successful therapy: Limitations and issues

The notion of stem cells as postnatal units of organ or tissue regeneration allows imagining therapies such as tissue engineering. A stem cell that could be expanded and modified *ex vivo,* and transplanted *in vivo* encourages attempts to treat severe or lethal diseases. However, even when the use of stem cells could replace the lost cells, it does not guarantee that the regenerated cells could circumvent the cell death caused by the disease. And replacing lost cells, even with cells expressing several specific cell markers, is far from a successful therapy with fully functional cells. Indeed, clinically successful translation of stem cell science into medicine has been conducted following a simple framework in which organ-specific stem cells were used for organ-specific diseases[6]. The roles of DPSC, and MSCs in general, as niche cells, tissue organizers and skeletal or neural progenitors open opportunities and pose challenges. The molecular mechanisms by which stem cells become functional are still largely unknown. Its comprehension and identification may involve new methods that go far beyond the empirical injection of poorly characterized cultured cell strains[6]. Such methods may involve modeling of disease mechanisms, identification of cell-derived bioactive factors and their use as drugs (including factors mediating the interactions with endothelial and hematopoietic cells), definition of targeted specific disease mechanisms and organ-specific strategies to deliver DPSC to a site of interest[127].

CONCLUSION

DPSC-based therapy is now entering into a new stage of development, shifting from initial *in vitro* and *in vivo* studies to optimization of therapeutic products for clinical applications. As for other MSC, there are still many challenges concerning stem cell potency, age-related and disease-related tissue impairment, and production of clinical grade stem cells lots. The strategies presented in this review emphasize the potential of DPSC to be used for innovative clinical trials based on rational DPSC therapy, following GMP conditions.

Indeed, dental pulp is a remarkable site of stem cells, and the collection of stem cells from dental pulp is a non-invasive practice that can be performed after routine wisdom teeth extraction. DPSC can be recovered in GMP conditions and cryopreserved for long periods, after HLA typing. However, in the perspective of therapeutic use, optimization and better methods are still necessary for DPSC isolation, expansion and banking.

Although producing and storing patient-specific stem cells could resolve immunological problems, this procedure would be costly, laborious, and time-consuming. Allogenic DPSC banking containing clinical grade stem cell lines offers an alternative and provides stem cell lines from which it will be possible to choose a HLA match for the patient to be treated. There are variations in national and international regulations for the collection and storage of human tissue, but ethical principles related to biobanks always include safety, informed consent and confidentiality. The recovery of DPSC doesn't involve any invasive procedure as they come from already extracted teeth. Thus, the main concerns are: (1) for the donor, clear explanations about the banking project and confidentiality of all personal and medical data; and (2) for the patient, safety of DPSC lots produced in accordance to guidelines. To date, despite promising preclinical data, clinical trials using DPSCs have not been widely reported. Allogenic biobanks represent a new strategy that aims to develop the clinical applications of the DPSC potential, involving both researchers and clinicians.

REFERENCES

1 **Robey PG**. Stem cells near the century mark. *J Clin Invest* 2000; **105**: 1489-1491 [PMID: 10841501 DOI: 10.1172/JCI10256]

2 **Caplan AI**. Review: mesenchymal stem cells: cell-based reconstructive therapy in orthopedics. *Tissue Eng* 2005; **11**: 1198-1211 [PMID: 16144456 DOI: 10.1089/ten.2005.11.1198]

3 **Dominici M**, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop Dj, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006; **8**: 315-317 [PMID: 16923606 DOI: 10.1080/14653240600855905]

4 **Horwitz EM**, Dominici M. How do mesenchymal stromal cells exert their therapeutic benefit? *Cytotherapy* 2008; **10**: 771-774 [PMID: 19089685 DOI: 10.1080/14653240802618085]

5 **Ma T**. Mesenchymal stem cells: From bench to bedside. *World J Stem Cells* 2010; **2**: 13-17 [PMID: 21607111 DOI: 10.4252/wjsc.v2.i2.13]

6 **Bianco P**, Cao X, Frenette PS, Mao JJ, Robey PG, Simmons PJ, Wang CY. The meaning, the sense and the significance: translating the science of mesenchymal stem cells into medicine. *Nat Med* 2013; **19**: 35-42 [PMID: 23296015 DOI: 10.1038/nm.3028]

7 **Mastri M**, Lin H, Lee T. Enhancing the efficacy of mesenchymal stem cell therapy. *World J Stem Cells* 2014; **6**: 82-93 [PMID: 24772236 DOI: 10.4252/wjsc.v6.i2.82]

8 **Crook JM**, Hei D, Stacey G. The International Stem Cell Banking Initiative (ISCBI): raising standards to bank on. *In Vitro Cell Dev Biol Anim* 2010; **46**: 169-172 [PMID: 20195786 DOI: 10.1007/s11626-010-9301-7]

9 **Borstlap J**, Luong MX, Rooke HM, Aran B, Damaschun A, Elstner A, Smith KP, Stein GS, Veiga A. International stem cell registries. *In Vitro Cell Dev Biol Anim* 2010; **46**: 242-246 [PMID: 20177992 DOI: 10.1007/s11626-010-9295-1]

10 **Kato K**, Kimmelman J, Robert J, Sipp D, Sugarman J. Ethical and policy issues in the clinical translation of stem cells: report of a focus session at the ISSCR Tenth Annual Meeting. *Cell Stem Cell* 2012; 11: 765-767 [PMID: 23217422]

11 **Sonoyama W**, Yamaza T, Gronthos S, Shi S. Multipotent Stem Cells in Dental Pulp. In: Freshney RI, Stacey GN, Auerbach JM. Culture of Human Stem Cells. Hoboken (NJ): John Wiley & Sons,

Inc., 2007: 187-206 [DOI: 10.1002/9780470167526.ch8]
12 **Gronthos S**, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. *Proc Natl Acad Sci USA* 2000; **97**: 13625-13630 [PMID: 11087820 DOI: 10.1073/pnas.240309797]
13 **d'Aquino R**, De Rosa A, Laino G, Caruso F, Guida L, Rullo R, Checchi V, Laino L, Tirino V, Papaccio G. Human dental pulp stem cells: from biology to clinical applications. *J Exp Zool B Mol Dev Evol* 2009; **312B**: 408-415 [PMID: 19065566 DOI: 10.1002/jez.b.21263]
14 **Goldberg M**, Smith AJ. Cells and extracellular matrices of dentin and pulp: a biological basis for repair and tissue engineering. *Crit Rev Oral Biol Med* 2004; **15**: 13-27 [PMID: 14761897 DOI: 10.1177/154411130401500103]
15 **Tirino V**, Paino F, De Rosa A, Papaccio G. Identification, isolation, characterization, and banking of human dental pulp stem cells. *Methods Mol Biol* 2012; **879**: 443-463 [PMID: 22610575 DOI: 10.1007/978-1-61779-815-3_26]
16 **Miura M**, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG, Shi S. SHED: stem cells from human exfoliated deciduous teeth. *Proc Natl Acad Sci USA* 2003; **100**: 5807-5812 [PMID: 12716973 DOI: 10.1073/pnas.0937635100]
17 **Laino G**, d'Aquino R, Graziano A, Lanza V, Carinci F, Naro F, Pirozzi G, Papaccio G. A new population of human adult dental pulp stem cells: a useful source of living autologous fibrous bone tissue (LAB). *J Bone Miner Res* 2005; **20**: 1394-1402 [PMID: 16007337 DOI: 10.1359/JBMR.050325]
18 **Sonoyama W**, Liu Y, Fang D, Yamaza T, Seo BM, Zhang C, Liu H, Gronthos S, Wang CY, Wang S, Shi S. Mesenchymal stem cell-mediated functional tooth regeneration in swine. *PLoS One* 2006; **1**: e79 [PMID: 17183711 DOI: 10.1371/journal.pone.0000079]
19 **Seo BM**, Miura M, Gronthos S, Bartold PM, Batouli S, Brahim J, Young M, Robey PG, Wang CY, Shi S. Investigation of multipotent postnatal stem cells from human periodontal ligament. *Lancet* 2004; **364**: 149-155 [PMID: 15246727 DOI: 10.1016/S0140-6736(04)16627-0]
20 **Huang AH**, Chen YK, Lin LM, Shieh TY, Chan AW. Isolation and characterization of dental pulp stem cells from a supernumerary tooth. *J Oral Pathol Med* 2008; **37**: 571-574 [PMID: 18331285 DOI: 10.1111/j.1600-0714.2008.00654.x]
21 **Morsczeck C**, Götz W, Schierholz J, Zeilhofer F, Kühn U, Möhl C, Sippel C, Hoffmann KH. Isolation of precursor cells (PCs) from human dental follicle of wisdom teeth. *Matrix Biol* 2005; **24**: 155-165 [PMID: 15890265 DOI: 10.1016/j.matbio.2004.12.004]
22 **Shi S**, Robey PG, Gronthos S. Comparison of human dental pulp and bone marrow stromal stem cells by cDNA microarray analysis. *Bone* 2001; **29**: 532-539 [PMID: 11728923 DOI: 10.1016/S8756-3282(01)00612-3]
23 **Gronthos S**, Brahim J, Li W, Fisher LW, Cherman N, Boyde A, DenBesten P, Robey PG, Shi S. Stem cell properties of human dental pulp stem cells. *J Dent Res* 2002; **81**: 531-535 [PMID: 12147742 DOI: 10.1177/154405910208100806]
24 **Huang GT**, Gronthos S, Shi S. Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine. *J Dent Res* 2009; **88**: 792-806 [PMID: 19767575 DOI: 10.1177/0022034509340867]
25 **d'Aquino R**, Graziano A, Sampaolesi M, Laino G, Pirozzi G, De Rosa A, Papaccio G. Human postnatal dental pulp cells co-differentiate into osteoblasts and endotheliocytes: a pivotal synergy leading to adult bone tissue formation. *Cell Death Differ* 2007; **14**: 1162-1171 [PMID: 17347663 DOI: 10.1038/sj.cdd.4402121]
26 **Almushayt A**, Narayanan K, Zaki AE, George A. Dentin matrix protein 1 induces cytodifferentiation of dental pulp stem cells into odontoblasts. *Gene Ther* 2006; **13**: 611-620 [PMID: 16319946 DOI: 10.1038/sj.gt.3302687]
27 **Arthur A**, Rychkov G, Shi S, Koblar SA, Gronthos S. Adult human dental pulp stem cells differentiate toward functionally active neurons under appropriate environmental cues. *Stem Cells* 2008; **26**: 1787-1795 [PMID: 18499892 DOI: 10.1634/stemcells.2007-0979]
28 **Iohara K**, Zheng L, Ito M, Tomokiyo A, Matsushita K, Nakashima M. Side population cells isolated from porcine dental pulp tissue with self-renewal and multipotency for dentinogenesis, chondrogenesis, adipogenesis, and neurogenesis. *Stem Cells* 2006; **24**: 2493-2503 [PMID: 16873765 DOI: 10.1634/stemcells.2006-0161]
29 **Alge DL**, Zhou D, Adams LL, Wyss BK, Shadday MD, Woods EJ, Gabriel Chu TM, Goebel WS. Donor-matched comparison of dental pulp stem cells and bone marrow-derived mesenchymal stem cells in a rat model. *J Tissue Eng Regen Med* 2010; **4**: 73-81 [PMID: 19842108 DOI: 10.1002/term.220]
30 **Yoo CH**, Na HJ, Lee DS, Heo SC, An Y, Cha J, Choi C, Kim JH, Park JC, Cho YS. Endothelial progenitor cells from human dental pulp-derived iPS cells as a therapeutic target for ischemic vascular diseases. *Biomaterials* 2013; **34**: 8149-8160 [PMID: 23896001 DOI: 10.1016/j.biomaterials.2013.07.001]
31 **Riegman PH**, Morente MM, Betsou F, de Blasio P, Geary P. Biobanking for better healthcare. *Mol Oncol* 2008; **2**: 213-222 [PMID: 19383342 DOI: 10.1016/j.molonc.2008.07.004]
32 **West CC**, Murray IR, González ZN, Hindle P, Hay DC, Stewart KJ, Péault B. Ethical, legal and practical issues of establishing an adipose stem cell bank for research. *J Plast Reconstr Aesthet Surg* 2014; **67**: 745-751 [PMID: 24529696 DOI: 10.1016/j.bjps.2014.01.030]
33 **Yuille M**, Dixon K, Platt A, Pullum S, Lewis D, Hall A, Ollier W. The UK DNA banking network: a "fair access" biobank. *Cell Tissue Bank* 2010; **11**: 241-251 [PMID: 19672698 DOI: 10.1007/s10561-009-9150-3]
34 **Hewitt RE**. Biobanking: the foundation of personalized medicine. *Curr Opin Oncol* 2011; **23**: 112-119 [PMID: 21076300 DOI: 10.1097/CCO.0b013e32834161b8]
35 **Knoppers BM**, Isasi R. Stem cell banking: between traceability and identifiability. *Genome Med* 2010; **2**: 73 [PMID: 20923580 DOI: 10.1186/gm194]
36 **Artene SA**, Ciurea ME, Purcaru SO, Tache DE, Tataranu LG, Lupu M, Dricu A. Biobanking in a constantly developing medical world. *ScientificWorldJournal* 2013; **2013**: 343275 [PMID: 24174912 DOI: 10.1155/2013/343275]
37 **Goh BC**, Thirumala S, Kilroy G, Devireddy RV, Gimble JM. Cryopreservation characteristics of adipose-derived stem cells: maintenance of differentiation potential and viability. *J Tissue Eng Regen Med* 2007; **1**: 322-324 [PMID: 18038424 DOI: 10.1002/term.35]
38 **Couble ML**, Farges JC, Bleicher F, Perrat-Mabillon B, Boudeulle M, Magloire H. Odontoblast differentiation of human dental pulp cells in explant cultures. *Calcif Tissue Int* 2000; **66**: 129-138 [PMID: 10652961 DOI: 10.1007/PL00005833]
39 **Perry BC**, Zhou D, Wu X, Yang FC, Byers MA, Chu TM, Hockema JJ, Woods EJ, Goebel WS. Collection, cryopreservation, and characterization of human dental pulp-derived mesenchymal stem cells for banking and clinical use. *Tissue Eng Part C Methods* 2008; **14**: 149-156 [PMID: 18489245 DOI: 10.1089/ten.tec.2008.0031]
40 **Woods EJ**, Perry BC, Hockema JJ, Larson L, Zhou D, Goebel WS. Optimized cryopreservation method for human dental pulp-derived stem cells and their tissues of origin for banking and clinical use. *Cryobiology* 2009; **59**: 150-157 [PMID: 19538953 DOI: 10.1016/j.cryobiol.2009.06.005]
41 **Taylor CJ**, Bolton EM, Bradley JA. Immunological considerations for embryonic and induced pluripotent stem cell banking. *Philos Trans R Soc Lond B Biol Sci* 2011; **366**: 2312-2322 [PMID: 21727137 DOI: 10.1098/rstb.2011.0030]
42 **Auchincloss H**, Bonventre JV. Transplanting cloned cells into therapeutic promise. *Nat Biotechnol* 2002; **20**: 665-666 [PMID: 12089546 DOI: 10.1038/nbt0702-665]
43 **Bradley JA**, Bolton EM, Pedersen RA. Stem cell medicine encounters the immune system. *Nat Rev Immunol* 2002; **2**: 859-871 [PMID: 12415309 DOI: 10.1038/nri934]
44 **Drukker M**, Katz G, Urbach A, Schuldiner M, Markel G, Itskovitz-Eldor J, Reubinoff B, Mandelboim O, Benvenisty N. Characterization of the expression of MHC proteins in human embryonic stem cells. *Proc Natl Acad Sci USA* 2002; **99**: 9864-9869

[PMID: 12114532 DOI: 10.1073/pnas.142298299]
45 **Montespan F**, Deschaseaux F, Sensébé L, Carosella ED, Rouas-Freiss N. Osteodifferentiated mesenchymal stem cells from bone marrow and adipose tissue express HLA-G and display immunomodulatory properties in HLA-mismatched settings: implications in bone repair therapy. *J Immunol Res* 2014; **2014**: 230346 [PMID: 24877156 DOI: 10.1155/2014/230346]
46 **Li Z**, Jiang CM, An S, Cheng Q, Huang YF, Wang YT, Gou YC, Xiao L, Yu WJ, Wang J. Immunomodulatory properties of dental tissue-derived mesenchymal stem cells. *Oral Dis* 2014; **20**: 25-34 [PMID: 23463961 DOI: 10.1111/odi.12086]
47 **Djouad F**, Plence P, Bony C, Tropel P, Apparailly F, Sany J, Noël D, Jorgensen C. Immunosuppressive effect of mesenchymal stem cells favors tumor growth in allogeneic animals. *Blood* 2003; **102**: 3837-3844 [PMID: 12881305 DOI: 10.1182/blood-2003-04-1193]
48 **Ghannam S**, Bouffi C, Djouad F, Jorgensen C, Noël D. Immunosuppression by mesenchymal stem cells: mechanisms and clinical applications. *Stem Cell Res Ther* 2010; **1**: 2 [PMID: 20504283 DOI: 10.1186/scrt2]
49 **Maumus M**, Guérit D, Toupet K, Jorgensen C, Noël D. Mesenchymal stem cell-based therapies in regenerative medicine: applications in rheumatology. *Stem Cell Res Ther* 2011; **2**: 14 [PMID: 21457518 DOI: 10.1186/scrt55]
50 **Uccelli A**, Pistoia V, Moretta L. Mesenchymal stem cells: a new strategy for immunosuppression? *Trends Immunol* 2007; **28**: 219-226 [PMID: 17400510 DOI: 10.1016/j.it.2007.03.001]
51 **Bartholomew A**, Sturgeon C, Siatskas M, Ferrer K, McIntosh K, Patil S, Hardy W, Devine S, Ucker D, Deans R, Moseley A, Hoffman R. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. *Exp Hematol* 2002; **30**: 42-48 [PMID: 11823036]
52 **Pierdomenico L**, Bonsi L, Calvitti M, Rondelli D, Arpinati M, Chirumbolo G, Becchetti E, Marchionni C, Alviano F, Fossati V, Staffolani N, Franchina M, Grossi A, Bagnara GP. Multipotent mesenchymal stem cells with immunosuppressive activity can be easily isolated from dental pulp. *Transplantation* 2005; **80**: 836-842 [PMID: 16210973]
53 **Zimmermann A**, Preynat-Seauve O, Tiercy JM, Krause KH, Villard J. Haplotype-based banking of human pluripotent stem cells for transplantation: potential and limitations. *Stem Cells Dev* 2012; **21**: 2364-2373 [PMID: 22559254 DOI: 10.1089/scd.2012.0088]
54 **Taylor CJ**, Bolton EM, Pocock S, Sharples LD, Pedersen RA, Bradley JA. Banking on human embryonic stem cells: estimating the number of donor cell lines needed for HLA matching. *Lancet* 2005; **366**: 2019-2025 [PMID: 16338451 DOI: 10.1016/S0140-6736(05)67813-0]
55 **Nakajima F**, Tokunaga K, Nakatsuji N. Human leukocyte antigen matching estimations in a hypothetical bank of human embryonic stem cell lines in the Japanese population for use in cell transplantation therapy. *Stem Cells* 2007; **25**: 983-985 [PMID: 17185611 DOI: 10.1634/stemcells.2006-0566]
56 **Nakatsuji N**, Nakajima F, Tokunaga K. HLA-haplotype banking and iPS cells. *Nat Biotechnol* 2008; **26**: 739-740 [PMID: 18612291 DOI: 10.1038/nbt0708-739]
57 **Faden RR**, Dawson L, Bateman-House AS, Agnew DM, Bok H, Brock DW, Chakravarti A, Gao XJ, Greene M, Hansen JA, King PA, O'Brien SJ, Sachs DH, Schill KE, Siegel A, Solter D, Suter SM, Verfaillie CM, Walters LB, Gearhart JD. Public stem cell banks: considerations of justice in stem cell research and therapy. *Hastings Cent Rep* 2003; **33**: 13-27 [PMID: 14983554 DOI: 10.2307/3527822]
58 **Lin G**, Xie Y, Ouyang Q, Qian X, Xie P, Zhou X, Xiong B, Tan Y, Li W, Deng L, Zhou J, Zhou D, Du L, Cheng D, Liao Y, Gu Y, Zhang S, Liu T, Sun Y, Lu G. HLA-matching potential of an established human embryonic stem cell bank in China. *Cell Stem Cell* 2009; **5**: 461-465 [PMID: 19896436 DOI: 10.1016/j.stem.2009.10.009]
59 **Tamaoki N**, Takahashi K, Tanaka T, Ichisaka T, Aoki H, Takeda-Kawaguchi T, Iida K, Kunisada T, Shibata T, Yamanaka S, Tezuka K. Dental pulp cells for induced pluripotent stem cell banking. *J Dent Res* 2010; **89**: 773-778 [PMID: 20554890 DOI: 10.1177/0022034510366846]
60 **Thirumala S**, Goebel WS, Woods EJ. Manufacturing and banking of mesenchymal stem cells. *Expert Opin Biol Ther* 2013; **13**: 673-691 [PMID: 23339745 DOI: 10.1517/14712598.2013.763925]
61 **Ancans J**. Cell therapy medicinal product regulatory framework in Europe and its application for MSC-based therapy development. *Front Immunol* 2012; **3**: 253 [PMID: 22912639 DOI: 10.3389/fimmu.2012.00253]
62 **La Noce M**, Paino F, Spina A, Naddeo P, Montella R, Desiderio V, De Rosa A, Papaccio G, Tirino V, Laino L. Dental pulp stem cells: state of the art and suggestions for a true translation of research into therapy. *J Dent* 2014; **42**: 761-768 [PMID: 24589847 DOI: 10.1016/j.jdent.2014.02.018]
63 **Tirino V**, Paino F, d'Aquino R, Desiderio V, De Rosa A, Papaccio G. Methods for the identification, characterization and banking of human DPSCs: current strategies and perspectives. *Stem Cell Rev* 2011; **7**: 608-615 [PMID: 21318597 DOI: 10.1007/s12015-011-9235-9]
64 **Bernardo ME**, Avanzini MA, Perotti C, Cometa AM, Moretta A, Lenta E, Del Fante C, Novara F, de Silvestri A, Amendola G, Zuffardi O, Maccario R, Locatelli F. Optimization of in vitro expansion of human multipotent mesenchymal stromal cells for cell-therapy approaches: further insights in the search for a fetal calf serum substitute. *J Cell Physiol* 2007; **211**: 121-130 [PMID: 17187344 DOI: 10.1002/jcp.20911]
65 **Govindasamy V**, Ronald VS, Totey S, Din SB, Mustafa WM, Totey S, Zakaria Z, Bhonde RR. Micromanipulation of culture niche permits long-term expansion of dental pulp stem cells--an economic and commercial angle. *In Vitro Cell Dev Biol Anim* 2010; **46**: 764-773 [PMID: 20725801 DOI: 10.1007/s11626-010-9332-0]
66 **Atari M**, Gil-Recio C, Fabregat M, García-Fernández D, Barajas M, Carrasco MA, Jung HS, Alfaro FH, Casals N, Prosper F, Ferrés-Padró E, Giner L. Dental pulp of the third molar: a new source of pluripotent-like stem cells. *J Cell Sci* 2012; **125**: 3343-3356 [PMID: 22467856 DOI: 10.1242/jcs.096537]
67 **Cyranoski D**. Stem-cell pioneer banks on future therapies. *Nature* 2012; **488**: 139 [PMID: 22874941 DOI: 10.1038/488139a]
68 **Eriksson S**, Helgesson G. Potential harms, anonymization, and the right to withdraw consent to biobank research. *Eur J Hum Genet* 2005; **13**: 1071-1076 [PMID: 15986039 DOI: 10.1038/sj.ejhg.5201458]
69 **De Robbio A**. Biobanks: Patents Or Open Science? 1st ed. Cambridge: Woodhead Publishing Limited, 2013: 1-70 [DOI: 10.1533/9781908818614]
70 **Graziano A**, d'Aquino R, Laino G, Proto A, Giuliano MT, Pirozzi G, De Rosa A, Di Napoli D, Papaccio G. Human CD34+ stem cells produce bone nodules in vivo. *Cell Prolif* 2008; **41**: 1-11 [PMID: 18211282 DOI: 10.1111/j.1365-2184.2007.00497.x]
71 **Huang AH**, Snyder BR, Cheng PH, Chan AW. Putative dental pulp-derived stem/stromal cells promote proliferation and differentiation of endogenous neural cells in the hippocampus of mice. *Stem Cells* 2008; **26**: 2654-2663 [PMID: 18687995 DOI: 10.1634/stemcells.2008-0285]
72 **Király M**, Porcsalmy B, Pataki A, Kádár K, Jelitai M, Molnár B, Hermann P, Gera I, Grimm WD, Ganss B, Zsembery A, Varga G. Simultaneous PKC and cAMP activation induces differentiation of human dental pulp stem cells into functionally active neurons. *Neurochem Int* 2009; **55**: 323-332 [PMID: 19576521 DOI: 10.1016/j.neuint.2009.03.017]
73 **Ikeda E**, Yagi K, Kojima M, Yagyuu T, Ohshima A, Sobajima S, Tadokoro M, Katsube Y, Isoda K, Kondoh M, Kawase M, Go MJ, Adachi H, Yokota Y, Kirita T, Ohgushi H. Multipotent cells from the human third molar: feasibility of cell-based therapy for liver disease. *Differentiation* 2008; **76**: 495-505 [PMID: 18093227 DOI: 10.1111/j.1432-0436.2007.00245.x]
74 **Gandia C**, Armiñan A, García-Verdugo JM, Lledó E, Ruiz A, Miñana MD, Sanchez-Torrijos J, Payá R, Mirabet V, Carbonell-Uberos F, Llop M, Montero JA, Sepúlveda P. Human dental pulp stem cells improve left ventricular function, induce angiogenesis,

and reduce infarct size in rats with acute myocardial infarction. *Stem Cells* 2008; **26**: 638-645 [PMID: 18079433 DOI: 10.1634/stemcells.2007-0484]
75 **Monteiro BG**, Serafim RC, Melo GB, Silva MC, Lizier NF, Maranduba CM, Smith RL, Kerkis A, Cerruti H, Gomes JA, Kerkis I. Human immature dental pulp stem cells share key characteristic features with limbal stem cells. *Cell Prolif* 2009; **42**: 587-594 [PMID: 19614680 DOI: 10.1111/j.1365-2184.2009.00623.x]
76 **Kerkis I**, Kerkis A, Dozortsev D, Stukart-Parsons GC, Gomes Massironi SM, Pereira LV, Caplan AI, Cerruti HF. Isolation and characterization of a population of immature dental pulp stem cells expressing OCT-4 and other embryonic stem cell markers. *Cells Tissues Organs* 2006; **184**: 105-116 [PMID: 17409736 DOI: 10.1159/000099617]
77 **Kerkis I**, Ambrosio CE, Kerkis A, Martins DS, Zucconi E, Fonseca SA, Cabral RM, Maranduba CM, Gaiad TP, Morini AC, Vieira NM, Brolio MP, Sant'Anna OA, Miglino MA, Zatz M. Early transplantation of human immature dental pulp stem cells from baby teeth to golden retriever muscular dystrophy (GRMD) dogs: Local or systemic? *J Transl Med* 2008; **6**: 35 [PMID: 18598348 DOI: 10.1186/1479-5876-6-35]
78 **Lombaert IM**, Brunsting JF, Wierenga PK, Faber H, Stokman MA, Kok T, Visser WH, Kampinga HH, de Haan G, Coppes RP. Rescue of salivary gland function after stem cell transplantation in irradiated glands. *PLoS One* 2008; **3**: e2063 [PMID: 18446241 DOI: 10.1371/journal.pone.0002063]
79 **Yamamura Y**, Yamada H, Sakurai T, Ide F, Inoue H, Muramatsu T, Mishima K, Hamada Y, Saito I. Treatment of salivary gland hypofunction by transplantation with dental pulp cells. *Arch Oral Biol* 2013; **58**: 935-942 [PMID: 23570866 DOI: 10.1016/j.archoralbio.2013.02.015]
80 **Collart-Dutilleul PY**, Secret E, Panayotov I, Deville de Périère D, Martín-Palma RJ, Torres-Costa V, Martin M, Gergely C, Durand JO, Cunin F, Cuisinier FJ. Adhesion and proliferation of human mesenchymal stem cells from dental pulp on porous silicon scaffolds. *ACS Appl Mater Interfaces* 2014; **6**: 1719-1728 [PMID: 24428409 DOI: 10.1021/am4046316]
81 **Haridas V**, Sadanandan S, Collart-Dutilleul PY, Gronthos S, Voelcker NH. Lysine-appended polydiacetylene scaffolds for human mesenchymal stem cells. *Biomacromolecules* 2014; **15**: 582-590 [PMID: 24364714 DOI: 10.1021/bm4015655]
82 **Panayotov IV**, Collart-Dutilleul PY, Salehi H, Martin M, Végh A, Yachouh J, Vladimirov B, Sipos P, Szalontai B, Gergely C, Cuisinier FJ. Sprayed cells and polyelectrolyte films for biomaterial functionalization: the influence of physical PLL-PGA film treatments on dental pulp cell behavior. *Macromol Biosci* 2014; **14**: 1771-1782 [PMID: 25212873 DOI: 10.1002/mabi.201400256]
83 **Moshaverinia A**, Chen C, Akiyama K, Xu X, Chee WW, Schricker SR, Shi S. Encapsulated dental-derived mesenchymal stem cells in an injectable and biodegradable scaffold for applications in bone tissue engineering. *J Biomed Mater Res A* 2013; **101**: 3285-3294 [PMID: 23983201 DOI: 10.1002/jbm.a.34546]
84 **Niu LN**, Sun JQ, Li QH, Jiao K, Shen LJ, Wu D, Tay F, Chen JH. Intrafibrillar-silicified collagen scaffolds enhance the osteogenic capacity of human dental pulp stem cells. *J Dent* 2014; **42**: 839-849 [PMID: 24705068 DOI: 10.1016/j.jdent.2014.03.016]
85 **Cummings BJ**, Uchida N, Tamaki SJ, Salazar DL, Hooshmand M, Summers R, Gage FH, Anderson AJ. Human neural stem cells differentiate and promote locomotor recovery in spinal cord-injured mice. *Proc Natl Acad Sci USA* 2005; **102**: 14069-14074 [PMID: 16172374 DOI: 10.1073/pnas.0507063102]
86 **Kumagai G**, Okada Y, Yamane J, Nagoshi N, Kitamura K, Mukaino M, Tsuji O, Fujiyoshi K, Katoh H, Okada S, Shibata S, Matsuzaki Y, Toh S, Toyama Y, Nakamura M, Okano H. Roles of ES cell-derived gliogenic neural stem/progenitor cells in functional recovery after spinal cord injury. *PLoS One* 2009; **4**: e7706 [PMID: 19893739 DOI: 10.1371/journal.pone.0007706]
87 **Hofstetter CP**, Schwarz EJ, Hess D, Widenfalk J, El Manira A, Prockop DJ, Olson L. Marrow stromal cells form guiding strands in the injured spinal cord and promote recovery. *Proc Natl Acad Sci USA* 2002; **99**: 2199-2204 [PMID: 11854516 DOI: 10.1073/pnas.042678299]
88 **Geffner LF**, Santacruz P, Izurieta M, Flor L, Maldonado B, Auad AH, Montenegro X, Gonzalez R, Silva F. Administration of autologous bone marrow stem cells into spinal cord injury patients via multiple routes is safe and improves their quality of life: comprehensive case studies. *Cell Transplant* 2008; **17**: 1277-1293 [PMID: 19364066]
89 **Sharp J**, Keirstead HS. Therapeutic applications of oligodendrocyte precursors derived from human embryonic stem cells. *Curr Opin Biotechnol* 2007; **18**: 434-440 [PMID: 18023336 DOI: 10.1016/j.copbio.2007.10.006]
90 **Dasari VR**, Veeravalli KK, Dinh DH. Mesenchymal stem cells in the treatment of spinal cord injuries: A review. *World J Stem Cells* 2014; **6**: 120-133 [PMID: 24772239 DOI: 10.4252/wjsc.v6.i2.120]
91 **Nosrat IV**, Widenfalk J, Olson L, Nosrat CA. Dental pulp cells produce neurotrophic factors, interact with trigeminal neurons in vitro, and rescue motoneurons after spinal cord injury. *Dev Biol* 2001; **238**: 120-132 [PMID: 11783998 DOI: 10.1006/dbio.2001.0400]
92 **Taghipour Z**, Karbalaie K, Kiani A, Niapour A, Bahramian H, Nasr-Esfahani MH, Baharvand H. Transplantation of undifferentiated and induced human exfoliated deciduous teeth-derived stem cells promote functional recovery of rat spinal cord contusion injury model. *Stem Cells Dev* 2012; **21**: 1794-1802 [PMID: 21970342 DOI: 10.1089/scd.2011.0408]
93 **Sakai K**, Yamamoto A, Matsubara K, Nakamura S, Naruse M, Yamagata M, Sakamoto K, Tauchi R, Wakao N, Imagama S, Hibi H, Kadomatsu K, Ishiguro N, Ueda M. Human dental pulp-derived stem cells promote locomotor recovery after complete transection of the rat spinal cord by multiple neuro-regenerative mechanisms. *J Clin Invest* 2012; **122**: 80-90 [PMID: 22133879 DOI: 10.1172/JCI59251]
94 **Young F**, Sloan A, Song B. Dental pulp stem cells and their potential roles in central nervous system regeneration and repair. *J Neurosci Res* 2013; **91**: 1383-1393 [PMID: 23996516 DOI: 10.1002/jnr.23250]
95 **Dafni UG**, Tzioufas AG, Staikos P, Skopouli FN, Moutsopoulos HM. Prevalence of Sjögren's syndrome in a closed rural community. *Ann Rheum Dis* 1997; **56**: 521-525 [PMID: 9370875]
96 **Voulgarelis M**, Dafni UG, Isenberg DA, Moutsopoulos HM. Malignant lymphoma in primary Sjögren's syndrome: a multicenter, retrospective, clinical study by the European Concerted Action on Sjögren's Syndrome. *Arthritis Rheum* 1999; **42**: 1765-1772 [PMID: 10446879 DOI: 10.1002/1529-0131(199908)42]
97 **Devauchelle-Pensec V**, Pennec Y, Morvan J, Pers JO, Daridon C, Jousse-Joulin S, Roudaut A, Jamin C, Renaudineau Y, Roué IQ, Cochener B, Youinou P, Saraux A. Improvement of Sjögren's syndrome after two infusions of rituximab (anti-CD20). *Arthritis Rheum* 2007; **57**: 310-317 [PMID: 17330280 DOI: 10.1002/art.22536]
98 **Tobón GJ**, Saraux A, Pers JO, Youinou P. Emerging biotherapies for Sjögren's syndrome. *Expert Opin Emerg Drugs* 2010; **15**: 269-282 [PMID: 20384543 DOI: 10.1517/14728211003702392]
99 **Xu J**, Wang D, Liu D, Fan Z, Zhang H, Liu O, Ding G, Gao R, Zhang C, Ding Y, Bromberg JS, Chen W, Sun L, Wang S. Allogeneic mesenchymal stem cell treatment alleviates experimental and clinical Sjögren syndrome. *Blood* 2012; **120**: 3142-3151 [PMID: 22927248 DOI: 10.1182/blood-2011-11-391144]
100 **Kamangar F**, Dores GM, Anderson WF. Patterns of cancer incidence, mortality, and prevalence across five continents: defining priorities to reduce cancer disparities in different geographic regions of the world. *J Clin Oncol* 2006; **24**: 2137-2150 [PMID: 16682732 DOI: 10.1200/JCO.2005.05.2308]
101 **Karamouzis MV**, Grandis JR, Argiris A. Therapies directed against epidermal growth factor receptor in aerodigestive carcinomas. *JAMA* 2007; **298**: 70-82 [PMID: 17609492 DOI: 10.1001/jama.298.1.70]
102 **Coppes RP**, Stokman MA. Stem cells and the repair of radiation-induced salivary gland damage. *Oral Dis* 2011; **17**: 143-153 [PMID: 20796229 DOI: 10.1111/j.1601-0825.2010.01723.x]
103 **Nanduri LS**, Lombaert IM, van der Zwaag M, Faber H, Brunsting JF, van Os RP, Coppes RP. Salisphere derived c-Kit+ cell

transplantation restores tissue homeostasis in irradiated salivary gland. *Radiother Oncol* 2013; **108**: 458-463 [PMID: 23769181 DOI: 10.1016/j.radonc.2013.05.020]

104 **Lim JY**, Yi T, Choi JS, Jang YH, Lee S, Kim HJ, Song SU, Kim YM. Intraglandular transplantation of bone marrow-derived clonal mesenchymal stem cells for amelioration of post-irradiation salivary gland damage. *Oral Oncol* 2013; **49**: 136-143 [PMID: 22981389 DOI: 10.1016/j.oraloncology.2012.08.010]

105 **Lim JY**, Ra JC, Shin IS, Jang YH, An HY, Choi JS, Kim WC, Kim YM. Systemic transplantation of human adipose tissue-derived mesenchymal stem cells for the regeneration of irradiation-induced salivary gland damage. *PLoS One* 2013; **8**: e71167 [PMID: 23951100 DOI: 10.1371/journal.pone.0071167]

106 **Sumita Y**, Liu Y, Khalili S, Maria OM, Xia D, Key S, Cotrim AP, Mezey E, Tran SD. Bone marrow-derived cells rescue salivary gland function in mice with head and neck irradiation. *Int J Biochem Cell Biol* 2011; **43**: 80-87 [PMID: 20933096 DOI: 10.1016/j.biocel.2010.09.023]

107 **Pihlstrom BL**, Michalowicz BS, Johnson NW. Periodontal diseases. *Lancet* 2005; **366**: 1809-1820 [PMID: 16298220 DOI: 10.1016/S0140-6736(05)67728-8]

108 **Hynes K**, Menicanin D, Gronthos S, Bartold PM. Clinical utility of stem cells for periodontal regeneration. *Periodontol 2000* 2012; **59**: 203-227 [PMID: 22507067 DOI: 10.1111/j.1600-0757.2012.00443.x]

109 **Gonçalves PF**, Gurgel BC, Pimentel SP, Sallum EA, Sallum AW, Casati MZ, Nociti FH. Effect of two different approaches for root decontamination on new cementum formation following guided tissue regeneration: a histomorphometric study in dogs. *J Periodontal Res* 2006; **41**: 535-540 [PMID: 17076778 DOI: 10.1111/j.1600-0765.2006.00902.x]

110 **Needleman IG**, Worthington HV, Giedrys-Leeper E, Tucker RJ. Guided tissue regeneration for periodontal infra-bony defects. *Cochrane Database Syst Rev* 2006; **(2)**: CD001724 [PMID: 16625546 DOI: 10.1002/14651858.CD001724.pub2]

111 **Kaigler D**, Cirelli JA, Giannobile WV. Growth factor delivery for oral and periodontal tissue engineering. *Expert Opin Drug Deliv* 2006; **3**: 647-662 [PMID: 16948560 DOI: 10.1517/17425247.3.5.647]

112 **Yamada Y**, Ueda M, Hibi H, Baba S. A novel approach to periodontal tissue regeneration with mesenchymal stem cells and platelet-rich plasma using tissue engineering technology: A clinical case report. *Int J Periodontics Restorative Dent* 2006; **26**: 363-369 [PMID: 16939018]

113 **Kawaguchi H**, Hirachi A, Hasegawa N, Iwata T, Hamaguchi H, Shiba H, Takata T, Kato Y, Kurihara H. Enhancement of periodontal tissue regeneration by transplantation of bone marrow mesenchymal stem cells. *J Periodontol* 2004; **75**: 1281-1287 [PMID: 15515346 DOI: 10.1902/jop.2004.75.9.1281]

114 **Ikeda E**, Hirose M, Kotobuki N, Shimaoka H, Tadokoro M, Maeda M, Hayashi Y, Kirita T, Ohgushi H. Osteogenic differentiation of human dental papilla mesenchymal cells. *Biochem Biophys Res Commun* 2006; **342**: 1257-1262 [PMID: 16516858 DOI: 10.1016/j.bbrc.2006.02.101]

115 **Flores MG**, Hasegawa M, Yamato M, Takagi R, Okano T, Ishikawa I. Cementum-periodontal ligament complex regeneration using the cell sheet technique. *J Periodontal Res* 2008; **43**: 364-371 [PMID: 18205734 DOI: 10.1111/j.1600-0765.2007.01046.x]

116 **Nakahara T**, Nakamura T, Kobayashi E, Kuremoto K, Matsuno T, Tabata Y, Eto K, Shimizu Y. In situ tissue engineering of periodontal tissues by seeding with periodontal ligament-derived cells. *Tissue Eng* 2004; **10**: 537-544 [PMID: 15165470 DOI: 10.1089/107632704323061898]

117 **Lin NH**, Menicanin D, Mrozik K, Gronthos S, Bartold PM. Putative stem cells in regenerating human periodontium. *J Periodontal Res* 2008; **43**: 514-523 [PMID: 18624941 DOI: 10.1111/j.1600-0765.2007.01061.x]

118 **Khorsand A**, Eslaminejad MB, Arabsolghar M, Paknejad M, Ghaedi B, Rokn AR, Moslemi N, Nazarian H, Jahangir S. Autologous dental pulp stem cells in regeneration of defect created in canine periodontal tissue. *J Oral Implantol* 2013; **39**: 433-443 [PMID: 23964777 DOI: 10.1563/AAID-JOI-D-12-00027]

119 **Ding G**, Liu Y, Wang W, Wei F, Liu D, Fan Z, An Y, Zhang C, Wang S. Allogeneic periodontal ligament stem cell therapy for periodontitis in swine. *Stem Cells* 2010; **28**: 1829-1838 [PMID: 20979138 DOI: 10.1002/stem.512]

120 **Collart Dutilleul PY**, Fonseca CG, Zimányi L, Romieu O, Pozos-Guillén AJ, Semetey V, Cuisinier F, Pérez E, Levallois B. Root canal hydrophobization by dentinal silanization: improvement of silicon-based endodontic treatment tightness. *J Biomed Mater Res B Appl Biomater* 2013; **101**: 721-728 [PMID: 23359546 DOI: 10.1002/jbm.b.32874]

121 **Iohara K**, Imabayashi K, Ishizaka R, Watanabe A, Nabekura J, Ito M, Matsushita K, Nakamura H, Nakashima M. Complete pulp regeneration after pulpectomy by transplantation of CD105+ stem cells with stromal cell-derived factor-1. *Tissue Eng Part A* 2011; **17**: 1911-1920 [PMID: 21417716 DOI: 10.1089/ten.TEA.2010.0615]

122 **Takahashi K**, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 2006; **126**: 663-676 [PMID: 16904174 DOI: 10.1016/j.cell.2006.07.024]

123 **Okita K**, Ichisaka T, Yamanaka S. Generation of germline-competent induced pluripotent stem cells. *Nature* 2007; **448**: 313-317 [PMID: 17554338 DOI: 10.1038/nature05934]

124 **Ramirez JM**, Bai Q, Dijon-Grinand M, Assou S, Gerbal-Chaloin S, Hamamah S, De Vos J. Human pluripotent stem cells: from biology to cell therapy. *World J Stem Cells* 2010; **2**: 24-33 [PMID: 21607113 DOI: 10.4252/wjsc.v2.i2.24]

125 **Yan X**, Qin H, Qu C, Tuan RS, Shi S, Huang GT. iPS cells reprogrammed from human mesenchymal-like stem/progenitor cells of dental tissue origin. *Stem Cells Dev* 2010; **19**: 469-480 [PMID: 19795982 DOI: 10.1089/scd.2009.0314]

126 **Beltrão-Braga PC**, Pignatari GC, Maiorka PC, Oliveira NA, Lizier NF, Wenceslau CV, Miglino MA, Muotri AR, Kerkis I. Feeder-free derivation of induced pluripotent stem cells from human immature dental pulp stem cells. *Cell Transplant* 2011; **20**: 1707-1719 [PMID: 21457612 DOI: 10.3727/096368911X566235]

127 **Sacchetti B**, Funari A, Michienzi S, Di Cesare S, Piersanti S, Saggio I, Tagliafico E, Ferrari S, Robey PG, Riminucci M, Bianco P. Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. *Cell* 2007; **131**: 324-336 [PMID: 17956733 DOI: 10.1016/j.cell.2007.08.025]

P- Reviewer: Jorge V, Politi LE **S- Editor**: Ji FF **L- Editor**: A **E- Editor**: Wu HL

WJSC
World Journal of Stem Cells

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4252/wjsc.v7.i7.1022

World J Stem Cells 2015 August 26; 7(7): 1022-1038
ISSN 1948-0210 (online)
© 2015 Baishideng Publishing Group Inc. All rights reserved.

REVIEW

Adoptive immunotherapy for acute leukemia: New insights in chimeric antigen receptors

Maël Heiblig, Mohamed Elhamri, Mauricette Michallet, Xavier Thomas

Maël Heiblig, Mohamed Elhamri, Mauricette Michallet, Xavier Thomas, Hematology Department, Lyon-Sud Hospital, 69495 Pierre Bénite cedex, France

Author contributions: Heiblig M designed and wrote this review; Elhamri M, Mauricette M and Thomas X provided a critical revision of the manuscript.

Conflict-of-interest statement: Authors declare that they have no competing interests.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: http://creativecommons.org/licenses/by-nc/4.0/

Correspondence to: Xavier Thomas, MD, PhD, Hematology Department, Lyon-Sud Hospital, 165 chemin du Grand Revoyet, 69495 Pierre Bénite cedex, France. xavier.thomas@chu-lyon.fr
Telephone: +33-04-78862235
Fax: +33-04-72678880

Received: September 5, 2014
Peer-review started: September 6, 2014
First decision: December 17, 2014
Revised: May 3, 2015
Accepted: June 18, 2015
Article in press: June 19, 2015
Published online: August 26, 2015

Abstract

Relapses remain a major concern in acute leukemia. It is well known that leukemia stem cells (LSCs) hide in hematopoietic niches and escape to the immune system surveillance through the outgrowth of poorly immunogenic tumor-cell variants and the suppression of the active immune response. Despite the introduction of new reagents and new therapeutic approaches, no treatment strategies have been able to definitively eradicate LSCs. However, recent adoptive immunotherapy in cancer is expected to revolutionize our way to fight against this disease, by redirecting the immune system in order to eliminate relapse issues. Initially described at the onset of the 90's, chimeric antigen receptors (CARs) are recombinant receptors transferred in various T cell subsets, providing specific antigens binding in a non-major histocompatibility complex restricted manner, and effective on a large variety of human leukocyte antigen-divers cell populations. Once transferred, engineered T cells act like an expanding "living drug" specifically targeting the tumor-associated antigen, and ensure long-term anti-tumor memory. Over the last decades, substantial improvements have been made in CARs design. CAR T cells have finally reached the clinical practice and first clinical trials have shown promising results. In acute lymphoblastic leukemia, high rate of complete and prolonged clinical responses have been observed after anti-CD19 CAR T cell therapy, with specific but manageable adverse events. In this review, our goal was to describe CAR structures and functions, and to summarize recent data regarding pre-clinical studies and clinical trials in acute leukemia.

Key words: Chimeric antigen receptors; Adoptive immunotherapy; Acute leukemia; T cells; Immune surveillance

© The Author(s) 2015. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Leukemia cells ultimately escape to the immune system, due to various mechanisms such as limited availability of tumor specific T cells or down-regulation in major histocompatibility complex expression. Chimeric antigen receptor (CAR) T cell technology redirects immune reactivity towards a broad variety of chosen antigens in a human leukocyte antigen-independent manner. Recent introduction of co-stimulatory domains

in the CAR construct enhances significantly *in vitro* and *in vivo* expansion and persistence of these genetically modified T cells. First clinical trials, especially with anti-CD19 CAR T cells, report promising results in acute lymphoblastic leukemia.

Heiblig M, Elhamri M, Mauricette M, Thomas X. Adoptive immunotherapy for acute leukemia: New insights in chimeric antigen receptors. *World J Stem Cells* 2015; 7(7): 1022-1038 Available from: URL: http://www.wjgnet.com/1948-0210/full/v7/i7/1022.htm DOI: http://dx.doi.org/10.4252/wjsc.v7.i7.1022

INTRODUCTION

Despite recent advances in therapeutics over the last decades, relapses remain a major concern in acute leukemia (AL). Despite complete remission (CR) achievement, leukemia stem cells (LSCs) resist to therapeutic strategies, hiding into bone marrow hematopoietic niches or other unknown sanctuaries[1]. More than evading apoptosis and self-sufficiency of growth signals, these leukemia cells are also characterized by their ability to evade the immune system. Malignant cells escape such immune surveillance through the outgrowth of poorly immunogenic tumor-cell variants, known as immune selection, and/or through disruption of the immune system[2,3]. A robust innate immune system is mandatory to avoid relapses by targeting chemoresistant malignant cells, underlining that bone marrow should be preserved as many as possible from aggressive chemotherapy agents. Allogeneic stem cell transplantation (ASCT) is a potential way to restore the tumor cell immunogenicity by transferring a brand new immune system. However, ASCT is largely unspecific and the benefit of graft versus leukemia is offset by the potential complications related to graft versus host disease (GVHD)[4].

In order to achieve long-term survival and good quality of life, other types of immunotherapy have been developed, such as treatments using tumor-associated antigen (TAA)-monoclonal antibodies (mAbs) and more recently adoptive cellular therapies. Adoptive transferred tumor reactive T cells compared favorably with mAbs. They display direct tumor lysis, enhanced bio-distribution and synergism with the immune system through release of cytokines, and long-term memory properties. Cytokine induced killer (CIK) cells are *in vitro* manufactured T lymphocytes with natural killer (NK) cell properties. They can be extracted from human peripheral blood, bone marrow or cord blood mononuclear cells[5]. They showed a non-major histocompatibility complex (MHC)-restricted lysis function on a broad spectrum of tumor targets *in vitro*, which was confirmed *in vivo*[6,7]. However, first clinical results were not convincing, probably due to a lack of specificity, with a limited basal anti-leukemia activity and a rapid exhaustion of these cells[8,9]. Adoptive transfer of autologous or allogeneic manipulated T cells has proven to be safe and extendable in clinical practice. In patients presenting prolonged lymphopenia, adoptive multi-virus specific T cell transfers have showed promising data in reconstituting anti-viral immunity after SCT or in patients infected by human immudeficiency virus[10,11]. Another approach is to genetically engineer lymphocyte subsets to redirect their natural immune response, correct impaired immunity, and improve T cell anti-tumor effector response.

First described in 1989, the concept of the genetic redirection of T cells is based on chimeric antigen receptors (CARs), which are recombinant receptor molecules genetically transferred, redirecting T cells against a specific TAA[12]. CARs are composed of 3 distinct domains, each displaying their own functions. The extra-cellular domain is generally constituted by a single chain variable fragment (scFv). Its targeting moiety derived from the fused variable heavy and light chains of an antibody. The trans-membrane domain is connected to the scFv through a "spacer" to provide flexibility and stable expression of the extracellular moiety. The intra-cellular signaling domain, usually derived from the CD3ζ-chain of the T cell receptor (TCR)-CD3 complex, mediates activation of CAR T cells. CAR T cells overcome some primordial limitations of TCR by targeting antigens in a non-MHC manner, and can recognize tumor independently of human leukocyte antigen (HLA) molecules[13]. After years of investigations to implement gene transfer tools and codify good manufacturing practices, CARs have been considered for human application. The first clinical trials have shown promising results in hematological diseases[14-17].

In this review, we focused on the CAR backbone technology and its application in the setting of human AL therapy.

CHIMERIC ANTIGEN RECEPTOR T CELLS: STRUCTURE AND FUNCTIONS

CAR is an artificial T cell surface receptor that simulates the physiological response of a T cell receptor and targets native cell surface antigens. However, CARs have the ability to target molecules that can be recognized without requiring peptide processing or HLA presentation. Unlike regular T cells, CAR T cells can restore immunogenicity of tumor cells. They recognize antigens in any HLA background, independently of the patient haplotype, and without any cross-reactive action toward endogenous antigens[18,19]. With a capacity of binding to any cell surface antigens, including proteins, carbohydrates and glycolipids, CARs can respond to a broader range of targets than native TCR. However, antibody-mediated recognition by CARs is not restricted to peptide antigens and does not exclude targeting MHC presented peptides. Engineered NK cells harboring a scFv specific for HLA*A2 (MHC class I) carying a peptide derived from the Epstein-Barr virus latent

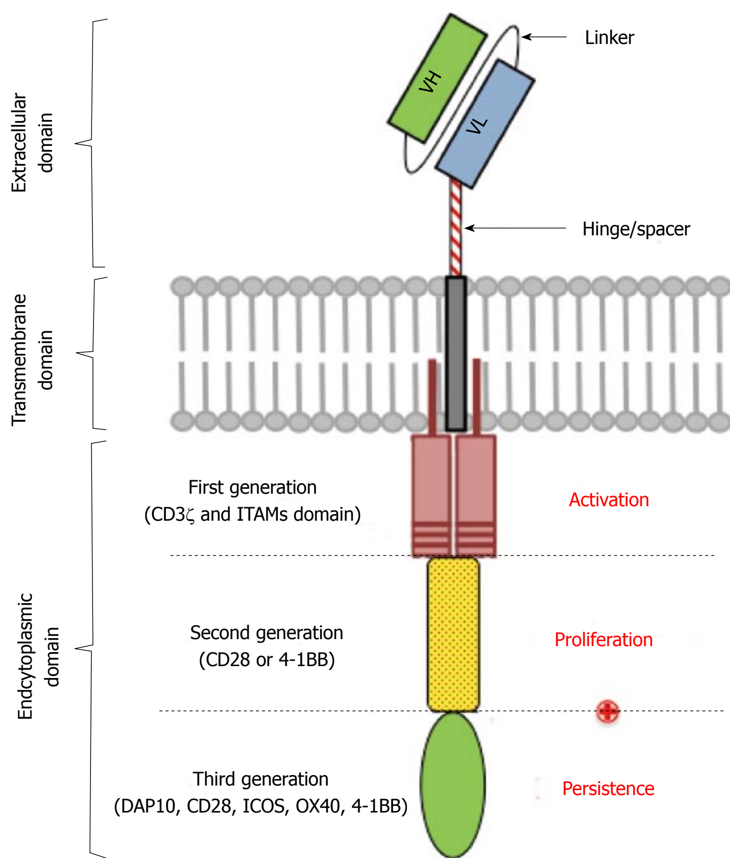


Figure 1 Schema of the general structure of a chimeric antigen receptor. The antigen recognition moiety is composed of the variable domain of heavy (VH) and light (VL) chains of the antibody, specific for a native tumor-associated antigen expressed on the surface of malignant cells. This structure is connected to the cytoplasm by a spacer, generally derived from CD8 or CD28 subunits, and a transmembrane domain. First generation chimeric antigen receptor T cells were solely composed of a single intracellular domain generally derived from CD3ζ subunit and its immunoreceptor tyrosine-based activation motifs (ITAMs), essential for Lck recruitment and full downstream T cell receptor-like signaling transduction. In order to improve engineered T cells proliferation and persistence, investigators introduced successively one (second generation) or ≥ 2 additional intracellular signaling domains (third generation). ICOS: Inducible costimulator.

protein EBNA3C have been recently developed[20]. These CAR NK cells showed substantial cytolytic activity against peptide-pulsed HLA-A2$^+$ antigen-presenting lymphoblastic B-cell line in a peptide-specific, HLA-restricted manner without cross reactivity with native HLA*A2, paving the way toward highly sensitive and specific target cell killing[21].

CARs engage the target *via* their extra-cellular recognition subunits, usually a scFv, but other strategies are actually explored, such as antigen-binding domains derived from natural ligand receptors (*i.e.*, NKG2D)[22,23]. These TAA extra-cellular moieties are either derived from murine or humanized Fab's (variable fragment of an antibody) or synthesized *via* phage display libraries (Figure 1). Because of their accessibility, murine scFvs are the most frequently used, but they are considered more immunogenic than those derived from human libraries. The major risk is to induce humoral and/or cell mediated immune responses as previously reported[24]. There is currently broad evidence that distinct epitopes of a same antigen, as well as their distance to the cell membrane, have not the same potential upon T cell activation. Based on the kinetic segregation model (KSM) relating TCR activation and ligands size-sensitivity, several reports support that this also occurs in CAR T cells[25]. The KSM implied that TCR engagement through distal epitopes binding creates larger TCR-MHC-peptide complexes and that close-contact zone displays the synapse to phosphatase action such as CD45 or CD148 repressing TCR signaling. Conversely, targeting more proximal epitopes favored more potent TCR-MHC interaction and more efficient downstream signaling[26]. In a study assessing the anti-leukemia effect of anti-CD22 CAR T cells, it was showed that proximal targets have superior anti-leukemia effects[27]. This was confirmed by further published data[28]. An increased affinity for the target was not necessarily associated with an increased cytotoxicity. CARs with high affinity for an antigen had a higher proliferation rate than CARs with lower affinity, although this increased affinity was not correlated with higher T cell effector functions[29,30]. Similarly, the effects of antigen density are not well understood but it seems that CARs exert various cytolytic activities according to antigen expression. In a CD123 CAR T cells model, it was demonstrated that engineered T cells eliminated CD123^{high} cells, while CD123^{low} targets survived in co-culture[31]. A high affinity and density regarding a specific antigen is not an absolute condition for an optimal anti-tumor activity, but this should be considered as an advantage in order to reduce the severity of the "on target/off tumor" effect. Despite technical considerations, identifying the perfect target remains a matter of debate, specifically in AL. The ideal antigen target should be homogenously expressed on malignant cells without ubiquitous expression on healthy tissues, and should be critical for tumor cell survival. CD19 is currently the best candidate in B cell precursor acute lymphoblastic leukemia (ALL). The situation is more complex in acute myeloid leukemia (AML), for which the best target still remains to be defined.

A short spacer region, which is the least aspect

of CAR design under discuss, generally follows the scFv's moieties. With the trans-membrane domain, these structures contribute to flexibility, accessibility and synapse formation of the extra-cellular complex. They also seem to influence CARs specificity[32,33]. The simplest form of spacer region is that derived from human immunoglobulin (Ig) Fc (constant fragment) (*i.e.*, IgG4) potentially linked with CH_2CH_3 molecule(s). However, constant regions from human CD8α and CD28 have been until now the most frequently used in pre-clinical assays (Figure 1). Following scFv binding moiety interaction with its target, CAR T cells need an active signaling transfer through their intra-cellular domain for proliferation, cytokine production, and acquisition of effector functions. In order to enhance T cell persistence and anti-tumor functions *in vivo*, research in CAR T cells has been focused on co-stimulation over the last decade.

CO-STIMULATORY DOMAINS IN CAR T CELLS: FIRST TO THIRD GENERATION

First generation CARs were composed only of one signaling domain such as the CD3ζ-chain of the TCR-CD3 complex or the γ-chain of the high affinity IgE Fc receptor (FcεRI) initially associated with antigen-specific scFv derived from a murine antibody (Figure 1). They were associated with the activation of the phosphatidyl inositol and tyrosine kinase pathways. However, they failed to mediate persistent and robust anti-tumor activity[15,34,35]. Clinical trials with first generation CAR T cells have been conducted in different settings. Some of them are still ongoing, but first results were rather disappointing (Table 1). In a study exploring CD20 CAR T cells in 7 patients with relapsed or refractory indolent lymphoma, only a clinical partial response was obtained in one patient. Moreover, after three infusions of autologous CD20 specific T cells, T cell persistence was less than 10 wk (15-65 d), even after interleukin (IL)-2 administrations[36]. In a cohort of 4 patients with non-Hodgkin lymphoma (NHL) treated by CD19-CD20 CAR T cells, no objective response and a very limited persistence of T cells was observed. In other patients, cellular anti-transgene immune rejection responses (specific immune response against transfected T cells) have been observed, showing that the immunogenicity of the transgene used is of major importance[37]. In ALL, there is no clinical data available with first generation CAR T cells. However, two clinical trials based on CD19 scFv-CD3ζ (19z1) CARs with or without IL-2 supplementation are currently recruiting (Table 1). Nevertheless, CD3ζ alone is able to induce a TCR-like signal through immunoreceptor tyrosine-kinase based activation motif (ITAM) phosphorylation and lymphocyte-specific protein tyrosine kinase (Lck) recruitment, which are indistinguishable from those generated by an intact TCR currently used to provide signal one.

It is well known that TCR engagement (signal one) without co-stimulatory signal (signal 2) lead to T cell anergy and rapid activated induced cell death (AICD). Signal 2 is provided by engagement of co-stimulatory domains, mediated essentially by the CD28 superfamily, involving receptors for the agonistic CD80 (B7.1) and CD86 (B7.2) ligands. However, these ligands are generally missing in most cancer cells. This plays a major role in the immune surveillance escape. In this setting, second generation CAR T cells have been designed by adding an additional intra-cellular co-stimulatory domain such as CD28 (forming at the end one polypeptide single chain) to the CAR backbone (Figure 1)[38,39]. CD28, a disulfide-linked homodimer of the immunoglobulin superfamily is activated by binding either B7.1 or B7.2, expressed on the surface of antigen presenting cells. This represents one of the most potent co-stimulatory pathways. It facilitates Lck recruitment to ITAMs and the formation of the linker activation (LAT) complex. This structure is necessary for signal transduction through phospholipase C gamma (PLCγ) leading to IL-2 promoter activation and full T cell activation. Other co-stimulatory domains have been tested, such as members of the tumor necrosis factor (TNF)-receptor family [4-1BB (CD137) or OX40 (CD134)], ICOS or DAP10[28,40-42]. Second generation CAR T cells generally show sustained polyclonal proliferation without B7-CD28 engagement, enhanced IL-2, interferon-gamma (IFNγ), TNF-alpha (α), and granulocyte-macrophage colony-stimulating factor (GM-CSF) cytokine production and enabled resting primary T cells to survive[43]. In two recent clinical studies, it was reported that CAR (scFv-4-1BB-CD3ζ) T cells undergo 1000-fold amplifications *in vivo* compared to only 3.75-fold reported in first generation CAR clinical trials[44,45]. This may be related directly on the co-stimulatory signal that seems to counteract the inhibition effect of tumor growth factor β1 (TGF-β1) secreted by regulator T cells (T_{reg}) on proliferating CAR T cells[46]. Generally, TGF-β1 represses IL-2-dependent T cell amplification, CD122 up-regulation (IL-2R-α), and IFNγ production. Dual signaling provided by second-generation CAR T cells enhanced engineered T cell persistence comparatively to their first generation counterparts. This was demonstrated in patients treated with either CD3ζ or CD3ζ-CD28 CARs[47]. Besides overcoming PD1-mediated inhibition (an activation-induced inhibitory receptor expressed on T cells), co-stimulation through CD28 induces intrinsic survival signals independently of the exogenous survival signals mediated by IL-2. *In vitro*, CD28-B7 interaction leads to an up-regulation of Bcl_{XL}, an anti-apoptotic protein of the Bcl2 family, and to resistance to apoptosis by Fas (CD95, APO-1) cross-linking[48]. This interaction enable protection from intrinsic and extrinsic cell death signals and promotes survival of the expanding lymphocyte population[49]. Surprisingly, it seems that the adjunction

Table 1 Ongoing clinical trials recruiting patients with acute leukemia

Clinical trial.gov ID	Center	Disease	Antigen (scFv)	CAR signaling domain	Vector	Transplantation history	Origin	Lymphocyte depletion	Patients age
Acute lymphoid malignancies									
NCT01044069	MSKCC	B-ALL	CD19	CD3ζ-CD28	γ-retrovirus	Autologous	Autologous	Chemo	≥ 18 yr
NCT01860937	MSKCC	B-ALL	CD19	CD3ζ-CD28	γ-retrovirus	Autologous	Autologous	Cy	up to 26 yr
NCT01430390	MSKCC	B-ALL	CD19 VST (EBV)	CD3ζ	Lentivirus	Relapse post-ASCT	Allogenic	Chemo	up to 19 yr
NCT01626495	CHOP	B-ALL	CD19	CD3ζ-4-1BB *vs* CD3ζ	Lentivirus	Relapse after ASCT	Autologous	Chemo	1-24 yr
NCT02030847	University of Pennsylvania	B-ALL	CD19	CD3ζ-4-1BB	Lentivirus	Relapse ± after ASCT	Autologous	NS	≥ 18 yr
NCT00840853	BCM	B-ALL/NHL/CLL	CD19 VST (EBV)	CD3ζ	γ-retrovirus	Post-ASCT	Allogenic	No	No limit
NCT00586391	BCM	B-ALL/NHL/CLL	CD19	CD3ζ-CD28	γ-retrovirus	No ASCT	Autologous	NS	≥ 18 yr
NCT02132624 (not open)	Uppsala University	B-ALL/NHL	CD19	CD3ζ-4-1BB-CD28	γ-retrovirus	Relapse ± after ASCT	Autologous	NS	≥ 18 yr
NCT01593696	NCI	B-ALL/NHL/CLL	CD19	CD3ζ	γ-retrovirus	Relapse ± after ASCT	Autologous	Flu/Cy	1-30 yr
NCT01087294	NCI	B-ALL/NHL/CLL	CD19 VST (EBV)	CD3ζ	γ-retrovirus	Relapse post-ASCT	Allogenic	Cy	18-75 yr
NCT01683279	Seattle Children Hospital	B-ALL	CD19 EGFRt[+]	CD3ζ-CD28	Lentivirus	No ASCT	Autologous	Cy	1-26 yr
NCT02028455	Seattle Children Hospital	B-ALL	CD19 EGFRt[+]	CD3ζ-4-1BB	Lentivirus	Relapse after ASCT *vs* no ASCT	Autologous	Cy/Flu/TBI	1-26 yr
NCT01865617	FHCRC	B-ALL/NHL/CLL	CD19	CD3ζ	Lentivirus	> 1st relapse	Autologous	NS	≥ 18 yr
NCT01475058	FHCRC	B-ALL/NHL/CLL	CD19 VST (CMV, EBV)	CD3ζ	Lentivirus	Post-ASCT	Allogenic	No	18-75 yr
NCT02051257	City of Hope Medical Center	B-ALL/NHL/CLL	CD19 EGFRt[+]	CD3ζ-CD28	Lentivirus	Post-ASCT	Autologous	Chemo	≥ 18 yr
NCT02146924 (not open)	City of Hope Medical Center	B-ALL	CD19 EGFRt[+]	CD3ζ-CD28	Lentivirus	Relapse/progression ± after ASCT	Autologous	Cy	≥ 18 yr
NCT01195480	London University College	B-ALL	CD19 (EBV-CTL)	CD3ζ	γ-retrovirus	Relapse after 1st ASCT	Allogenic	NS	Up to 18 yr
NCT01864889	Chinese PLA General Hospital	B-ALL/NHL/CLL	CD19	CD3ζ-4-1BB *vs* CD3ζ	γ-retrovirus	Relapse ± after ASCT	Autologous	NS	5-90 yr
NCT01735604	Chinese PLA General Hospital	B-ALL/NHL/CLL	CD20	CD3ζ-4-1BB *vs* CD3ζ	γ-retrovirus	Relapse ± after ASCT	Autologous	NS	18-90 yr
NCT01362452	MDACC	B-ALL/NHL/CLL	CD19 (cord blood derived)	CD3ζ	Transposon	Relapse post-ASCT	Allogenic	No	1-75 yr
NCT01497184	MDACC	B-ALL/NHL/CLL	CD19	CD3ζ	Transposon	Post-ASCT	Allogenic	No	1-65 yr
Acute myeloid malignancies									
NCT01864902	Chinese PLA General Hospital	AML	CD33	CD3ζ-4-1BB *vs* CD3ζ	γ-retrovirus	Relapse ± after ASCT	Autologous/Allogenic	NS	5-90 yr
NCT02159495	City of Hope Medical Center	AML	CD123 EGFRt[+]	CD3ζ-CD28	γ-retrovirus	Relapse/refractory AML	Autologous	Cy	≥ 18 yr

CAR: Chimeric antigen receptor; AML: Acute myeloid leukemia; ASCT: Allogeneic stem cell transplantation; B-ALL: B-lineage acute lymphoblastic leukemia; BCM: Baylor College of Medecine; CHOP: Children Hospital of Philadelphia; Chemo: Chemotherapy prior CAR T cells infusion; CLL: Chronic lymphoid leukemia; Cy: Cyclophosphamide; FHCRC: Fred Hutchinson Cancer Research Center; Flu: Fludarabine; MDACC: MD Anderson Cancer Center; MSKCC: Memorial Sloan-Kettering Clinical Center; NCI: National Center Institute; NS: Not specified; NHL: Non Hodgkin lymphoma; VST: Virus specific T lymphocytes; EBV: Epstein-barr virus; CTL: Cytotoxic T-cell lymphoma.

of a co-stimulatory domain (*i.e.*, CD28) do not modify the threshold of antigen-dependent MHC-independent T cell activation[50]. It seems therefore that incorporation of at least one co-stimulatory domain in a CAR cons-

truct is mandatory for complete T cell activation. However, a major remaining issue concerns the potential superiority of one endodomain comparatively to another. Because of their heterogeneity, some CD28 or 4-1BB-based second generation CARs have been compared, essentially in mice models, and showed contradictory results. Regarding cell proliferation and cytokine production, second generation CD28 transfected CAR T cells were superior to CD19z1 (first generation CAR T cells), but also to constructs with 4-1BB, OX40 or DAP10 endodomains[51]. Conversely, it was showed that CD28⁻ and 4-1BB-based CAR T cells, directed against the same epitope (SS1 scFv-based chimeric receptor), have the same anti-tumor activity[52]. Other pre-clinical studies, containing 4-1BB signal transduction endodomain, exhibited a greatest anti-leukemia activity and prolonged *in vivo* survival[53]. In AL, most of the current clinical trials use indistinctly CD28 or 4-1BB endodomains. Comparisons between intra-cellular signaling domains should take into account all representatives of distinct antigen epitopes, their location, their density, and their affinity for the target in order to clarify their exact contribution in the anti-tumor activity.

More recently, third generation CAR T cells have been developed, including more complex structures with three or more signaling domains, enabling wider T cell effector function in a specific fashion (Figure 1). In mice models, the inclusion of multiple endodomains showed enhanced functionality and greater potency of tumor targeted T cells *in vivo* and *in vitro.* However, this was not confirmed in other models[54-56]. In a study comparing the consequences of CD28, OX40 and 4-1BB co-stimulation, it was showed that third generation CD3ζ-CD28-OX40 CARs prevent AICD in memory T cells, and substantially improve effector functions in both naive and memory CD4 and CD8 T cells. They also increase cytokine production comparatively to second generation CAR T cells combination[57]. Considering these results and the substantial improvement in terms of efficacy, the addition of a third endomain should be associated with increased long-term persistence and should delay anergy. However, a pilot study, using CD28-4-1BB-based CARs in 4 NHL patients, reported one partial response, one disease progression, and 2 cases free of disease at 12 and 24 mo, respectively. In this study, engineered T cells were detectable up to 12 mo after infusions by quantitative polymerase chain reaction (PCR) at low levels (< 1%)[58]. Because of discrepancies among pre-clinical and clinical results, further investigations are warranted to optimize our understanding regarding CAR T cell signaling. Because massive activation and uncontrolled proliferation of genetically modified T cells has been associated with serious adverse events, investigators should take into account these features and find the right balance between efficiency of these engineered T cells and an acceptable tolerance.

CARS MANUFACTURING: A PERSONALIZED ASSEMBLY-LINE WORK

Investigators developed distinct approaches to introduce efficiently CAR constructs into T cells. Actually, most of them utilized viral transduction systems (mostly γ-retroviral) leading to permanent sequence integration into T-cell DNA. However, this kind of vehicle presents disadvantages: (1) high costs related to the production process; (2) risks of CAR expression silencing due to terminal repeat alterations; (3) risks of insertional mutagenesis; and (4) production of replication competent virus. Alternatively, lentiviral vectors, which are theoretically less genotoxic, can permanently transduce T cells, but display inferior yields of transgene integration[59,60]. However, no genotoxic-related events related to viral vectors have been reported until now in human clinical trials using manipulated T cells[61]. Moreover, residual vector sequences present in the genome lead to immunogenic epitope expression and can increase anti-CAR mediated response. First clinical trials in B-cell lineage ALL using viral vectors reported very limited gene transfection efficiency (5%-15%), without impairing anti-leukemia effect[62,63].

Non-viral based DNA transfection represents an interesting alternative because of its low cost and its theoretically limited insertional mutagenesis [insertion at thymidine-adenosine (TA) dinucleotide base pairs and non-preference of integration into transcriptional units]. DNA plasmids were the first non-viral vehicles that have been tested. They seemed less immunogenic and independent of the sequence size comparatively to viral vectors. However, because of low chromosomal intake and sustained transgene expression, long-term cultures are required to obtain a sufficient number of CAR-modified T cells, despite a potential negative effect on T cell activity and *in vivo* expansion. One major issue with plasmid vectors is represented by the genomic integration of multiple copies and transfected DNA hypermethylation, leading to transgene silencing[64]. Originally described in 1996, "Sleeping Beauty (SB)" transposase/transposon, based on the concept of "jumping genes" discovered by McClintock, enabled to overcome these issues and to restart non-viral DNA vectors[65]. Generally, one plasmid is loaded with a transgene, named transposon, surrounded by inverted repeats that contain short direct repeats (Figure 2). These sequences are recognized by an enzyme (transposase) transported in a second plasmid, which cut the transposon out of the plasmid. The genetic cargo is then delivered into the targeted cell cytoplasm by any of the established non-viral delivery techniques, and inserted randomly into TA dinucleotide base pairs of the recipient (Figure 2). Costly but safer, the SB platform displays attractive features for human gene therapy, to such an extent that many protocols for manufacturing clinical grade T cells recently came to light[66]. At the last European Bone Marrow Transplantation (EBMT)

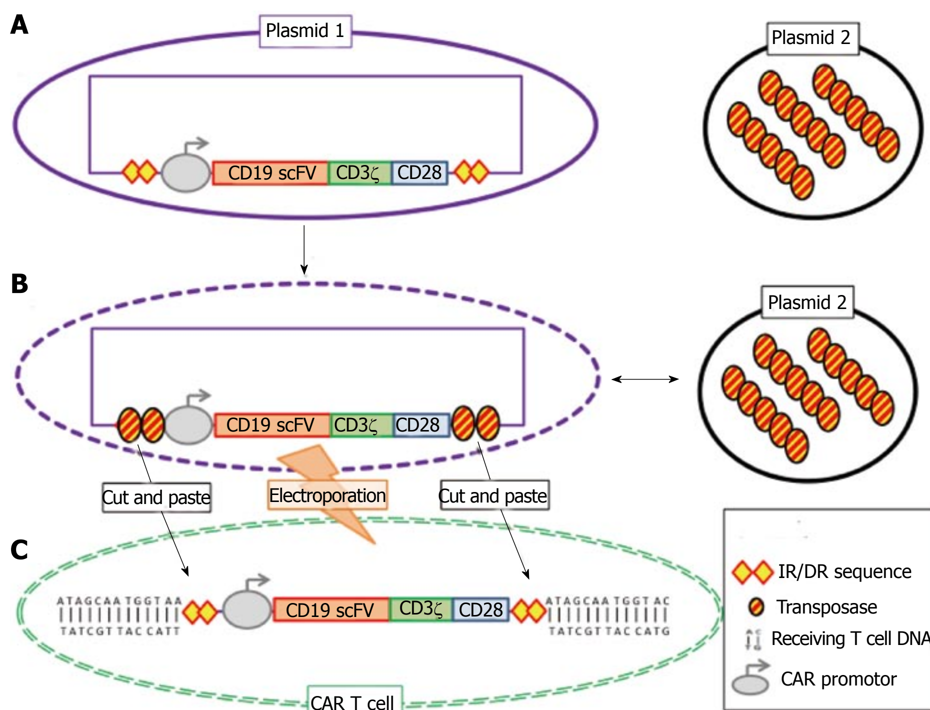


Figure 2 Schema of the transposon/transposase "sleeping beauty" system. A: The genetic cargo of a first plasmid (plasmid 1) is the chimeric antigen receptor (CAR) (anti-CD19 CD3ζ-CD28) flanked by inverted/short direct repeats (IR/DR). The whole set composed the transposon. The enzyme transposase is loaded in a second plasmid, which is specific of the IR/DR sequence; B: Materials of the two plasmids are fused together by electroporation, an electric current opening pores or channels at the cell surface. Transposase binds to the IR/DR sequences; C: The enzyme cut out of the plasmid the flanked sequences (CAR transgene with its promotor) and transfect the genetic cargo into a random DNA sequence of the recipient (T cell).

meeting, promising results were reported about the production of third generation CD19/CD123 CIK CAR cells. With about 50% CAR transfection yields, investigators are now able to greatly expand CAR T cells and produce clinical grade within 3 wk (*vs* 90 d with retroviral vectors). To our knowledge, two human clinical trials using the SB system are currently ongoing at the MD Anderson Cancer Center (Table 1)[67].

Clinical trials have reported serious adverse events during CAR T cells therapy in early clinical phase trials, which were related to uncontrolled T cells proliferation, cytokine storm, or "off tumor" effect[68,69]. In this context, strategies to incorporate abortive mechanisms inside the genomic load have emerged. Based on *ex vivo* genetic modification of donor T lymphocytes, suicide gene therapy with the Herpes simplex thymidine kinase (Hsv-tk)/ganciclovir (GCV) suicide system in the context of ASCT has demonstrated its safety and efficacy[70]. In order to facilitate its clinical development, this technology has been applied to CAR T cell development. Despite successes in the treatment of severe GVHD, Hsv-tk/GCV system showed some limitations: (1) the HSV-TK protein can be immunogenic in an immunocompetent situation; (2) the system can lead to unexpected elimination of modified T-cells in case of treatment with GCV; and (3) the system can show efficacy only on dividing cells[71]. Other non-immunogenic suicide systems have been developed, especially through a modified caspase 9 (iCasp9) member of the intrinsic apoptosis pathway[72]. A modified FK506-binding protein (humanized FKBP12), belonging to a family of protein which display a propyl isomerase activity, has been combined with caspase 9. Infusion of a synthetic drug, AP1903, allows the dimerization of iCasp9 and activates the apoptosis cascade. Beside optimal bio-distribution of the dimerization inducer, iCasp9-based cell safety switch offers superior pharmacodynamical properties than the HSC-tk system, and leaves GCV available for antiviral therapy[73]. The major issue of this suicide gene strategy is that anti-tumor T cells could be definitely eliminated, impairing the efficacy of cellular therapy. However, to date, no suicide gene therapy has been already used in human clinical trials with engineered CAR T cells. Although not as effective as CAR using integrating vectors technology, transient CAR expression is an interesting alternative approach currently under investigation. According to a recent study published by the University of Pennsylvania, CAR mRNA can be efficiently transduced into T cells by *in vitro* repeated electroporation procedure and administrated repeatedly safely, avoiding the risk of CAR T cells accumulation. This approach precludes transgene persistence for one week or two, and a rapid decrease of toxicity in case of adverse events after T cell discontinuation[73]. The current stocks and production rates of adequate serum for good manufacturing procedure (GMP) are insufficient regarding the outgrowing demand, suggesting a further development of serum-free conditions for cell therapy cultures[74].

CAR'S CULTURE: EXPANSION, PERSISTENCE AND TRAFFICKING

The technique and the duration of CAR T cell cultures prior CAR transfection and infusion of the product may be also critical in the setting of clinical activity. Autologous/allogeneic non modified-T cells are harvested from peripheral blood by leukapheresis, with heterogeneous threshold according to the underlying pathology. Then, naive T cells are expanded *ex vivo* generally with an artificial-antigen presenting cell system or anti-CD3/CD28 coated beads in combination with various cytokines (IL-2, IL-15, IL-17, IFNγ). As T cells are grown under GMP, expansion protocols and manufacturing input may vary among centers, depending on transduction efficiency yields, CD4/CD8 ratio, and final T cell phenotype. Most of culture protocols support the acquisition of a central memory phenotype ($CD45RA^+/CD122^{low}/CD62L^{low}/CCR7^+$), which is associated with self-renewal, high proliferation potential, and increased longevity. However, it was highlighted that proliferation, cytolytic activity, and persistence are markedly hampered by long-term *ex vivo* cultures, likely due to T cell exhaustion and telomere length shortening[36,58]. In addition, the T cell subtype transduced with CARs seems to play a key role in the clinical efficacy of adoptive immunotherapy. Due to their well-known cytolytic activity through granzyme/perforine and Fas/FasL complexes, αβ $CD8^+$ CAR T cells have been considered as the effective component of CAR T cell-based therapy and have been the major T cell subset used in pre-clinical trials. However, recent data revealed that $CD4^+$ T cell subset transfected with CARs showed cytolytic activity similar to that of $CD8^+$ T cells toward their targeted antigen. They are also useful on CAR mediated activation[75,76]. All T cell subsets play a key role in the anti-tumor immune response. The most powerful subset depends likely on tumor phenotype, its accessibility, and the tumor-cytokine microenvironment. In this setting, γδ, Th17, central memory, stem cell like memory, virus specific T cells and hematopoietic stem cells are also a part of CAR-based therapy[77-80].

For a long-term disease control by adoptive immune surveillance, engineered T cell persistence is highly warranted. Preclinical studies indicate that tumor burden and the degree of lymphocyte depletion prior T cell infusion are likely to be critical requirements for proper T cell expansion and persistence. First clinical trials indicate that persistence and *in vivo* expansion of adoptively transferred T cells is strongly correlated with treatment outcome, as previously documented with tumor-infiltrating lymphocytes (TILs) [45,69]. Conditioning chemotherapy is a way to enhance persistence and expansion of transferred CAR T cells. However, the optimal regimen is still a matter of debate. Although underlying mechanisms are not fully understood, this model probably involves homeostatic proliferation. Actually, T cell populations are tightly regulated by homeostatic mechanisms that maintain the T cell pool at a near-constant level. These mechanisms mimic chronic T cell activation, depend upon TCR/MHC self-peptide interaction, and involve cytokines, such as IL-7 and IL-15. During the first clinical trials in chronic lymphoid leukemia (CLL) patients, anti-CD19 CAR T cell infusions without conditioning chemotherapy showed limited T cells persistence and disappointing results[45]. Conversely, in a cohort of 5 relapsed ALL patients treated with 19-28z CAR T cells after lymphocyte depletion by cyclophosphamide, it was reported long-term CAR modified T cells persistence for 3 to 8 wk. Four of the five patients underwent ASCT and remained in minimal residual disease (MRD)-negative status. This study was the first to suggest an inverse relationship between CAR T cells clinical efficiency and initial tumor burden at the time of infusion. It was also shown that lymphocyte depletion prior CAR T cells infusion enhanced CAR T cells persistence[62]. Lymphocyte depletion following chemotherapy (cyclophosphamide/fludarabine) or total body irradiation (TBI) suppresses repressive cell populations, such as T_{reg}, and cells competing for stimulatory cytokines (IL-2, IL-7, IL-15, IL-21), preventing rapid anergy of adoptively transferred T cells and transient "cytokine sink". "Cytokine sink" corresponds to the competition between transferred and host T cell subsets regarding homeostatic cytokines, and can decrease *in vivo* T cell expansion[81]. IL-2, which has been widely used for adoptive T cell expansion *in vivo*, is essential for the maintenance of peripheral self-tolerance and is able to promote T cell effector functions. However, the administration of exogenous cytokines remains highly controversial. It has been shown that homeostasis and efficient suppressor functions of T_{reg} were essentially mediated by IL-2 receptor signaling. The infusion of anti-IL-2 plus IL-15 in tumor bearing mice increases effector functions of adoptively transferred T cells[82]. Although IL-2 represents a key factor in the induction of terminal differentiation of effector T cells, its use for *in vivo* expansion may impair anti-tumor immunity. More recently, cyclophosphamide lymphocyte depletion has been shown to significantly reduce FoxP3 T_{reg} and to induce IL-12 and IFNγ secretion[83]. IL-12 is a heterodimeric cytokine secreted by APCs, known to enhance T cell clonal expansion and T cell effector function in concert with TCR complex signaling (signal 1 and 2), serving as signal 3. This cytokine favors NK recruitment, but also avoid T cell anergy and T_{reg} action on effector T cells. This was illustrated by CAR targeted T cells (first generation 19z1) modified to produce autocrine IL-12 and to manage B lymphocyte depletion associated with tumor eradication[84]. The exact schedule for infusing CAR T cells after lymphocyte depletion is still a matter of debate. Nevertheless, recent evidences support the superiority of a rapid transfer in term of T cell engraftment and immune reconstitution[85,86].

AL is defined by an erratic clonal proliferation of immature hematopoietic stem cells, essentially localized in the bone marrow. Despite 60%-80% of CR

achievement after a first induction course of chemotherapy, most of AL patients will ultimately relapse, due to LSCs or persistence of resistant clones in unreachable reservoirs to standard chemotherapy. CAR T cells have to traffic in the entire body, after *ex-vivo/in vivo* activation, to clear central (bone marrow) and peripheral reservoirs. In solid tumors and lymphomas, preclinical studies and clinical trials have established that CAR T cells, specifically when using second generation CARs, can accumulate and persist over time at the site of disease[47,62]. In a recent phase I study using anti-LeY CAR T cell therapy in AML, it was demonstrated by detection of a transgene derived PCR signal and by radio-labeled cells that CAR T cells have a systemic distribution and migrate to the bone marrow[87]. Genetically engineered T cells seem to naturally traffic to the bone marrow, but also to the central nervous system (CNS), with however 5 to 10-folds less than what is observed in blood[17,44]. The reasons why CAR T cells naturally cross the meningeal barrier remain intriguing. However, this is a major concern regarding B cell lineage-ALL, for which CNS involvement could have dramatic repercussions on treatment outcome. Lymphocyte depletion by cyclophosphamide or TBI stimulate the production of chemo-attractants by the microenvironment, and thus favor CAR T cells migration and engraftment in the bone marrow[88]. Because chronic activation is associated with down-regulation of homing receptors such as CD62L, investigators are currently exploring genetic expression of chemokine/cytokine receptors (*i.e.*, CXCR2) and *ex vivo* cell surface glycan engineering (*i.e.*, modifications of fucosyltransferase, an enzyme involved in hematopoietic stem cell homing) in order to enhance bone marrow trafficking, which is critical for successful leukemia cell eradication[89,90].

CARS IN ACUTE LEUKEMIA: A MATTER OF TARGET

Acute lymphoblastic leukemia

Historically, the first hematological malignancies treated with CAR T cells were CLL and lymphomas from the B cell lineage. As mentioned above, several issues have to be considered to select a target for CAR-mediated tumor cell recognition and unfortunately most of the TAA are self-antigens expressed on healthy tissues. In this setting, CD19 arise as a perfect target antigen, since it is expressed in almost all B cell malignancies (except 5% of undifferentiated immature B cell lineage-ALL) and long-term B cell depletion is generally well tolerated (*i.e.*, chimeric monoclonal antibody rituximab). CD19 is expressed on normal B lineage cells from pro-B-cells to mature B cells and plasma cells, but hematopoietic stem cells and other tissues lack this antigen expression. CD19 is thought to play a role in the balance between self-tolerance and antigen activation of the B cell receptor (BCR) complex in a specific and sensitive manner. Another hypothetical advantage of CD19 is that CD19-positive cells are constantly produced in the bone marrow, thus providing an inexhaustible source of antigen stimulation. It was shown that CD19 was involved in Myc driven B cell oncogenesis in a BCR-independent manner, through paired box protein 5[91]. Although they are sharing the same target, B cell lineage malignancies may respond differentially to adoptively transferred T cells. Based on promising pre-clinical data with first and second-generation CARs, first trials with anti-CD19 CARs were designed in patients with recurrent indolent NHL or CLL. They showed promising results with prolonged CR[37,47,69]. However, the initial enthusiasm was hampered by further trials showing no CR achievement and very limited CAR T cells persistence[47,62]. Discrepancies among studies could be attributed to the suppressor role of the tumor microenvironment, differences in treatment history, pretreatment tumor burden, potential tumor resistance to the lymphocyte depleting agent, and/or impaired T cell immunological function in lymphomas[92,93]. CD-19-CAR T cells appeared as the best choice for the treatment of B-cell lineage-ALL with an overall CR rate of 80% (Table 2). The first case of treatment by CD19-CAR T cells in B cell lineage-ALL has been published by the Memorial Sloan Kettering Cancer Center. The patient was in early relapse and has been treated with second generation 1928z CAR T cells (second generation CAR T cells anti-CD19 scFv CD28-CD3ζ). After CR achievement, the patient received cyclophosphamide (3.0 g/m^2) followed after 2 d by a split dose of autologous 1928z T cells (1.8 × 10^8 cells/kg). He underwent ASCT 8 wk after T cell infusion. Prolonged B lymphocyte depletion was observed and related to 1928z CAR T cells persistence confirmed by immunohistochemistry on bone marrow aspirates through 6 to 8 wk post-infusion[62]. This trial has been recently updated reporting about 13 patients (including the first one) with relapsed/refractory B cell lineage-ALL (of whom 3 patients with Philadelphia-positive B cell lineage-ALL). CR rate was 85% (10/13 patients) with complete molecular responses obtained 7 to 14 d after T cell infusion. Cytokine release syndrome (CRS) was observed in 6/13 patients and was controlled by corticosteroids or anti-IL-6R antibody therapy. Nine of the 13 patients underwent ASCT[94]. Another phase 1 trial was reported in 5 adults with relapsed/refractory B cell lineage-ALL. They received one single dose of autologous 1928z-CAR T cells (1.5 to 3 × 10^6 cells/kg) after administration of high-dose cyclophosphamide. All patients became MRD-negative after CAR T cell infusion. Four of them underwent ASCT and were still MRD-negative at the time of the last report. The last patient, ineligible for ASCT and further CAR T cell infusion, relapsed 13 wk after T cell administration. Relapse was due to abrogation of CAR T cells persistence[63]. Another phase 1 study, using virus-specific 1928z-CAR T cells (CD19 CAR-VSTs, cytotoxic T cells with a native receptor specificity directed to persistent human viruses) in patients relapsing after ASCT or with high-risk B cell malignancies, included 3 patients with B cell lineage-

Table 2 Completed chimeric antigen receptor T cells trials including acute leukemia patients

Indications	CAR construct	Vector	Cell dose	Pre-treatment	Patients	Responses	Relapses	CAR persistence (days median)	Toxicities	Ref.
Acute lymphoid malignancies										
Relapsed B-ALL ± post-ASCT	Anti-CD19 scFv 4-1BB-CD3ζ	Lentiviral	10^6-10^7 cells/kg	Cy	30	90% CR (27/30)	6 relapses (one CD19$^-$)	145	CRS for all responding patients (fever, ARDS. MODS)	[17,97,98]
Relapsed B-ALL post-ASCT	Anti-CD19 scFv CD28-CD3ζ	γ-retroviral	10^6 cells/kg	Cy + flu	2	100% CR (5/5)	1 transient CR	ND	Mild CRS, no GVHD	[117]
B-ALL relapsed	Anti-CD19 scFv CD28-CD3ζ	γ-retroviral	3×10^6 cells/kg	Chemo	13 (3 Ph$^+$ B-ALL)	85% CR (10/13)	1 NR, 1 relapse	ND	6/13 CRS	[62,94]
B-ALL relapsed without prior ASCT	Anti-CD19 scFv CD28-CD3ζ	γ-retroviral	1.5-3×10^6 cells/kg	Cy	5	100% CR (5/5)	1 relapse (no ASCT)	ND	3/5 mild CRS (MRD$^+$ or bulk)	[63]
Relapsed B-ALL/ CLL post-ASCT	Anti-CD19 scFv CD28-CD3ζ VST (CMV, EBV, ADV)	γ-retroviral	1.5×10^7-1.2×10^8 cells/m^2	No	4 (4/8)	75% CR (3/4), 25% PD (1/4)	1 relapse (no ASCT)	80	No CRS, No GVHD	[95]
Acute myeloid malignancies										
Relapsed AML	Anti-LeY scFv CD28-CD3ζ	γ-retroviral	1.3×10^9 cells	Chemo	4	25% CR, 50% SD	4 relapses	14-120	Neutropenia, skin "flare" reaction, fever, rigors	[87]

CAR: Chimeric antigen receptor; ADV: Adenovirus; AML: Acute myeloid leukemia; ARDS: Acute respiratory distress syndrom; ASCT: Allogeneic stem cell transplantation; B-ALL: B-lineage acute lymphoblastic leukemia; Chemo: Chemotherapy prior CAR T cells infusion; CLL: Chronic lymphoid leukemia; CMV: Cytomegalovirus; CR: Complete remission; CRS: Cytokine release syndrome; Cy: Cyclophosphamide; EBV: Epstein Barr virus; GVHD: Graft versus host disease; MODS: Multi-organ dysfunction syndrome; ND: Not determined; NR: No response; PD: Progressive disease; SD: Stable disease; VST: Virus specific T lymphocytes.

ALL. No pre-conditioning regimen was administered. CD19 CAR-VSTs required several rounds of expansion (5-6 wk of cell culture) before infusion in order to reach clinical CAR T cell threshold. No GVHD was observed and all B ALL patients achieved CR, but relapsed between 2 to 8 mo after T cell infusion (Table 2)[95]. The largest cohort was initially reported by the Children Hospital of Philadelphia in 2013 and updated at the 2014 EBMT meeting. The study included 30 patients (25 children and 5 adults) with chemo-refractory B cell lineage-ALL or ALL relapsing after ASCT. Patients received CTL019 CARs (second generation anti-CD19-scFv CAR T cells) coupled with 4-1BB endodomain and lentivirus transfected. The median dose of CTL019 was 3.7×10^6 cells/kg administrated in 3 doses with 5×10^9 total cells as a target dose. Lymphocyte depletion regimens varied among patients, but were mostly based on cyclophosphamide administered during the week prior T cell infusion. Patients were at least in second relapse or were refractory to several lines of treatment. Overall, 90% of them achieved CR, and half of them underwent subsequently ASCT. With a median follow-up of 3.4 mo (range: 2-18 mo), only 6 patients relapsed. In one case, relapse occurred with emergence of CD19 negative blast cells. As previously reported with blinatumomab (a bi-specific T-cell engager antibody designed to redirect CD3$^+$ cytotoxic T cells to CD19$^+$ malignant B cells), this relapse from CD19 negative leukemia cells illustrates the impact of such a targeted therapy on leukemia sub-clones[96]. No such cases have been reported in CLL[93]. CRs were observed independently of the level of tumor burden before T cell infusion. Among the 16 patients treated after ASCT, T cells were efficiently collected from recipient. No GVHD recurrence was observed after CAR T cell infusions. CTL019 cells expanded to levels that were more than 1000 to 10000 times as high as the initial levels. CAR T cell persistence was observed until 6 to 18 mo. This was concomitant of B lymphocyte depletion in responding patients, as previously reported in CLL patients treated with CTL019. Long-term persistence ($\geqslant$ 145 d) was significantly associated with CR achievement. All responding patients developed some degree of delayed CRS, which was concomitant to T cell expansion and increased levels of IL-6. This seemed in relationship with the tumor burden prior CAR T cell infusion (Table 2)[17,97,98]. Overall, these first results suggest that CD19 gene-modified T cell therapy is likely not enough efficient by itself, but it should be considered as a potentially life-saving bridge to ASCT. Recently, a multi-center clinical consortium was proposed in order

to harmonize practices and further export this new technology to academic institutions.

Alternative targets are likely to be developed. CD22, a type I trans-membrane sialo-glycoprotein expressed specifically on B lineage cells, is closely related to the BCR pathway. Moxetumomab pasudotox, an anti-CD22 covalently fused to a pseudomonas exotoxin, has shown promising results[99], and could be considered as a suitable possible choice. On the other hand, in order to prevent antigen loss through selective clonal pressure and to improve tumor specificity, investigators are developing combinatorial antigen recognition strategy, and oncogene targeted CARs[100,101].

Acute myeloid leukemia

Treatment of AML remains a great challenge. Whereas combined efforts in the field of intensified chemotherapy, SCT, and supportive care have yielded to improve survival, more than 50% of patients will relapse. Because 70% of AML patients are over 60 years, many of them are not eligible for ASCT[102]. Recent preclinical reports demonstrated that CAR T cells have the potential to effectively and durably eradicate primitive myeloid blast cells[103,104]. Although two trials are currently recruiting in China and in Australia, only one phase 1 trial, targeting Lewis Y (LeY) antigen coupled with cytoplasmic CD28 and CD3ζ chain, has been reported so far in the literature regarding AML (Table 1). LeY antigen is a difucosylated carbohydrate antigen, part of the human blood system. It is widely expressed on AML cells, but has a limited expression in healthy tissues. However, its exact role and its functional significance for survival of the leukemia cells have to be elucidated. Four patients received 1.5 to 4.7 $\times$ 10^6 cells/kg CAR T cells 6 wk after fludarabine-based chemotherapy regimen. A modest T cell expansion and persistence (1 to 10 mo) was observed and resulted in limited advantages. Only 2 out of four treated patients showed a reduction of leukemia cells. Beside these disappointing results, this study conveys major endpoints. Infusions were well tolerated with no CRS even in cases presenting with a high tumor burden. Second, it was demonstrated by SPECT imagery that anti-LeY CAR T cells can target bone marrow leukemia cells, but also peripheral lesions (leukemia cutis). The absence of down-regulation of LeY antigen after CAR T cell infusions suggests that this target is suitable for long-term immune control of the disease[87]. However, the weak anti-leukemia activity of this antigen-based therapy should be balanced with combinatorial recognition strategy or loaded in more potent effectors such as CIK cells. Other antigens are also on the bench, such as CD33, CD123 or CD44v6, but only pre-clinical data are currently available, demonstrating a potential efficacy against AML blasts. CD33 is a member of the sialic acid-binding receptor family and is highly expressed on myeloid progenitor cells, and on the surface of 90% of AML blasts. Gemtuzumab ozogamicin (Mylotarg), an anti-CD33 humanized mAb conjugated to calicheamicin, was the first agent of its class and one of the first targeted therapies in AML. However, initial enthusiasm was tempered by inadequate efficacy, severe hepatotoxicity (*i.e.*, veino-occlusive disease) related to accumulation of the drug acting like an intercalating agent, and prolonged neutropenia[105]. Moreover, drug resistance rapidly may occur by active efflux from leukemia cells through the P-glycoprotein pump. Because of the very strong non HLA-restricted NK-like cytotoxicity and the lack of allogeneic activity in the ASCT setting, CIK have been largely used in pre-clinical studies of CAR redirected T cells in AML. Anti-CD33 CAR-T cells might have several potential advantages over gemtuzumab ozogamicin. First pre-clinical data using CIK and EBV-specific T cells endowed promising features[104,106]. Beside the anti-leukemia activity and its expected myelotoxicity, *in vitro* colony forming unit (CFU) assays showed remnant clonogenic activity of hematopoietic progenitors suggesting that toxicity is reversible. Furthermore, it was shown that anti-CD33 CAR T cells with CD28-OX40 endodomains exert cytolytic activity on KG-1 cell line, known to be resistant to gemtuzumab ozogamicin[106]. In order to avoid off-target toxic effect on hematopoietic stem cells (HSC), CD123, also known as IL-3 receptor α-subunit, appears as an attractive target. Its functional role in AML is still unknown. CD123 is widely over-expressed among AML blasts and LSCs, while its expression is lower on HSCs, monocytes, and endothelial cells. The expression of CD123 at the time of diagnosis has been associated with a poor prognosis and resistance to apoptosis. Preliminary *in vivo* data showed that one single administration of anti-CD123 CAR T cells led to an immunologic memory associated with a specific anti-tumor response, yielding to long-term survival in mice engrafted with AML cell lines or fresh AML cells[107]. Results of CFUs, regarding distinct anti-CD123 CAR constructs, were heterogeneous. However, one study revealed a limited activity against normal CD123^{low} expression on endothelial cells and monocytes[31,103,104]. It is therefore hypothesized that suboptimal affinity of the scFv for CD123 allows recognition of targeted cells by CAR T cells, according to their antigen density and their expression of co-stimulatory molecules[108]. The hyaluronase receptor CD44, a ligand for E-selectin, is broadly expressed on malignant cells in hematological malignancies. It plays a crucial role in the bone marrow homing of initiating leukemia cells and in interactions with the microenvironment. It was recently showed that CD44 inhibition drives leukemia cells into differentiation and apoptosis by dislodging them from the osteogenic niche[109]. The isoform variant 6 (CD44v6) recently emerged as one of the most promising TAA for AML. It is absent on normal HSCs, but is over-expressed in 80% of AML cells[110,111]. It has been demonstrated that anti-CD44v6 redirect T cells were able to efficiently kill leukemia cells, while sparing HSCs and keratinocytes that expressed low levels of CD44v6[72]. These findings suggest that "off tumor" target expression levels do

not accurately predict susceptibility to CAR T cells[72]. All together, these results suggest that anti-CD123 and anti-CD44v6 CARs are safer than anti-CD33, but *in vitro* data have to be confirmed in clinical practice (Table 1).

TOXICITY: NEW INSIGHTS IN CYTOKINE RELEASE SYNDROME MANAGEMENT

With the development of new therapeutic reagents, physicians have to face previously unknown toxicities. Immunotherapy adverse events are mainly related to autoimmune direct toxicity, known as "on tumor/off target effect", and to an indirect cytokine-associated toxicity, called CRS.

Indirect toxicity: Cytokine release syndrome

CRS has been initially described following mAb infusions (*i.e.*, anti-CD52 or anti-CD20) and more recently with bi-specific antibodies and CARs[112,113]. This syndrome is characterized by a massive non-antigen specific inflammatory response. CRS shares features with macrophage activation syndrome, such as cytopenia, fever, hyperferritinemia and hypofibrinogenemia. Regardless the TAA target, it seems that T cell expansion activates other hematological effectors (B cells, neutrophils, macrophages), and favors release of inflammatory cytokines, such as TNF-α, IL-2, IL-6, IL-13 and IFNγ[44,98]. Symptom onset has been reported with a widely variable timing among clinical trials, ranging from 1 d to 3 wk for anti-CD19 CARs. All CRS reported with anti-CD19 in ALL trials were related to the tumor burden prior the first infusion and corresponded with maximal *in vivo* T cell expansion. Symptoms of CRS were drastically mild or even absent with anti-LeY in AML and with anti-CD19 CARs in CLL[45,87]. The magnitude of immune activation after engineered T cell infusion is therefore dependent upon the underlying condition, the remaining lymphocyte pool, and CAR T cell features. Symptoms are not specific and confusion can be made with those of infections. Fever appears as the hallmark of CRS, and arises generally concomitantly to rigors, myalgia, and gut disorders. However, other life threatening complications can occur. Recently, IL-6, a pleiotropic cytokine with ambivalent functions, emerged as the gatekeeper in the pathophysiology of CRS. In this setting, tocilizumab, an anti-IL-6R mAb initially developed for rheumatologic autoimmune diseases, has proven its efficacy in severe CRS. This agent showed a safe profile with few side effects at the recommended dose (one injection at 8 mg/kg for children and 4 mg/kg for adults). It did not seem to alter anti-leukemia functions of the transferred T cells. Alternative therapy in non-responders could involve anti-TNF-α mAb (infliximab), soluble TNF-α receptor (etanercept), or corticosteroids especially in patients presenting neurological symptoms[114]. Cardiac complications (similar to stress cardiomyopathies) are non frequent and generally reversible events, although potentially fatal. Neurological symptoms include mainly headache, dysphasia, confusion, seizure. Magnetic resonance imaging has shown abnormalities consistent with a mild encephalopathy with reversible splenial lesion syndrome, as observed in severe viral infections. The exact pathophysiology of neurological features during CRS is not fully understood, but seems to be related to a direct neurotoxicity of IL-6[115]. IL-6 levels in cerebrospinal fluid have been monitored during CRS and have shown high levels. It can be hypothesized that systemic inflammatory response after CAR T cells infusion could lead to permeable blood-brain barrier, yielding trafficking of IL-6 and activated immune cells to CNS. At least for CTL019, CAR T cells revealed to cross the blood-brain barrier and to be evolve in the neurological symptoms, which can be overcome by tocilizumab therapy. However, the occurrence of neurological symptoms can be enhanced by the IL-6R inhibitor. Tocilizumab may inhibit the IL-6 receptor mediated clearance and may allow transient increase of IL-6 levels. In patients with severe neurological symptoms, but without other life-threatening organ failure, it has been recommended to treat with corticosteroids (especially dexamethasone)[116]. Acute respiratory distress syndrome, hepatic/renal failure, disseminated coagulopathy have also been reported.

Direct toxicity: "On tumor/off target effect"

Prolonged B lymphocyte depletion illustrates perfectly the antigen driven direct toxicity, provided by anti-CD19 CAR T cells and hence a theoretical immunodeficiency. Although B lymphocyte depletion has been profound in reported clinical studies, its durability appears as a sign of prolonged anti-tumor response. This condition is very close to that of patients with X-linked agammaglobulinemia[97]. While B lymphocyte depletion increases the risk of opportunistic infections, this may be improved by intravenous immunoglobulin therapy. Although patient follow-up is relatively short, patients treated with anti-CD19 CAR T cells did not show until now any increase of bacterial infections. However, the development of this new technique will certainly be accompanied by the description of further new adverse events.

CONCLUSION

Immunotherapy represents certainly a great step forward in the treatment of AL. Because of the complexity of T cell repertory and its interaction with the immune system, there is, however, a long way to go before achievement of a complete and optimal understanding of this technology, as illustrated by the "on tumor/off target" paradigm. Furthermore, production of CAR T cells is time consuming, and still requires tremendous financial resources. The next challenges will be to improve cell isolation and culture of CAR-modified T cell production, to reduce the cost of the technique,

and therefore facilitate CAR T cells production and delivery in most institutions, beyond the academic research environment.

REFERENCES

1 **Nwajei F**, Konopleva M. The bone marrow microenvironment as niche retreats for hematopoietic and leukemic stem cells. *Adv Hematol* 2013; **2013**: 953982 [PMID: 23365579 DOI: 10.1155/2013/953982]

2 **Riether C**, Schürch CM, Ochsenbein AF. Regulation of hematopoietic and leukemic stem cells by the immune system. *Cell Death Differ* 2015; **22**: 187-198 [PMID: 24992931 DOI: 10.1038/cdd.2014.89]

3 **Zitvogel L**, Tesniere A, Kroemer G. Cancer despite immunosurveillance: immunoselection and immunosubversion. *Nat Rev Immunol* 2006; **6**: 715-727 [PMID: 16977338 DOI: 10.1038/nri1936]

4 **Weiden PL**, Sullivan KM, Flournoy N, Storb R, Thomas ED. Antileukemic effect of chronic graft-versus-host disease: contribution to improved survival after allogeneic marrow transplantation. *N Engl J Med* 1981; **304**: 1529-1533 [PMID: 7015133 DOI: 10.1056/NEJM198106183042507]

5 **Introna M**, Franceschetti M, Ciocca A, Borleri G, Conti E, Golay J, Rambaldi A. Rapid and massive expansion of cord blood-derived cytokine-induced killer cells: an innovative proposal for the treatment of leukemia relapse after cord blood transplantation. *Bone Marrow Transplant* 2006; **38**: 621-627 [PMID: 16980990 DOI: 10.1038/sj.bmt.1705503]

6 **Verneris MR**, Baker J, Edinger M, Negrin RS. Studies of ex vivo activated and expanded CD8+ NK-T cells in humans and mice. *J Clin Immunol* 2002; **22**: 131-136 [PMID: 12078854]

7 **Linn YC**, Lau LC, Hui KM. Generation of cytokine-induced killer cells from leukaemic samples with in vitro cytotoxicity against autologous and allogeneic leukaemic blasts. *Br J Haematol* 2002; **116**: 78-86 [PMID: 11841399]

8 **Introna M**, Borleri G, Conti E, Franceschetti M, Barbui AM, Broady R, Dander E, Gaipa G, D'Amico G, Biagi E, Parma M, Pogliani EM, Spinelli O, Baronciani D, Grassi A, Golay J, Barbui T, Biondi A, Rambaldi A. Repeated infusions of donor-derived cytokine-induced killer cells in patients relapsing after allogeneic stem cell transplantation: a phase I study. *Haematologica* 2007; **92**: 952-959 [PMID: 17606446]

9 **Laport GG**, Sheehan K, Baker J, Armstrong R, Wong RM, Lowsky R, Johnston LJ, Shizuru JA, Miklos D, Arai S, Benjamin JE, Weng WK, Negrin RS. Adoptive immunotherapy with cytokine-induced killer cells for patients with relapsed hematologic malignancies after allogeneic hematopoietic cell transplantation. *Biol Blood Marrow Transplant* 2011; **17**: 1679-1687 [PMID: 21664472 DOI: 10.1016/j.bbmt.2011.05.012]

10 **Doubrovina E**, Oflaz-Sozmen B, Prockop SE, Kernan NA, Abramson S, Teruya-Feldstein J, Hedvat C, Chou JF, Heller G, Barker JN, Boulad F, Castro-Malaspina H, George D, Jakubowski A, Koehne G, Papadopoulos EB, Scaradavou A, Small TN, Khalaf R, Young JW, O'Reilly RJ. Adoptive immunotherapy with unselected or EBV-specific T cells for biopsy-proven EBV+ lymphomas after allogeneic hematopoietic cell transplantation. *Blood* 2012; **119**: 2644-2656 [PMID: 22138512 DOI: 10.1182/blood-2011-08-371971]

11 **Leen AM**, Myers GD, Sili U, Huls MH, Weiss H, Leung KS, Carrum G, Krance RA, Chang CC, Molldrem JJ, Gee AP, Brenner MK, Heslop HE, Rooney CM, Bollard CM. Monoculture-derived T lymphocytes specific for multiple viruses expand and produce clinically relevant effects in immunocompromised individuals. *Nat Med* 2006; **12**: 1160-1166 [PMID: 16998485 DOI: 10.1038/nm1475]

12 **Gross G**, Waks T, Eshhar Z. Expression of immunoglobulin-T-cell receptor chimeric molecules as functional receptors with antibody-type specificity. *Proc Natl Acad Sci USA* 1989; **86**: 10024-10028 [PMID: 2513569]

13 **Sadelain M**, Rivière I, Brentjens R. Targeting tumours with genetically enhanced T lymphocytes. *Nat Rev Cancer* 2003; **3**: 35-45 [PMID: 12509765 DOI: 10.1038/nrc971]

14 **Lamers CH**, Sleijfer S, Vulto AG, Kruit WH, Kliffen M, Debets R, Gratama JW, Stoter G, Oosterwijk E. Treatment of metastatic renal cell carcinoma with autologous T-lymphocytes genetically retargeted against carbonic anhydrase IX: first clinical experience. *J Clin Oncol* 2006; **24**: e20-e22 [PMID: 16648493 DOI: 10.1200/JCO.2006.05.9964]

15 **Kershaw MH**, Westwood JA, Parker LL, Wang G, Eshhar Z, Mavroukakis SA, White DE, Wunderlich JR, Canevari S, Rogers-Freezer L, Chen CC, Yang JC, Rosenberg SA, Hwu P. A phase I study on adoptive immunotherapy using gene-modified T cells for ovarian cancer. *Clin Cancer Res* 2006; **12**: 6106-6115 [PMID: 17062687 DOI: 10.1158/1078-0432.CCR-06-1183]

16 **Park JR**, Digiusto DL, Slovak M, Wright C, Naranjo A, Wagner J, Meechoovet HB, Bautista C, Chang WC, Ostberg JR, Jensen MC. Adoptive transfer of chimeric antigen receptor re-directed cytolytic T lymphocyte clones in patients with neuroblastoma. *Mol Ther* 2007; **15**: 825-833 [PMID: 17299405 DOI: 10.1038/sj.mt.6300104]

17 **Grupp SA**, Kalos M, Barrett D, Aplenc R, Porter DL, Rheingold SR, Teachey DT, Chew A, Hauck B, Wright JF, Milone MC, Levine BL, June CH. Chimeric antigen receptor-modified T cells for acute lymphoid leukemia. *N Engl J Med* 2013; **368**: 1509-1518 [PMID: 23527958 DOI: 10.1056/NEJMoa1215134]

18 **Sadelain M**, Brentjens R, Rivière I. The promise and potential pitfalls of chimeric antigen receptors. *Curr Opin Immunol* 2009; **21**: 215-223 [PMID: 19327974 DOI: 10.1016/j.coi.2009.02.009]

19 **Zhou G**, Levitsky H. Towards curative cancer immunotherapy: overcoming posttherapy tumor escape. *Clin Dev Immunol* 2012; **2012**: 124187 [PMID: 22778760 DOI: 10.1155/2012/124187]

20 **Tassev DV**, Cheng M, Cheung NK. Retargeting NK92 cells using an HLA-A2-restricted, EBNA3C-specific chimeric antigen receptor. *Cancer Gene Ther* 2012; **19**: 84-100 [PMID: 21979579 DOI: 10.1038/cgt.2011.66]

21 **Stewart-Jones G**, Wadle A, Hombach A, Shenderov E, Held G, Fischer E, Kleber S, Nuber N, Stenner-Liewen F, Bauer S, McMichael A, Knuth A, Abken H, Hombach AA, Cerundolo V, Jones EY, Renner C. Rational development of high-affinity T-cell receptor-like antibodies. *Proc Natl Acad Sci USA* 2009; **106**: 5784-5788 [PMID: 19307587 DOI: 10.1073/pnas.0901425106]

22 **Spear P**, Barber A, Rynda-Apple A, Sentman CL. NKG2D CAR T-cell therapy inhibits the growth of NKG2D ligand heterogeneous tumors. *Immunol Cell Biol* 2013; **91**: 435-440 [PMID: 23628805 DOI: 10.1038/icb.2013.17]

23 **Zhang T**, Lemoi BA, Sentman CL. Chimeric NK-receptor-bearing T cells mediate antitumor immunotherapy. *Blood* 2005; **106**: 1544-1551 [PMID: 15890688 DOI: 10.1182/blood-2004-11-4365]

24 **Lamers CH**, Gratama JW, Warnaar SO, Stoter G, Bolhuis RL. Inhibition of bispecific monoclonal antibody (bsAb)-targeted cytolysis by human anti-mouse antibodies in ovarian carcinoma patients treated with bsAb-targeted activated T-lymphocytes. *Int J Cancer* 1995; **60**: 450-457 [PMID: 7829257]

25 **Davis SJ**, van der Merwe PA. The kinetic-segregation model: TCR triggering and beyond. *Nat Immunol* 2006; **7**: 803-809 [PMID: 16855606 DOI: 10.1038/ni1369]

26 **Guest RD**, Hawkins RE, Kirillova N, Cheadle EJ, Arnold J, O'Neill A, Irlam J, Chester KA, Kemshead JT, Shaw DM, Embleton MJ, Stern PL, Gilham DE. The role of extracellular spacer regions in the optimal design of chimeric immune receptors: evaluation of four different scFvs and antigens. *J Immunother* 2005; **28**: 203-211 [PMID: 15838376]

27 **James SE**, Greenberg PD, Jensen MC, Lin Y, Wang J, Till BG, Raubitschek AA, Forman SJ, Press OW. Antigen sensitivity of CD22-specific chimeric TCR is modulated by target epitope distance from the cell membrane. *J Immunol* 2008; **180**: 7028-7038 [PMID: 18453625]

28 **Haso W**, Lee DW, Shah NN, Stetler-Stevenson M, Yuan CM, Pastan IH, Dimitrov DS, Morgan RA, FitzGerald DJ, Barrett DM, Wayne AS, Mackall CL, Orentas RJ. Anti-CD22-chimeric antigen

receptors targeting B-cell precursor acute lymphoblastic leukemia. *Blood* 2013; **121**: 1165-1174 [PMID: 23243285 DOI: 10.1182/blood-2012-06-438002]
29 **Chmielewski M**, Hombach A, Heuser C, Adams GP, Abken H. T cell activation by antibody-like immunoreceptors: increase in affinity of the single-chain fragment domain above threshold does not increase T cell activation against antigen-positive target cells but decreases selectivity. *J Immunol* 2004; **173**: 7647-7653 [PMID: 15585893]
30 **Hudecek M**, Lupo-Stanghellini MT, Kosasih PL, Sommermeyer D, Jensen MC, Rader C, Riddell SR. Receptor affinity and extracellular domain modifications affect tumor recognition by ROR1-specific chimeric antigen receptor T cells. *Clin Cancer Res* 2013; **19**: 3153-3164 [PMID: 23620405 DOI: 10.1158/1078-0432.CCR-13-0330]
31 **Mardiros A**, Dos Santos C, McDonald T, Brown CE, Wang X, Budde LE, Hoffman L, Aguilar B, Chang WC, Bretzlaff W, Chang B, Jonnalagadda M, Starr R, Ostberg JR, Jensen MC, Bhatia R, Forman SJ. T cells expressing CD123-specific chimeric antigen receptors exhibit specific cytolytic effector functions and antitumor effects against human acute myeloid leukemia. *Blood* 2013; **122**: 3138-3148 [PMID: 24030378 DOI: 10.1182/blood-2012-12-474056]
32 **Hombach A**, Hombach AA, Abken H. Adoptive immunotherapy with genetically engineered T cells: modification of the IgG1 Fc 'spacer' domain in the extracellular moiety of chimeric antigen receptors avoids 'off-target' activation and unintended initiation of an innate immune response. *Gene Ther* 2010; **17**: 1206-1213 [PMID: 20555360 DOI: 10.1038/gt.2010.91]
33 **Hudecek M**, Silva A, Kosasih PL, Chen YY, Turtle CJ, Jensen MC, Riddell SR. The non-signaling extracellular spacer domain of CD19-specific chimeric antigen receptors is decisive for in vivo anti-tumor activity. *Blood* 2012; **120**: 951
34 **Louis CU**, Savoldo B, Dotti G, Pule M, Yvon E, Myers GD, Rossig C, Russell HV, Diouf O, Liu E, Liu H, Wu MF, Gee AP, Mei Z, Rooney CM, Heslop HE, Brenner MK. Antitumor activity and long-term fate of chimeric antigen receptor-positive T cells in patients with neuroblastoma. *Blood* 2011; **118**: 6050-6056 [PMID: 21984804 DOI: 10.1182/blood-2011-05-354449]
35 **Pule MA**, Savoldo B, Myers GD, Rossig C, Russell HV, Dotti G, Huls MH, Liu E, Gee AP, Mei Z, Yvon E, Weiss HL, Liu H, Rooney CM, Heslop HE, Brenner MK. Virus-specific T cells engineered to coexpress tumor-specific receptors: persistence and antitumor activity in individuals with neuroblastoma. *Nat Med* 2008; **14**: 1264-1270 [PMID: 18978797 DOI: 10.1038/nm.1882]
36 **Till BG**, Jensen MC, Wang J, Chen EY, Wood BL, Greisman HA, Qian X, James SE, Raubitschek A, Forman SJ, Gopal AK, Pagel JM, Lindgren CG, Greenberg PD, Riddell SR, Press OW. Adoptive immunotherapy for indolent non-Hodgkin lymphoma and mantle cell lymphoma using genetically modified autologous CD20-specific T cells. *Blood* 2008; **112**: 2261-2271 [PMID: 18509084 DOI: 10.1182/blood-2007-12-128843]
37 **Jensen MC**, Popplewell L, Cooper LJ, DiGiusto D, Kalos M, Ostberg JR, Forman SJ. Antitransgene rejection responses contribute to attenuated persistence of adoptively transferred CD20/CD19-specific chimeric antigen receptor redirected T cells in humans. *Biol Blood Marrow Transplant* 2010; **16**: 1245-1256 [PMID: 20304086 DOI: 10.1016/j.bbmt.2010.03.014]
38 **Hombach A**, Wieczarkowiecz A, Marquardt T, Heuser C, Usai L, Pohl C, Seliger B, Abken H. Tumor-specific T cell activation by recombinant immunoreceptors: CD3 zeta signaling and CD28 costimulation are simultaneously required for efficient IL-2 secretion and can be integrated into one combined CD28/CD3 zeta signaling receptor molecule. *J Immunol* 2001; **167**: 6123-6131 [PMID: 11714771]
39 **Hombach A**, Abken H. Costimulation tunes tumor-specific activation of redirected T cells in adoptive immunotherapy. *Cancer Immunol Immunother* 2007; **56**: 731-737 [PMID: 17143613 DOI: 10.1007/s00262-006-0249-0]
40 **Hombach AA,** Heiders J, Foppe M, Chmielewski M, Abken H. OX40 costimulation by a chimeric antigen receptor abrogates CD28 and IL-2 induced IL-10 secretion by redirected CD4(+) T cells. *Oncoimmunology* 2012; **1**: 458-466 [PMID: 22754764]
41 **Shen CJ**, Yang YX, Han EQ, Cao N, Wang YF, Wang Y, Zhao YY, Zhao LM, Cui J, Gupta P, Wong AJ, Han SY. Chimeric antigen receptor containing ICOS signaling domain mediates specific and efficient antitumor effect of T cells against EGFRvIII expressing glioma. *J Hematol Oncol* 2013; **6**: 33 [PMID: 23656794 DOI: 10.1186/1756-8722-6-33]
42 **Duong CP**, Westwood JA, Yong CS, Murphy A, Devaud C, John LB, Darcy PK, Kershaw MH. Engineering T cell function using chimeric antigen receptors identified using a DNA library approach. *PLoS One* 2013; **8**: e63037 [PMID: 23667569 DOI: 10.1371/journal.pone.0063037]
43 **Beecham EJ**, Ma Q, Ripley R, Junghans RP. Coupling CD28 co-stimulation to immunoglobulin T-cell receptor molecules: the dynamics of T-cell proliferation and death. *J Immunother* 2000; **23**: 631-642 [PMID: 11186151]
44 **Kalos M**, Levine BL, Porter DL, Katz S, Grupp SA, Bagg A, June CH. T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. *Sci Transl Med* 2011; **3**: 95ra73 [PMID: 21832238 DOI: 10.1126/scitranslmed.3002842]
45 **Porter DL**, Levine BL, Kalos M, Bagg A, June CH. Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. *N Engl J Med* 2011; **365**: 725-733 [PMID: 21830940 DOI: 10.1056/NEJMoa1103849]
46 **Koehler H**, Kofler D, Hombach A, Abken H. CD28 costimulation overcomes transforming growth factor-beta-mediated repression of proliferation of redirected human CD4+ and CD8+ T cells in an antitumor cell attack. *Cancer Res* 2007; **67**: 2265-2273 [PMID: 17332357 DOI: 10.1158/0008-5472.CAN-06-2098]
47 **Savoldo B**, Ramos CA, Liu E, Mims MP, Keating MJ, Carrum G, Kamble RT, Bollard CM, Gee AP, Mei Z, Liu H, Grilley B, Rooney CM, Heslop HE, Brenner MK, Dotti G. CD28 costimulation improves expansion and persistence of chimeric antigen receptor-modified T cells in lymphoma patients. *J Clin Invest* 2011; **121**: 1822-1826 [PMID: 21540550 DOI: 10.1172/JCI46110]
48 **Chambers CA**, Allison JP. Co-stimulation in T cell responses. *Curr Opin Immunol* 1997; **9**: 396-404 [PMID: 9203422]
49 **Bour-Jordan H**, Bluestone JA. Regulating the regulators: costimulatory signals control the homeostasis and function of regulatory T cells. *Immunol Rev* 2009; **229**: 41-66 [PMID: 19426214 DOI: 10.1111/j.1600-065X.2009.00775.x]
50 **Chmielewski M**, Hombach AA, Abken H. CD28 cosignalling does not affect the activation threshold in a chimeric antigen receptor-redirected T-cell attack. *Gene Ther* 2011; **18**: 62-72 [PMID: 20944680 DOI: 10.1038/gt.2010.127]
51 **Brentjens RJ**, Santos E, Nikhamin Y, Yeh R, Matsushita M, La Perle K, Quintás-Cardama A, Larson SM, Sadelain M. Genetically targeted T cells eradicate systemic acute lymphoblastic leukemia xenografts. *Clin Cancer Res* 2007; **13**: 5426-5435 [PMID: 17855649 DOI: 10.1158/1078-0432.CCR-07-0674]
52 **Carpenito C**, Milone MC, Hassan R, Simonet JC, Lakhal M, Suhoski MM, Varela-Rohena A, Haines KM, Heitjan DF, Albelda SM, Carroll RG, Riley JL, Pastan I, June CH. Control of large, established tumor xenografts with genetically retargeted human T cells containing CD28 and CD137 domains. *Proc Natl Acad Sci USA* 2009; **106**: 3360-3365 [PMID: 19211796 DOI: 10.1073/pnas.0813101106]
53 **Milone MC**, Fish JD, Carpenito C, Carroll RG, Binder GK, Teachey D, Samanta M, Lakhal M, Gloss B, Danet-Desnoyers G, Campana D, Riley JL, Grupp SA, June CH. Chimeric receptors containing CD137 signal transduction domains mediate enhanced survival of T cells and increased antileukemic efficacy in vivo. *Mol Ther* 2009; **17**: 1453-1464 [PMID: 19384291 DOI: 10.1038/mt.2009.83]
54 **Zhong XS**, Matsushita M, Plotkin J, Riviere I, Sadelain M. Chimeric antigen receptors combining 4-1BB and CD28 signaling domains augment PI3kinase/AKT/Bcl-XL activation and CD8+

T cell-mediated tumor eradication. *Mol Ther* 2010; **18**: 413-420 [PMID: 19773745 DOI: 10.1038/mt.2009.210]
55 **Pulè MA**, Straathof KC, Dotti G, Heslop HE, Rooney CM, Brenner MK. A chimeric T cell antigen receptor that augments cytokine release and supports clonal expansion of primary human T cells. *Mol Ther* 2005; **12**: 933-941 [PMID: 15979412 DOI: 10.1016/j.ymthe.2005.04.016]
56 **Abate-Daga D**, Lagisetty KH, Tran E, Zheng Z, Gattinoni L, Yu Z, Burns WR, Miermont AM, Teper Y, Rudloff U, Restifo NP, Feldman SA, Rosenberg SA, Morgan RA. A novel chimeric antigen receptor against prostate stem cell antigen mediates tumor destruction in a humanized mouse model of pancreatic cancer. *Hum Gene Ther* 2014; **25**: 1003-1012 [PMID: 24694017 DOI: 10.1089/hum.2013.209]
57 **Hombach AA**, Abken H. Costimulation by chimeric antigen receptors revisited the T cell antitumor response benefits from combined CD28-OX40 signalling. *Int J Cancer* 2011; **129**: 2935-2944 [PMID: 22030616 DOI: 10.1002/ijc.25960]
58 **Till BG**, Jensen MC, Wang J, Qian X, Gopal AK, Maloney DG, Lindgren CG, Lin Y, Pagel JM, Budde LE, Raubitschek A, Forman SJ, Greenberg PD, Riddell SR, Press OW. CD20-specific adoptive immunotherapy for lymphoma using a chimeric antigen receptor with both CD28 and 4-1BB domains: pilot clinical trial results. *Blood* 2012; **119**: 3940-3950 [PMID: 22308288 DOI: 10.1182/blood-2011-10-387969]
59 **Montini E**, Cesana D, Schmidt M, Sanvito F, Ponzoni M, Bartholomae C, Sergi Sergi L, Benedicenti F, Ambrosi A, Di Serio C, Doglioni C, von Kalle C, Naldini L. Hematopoietic stem cell gene transfer in a tumor-prone mouse model uncovers low genotoxicity of lentiviral vector integration. *Nat Biotechnol* 2006; **24**: 687-696 [PMID: 16732270 DOI: 10.1038/nbt1216]
60 **Davila ML**, Kloss CC, Gunset G, Sadelain M. CD19 CAR-targeted T cells induce long-term remission and B Cell Aplasia in an immunocompetent mouse model of B cell acute lymphoblastic leukemia. *PLoS One* 2013; **8**: e61338 [PMID: 23585892 DOI: 10.1371/journal.pone.0061338]
61 **Scholler J**, Brady TL, Binder-Scholl G, Hwang WT, Plesa G, Hege KM, Vogel AN, Kalos M, Riley JL, Deeks SG, Mitsuyasu RT, Bernstein WB, Aronson NE, Levine BL, Bushman FD, June CH. Decade-long safety and function of retroviral-modified chimeric antigen receptor T cells. *Sci Transl Med* 2012; **4**: 132ra53 [PMID: 22553251 DOI: 10.1126/scitranslmed.3003761]
62 **Brentjens RJ**, Rivière I, Park JH, Davila ML, Wang X, Stefanski J, Taylor C, Yeh R, Bartido S, Borquez-Ojeda O, Olszewska M, Bernal Y, Pegram H, Przybylowski M, Hollyman D, Usachenko Y, Pirraglia D, Hosey J, Santos E, Halton E, Maslak P, Scheinberg D, Jurcic J, Heaney M, Heller G, Frattini M, Sadelain M. Safety and persistence of adoptively transferred autologous CD19-targeted T cells in patients with relapsed or chemotherapy refractory B-cell leukemias. *Blood* 2011; **118**: 4817-4828 [PMID: 21849486 DOI: 10.1182/blood-2011-04-348540]
63 **Brentjens RJ**, Davila ML, Riviere I, Park J, Wang X, Cowell LG, Bartido S, Stefanski J, Taylor C, Olszewska M, Borquez-Ojeda O, Qu J, Wasielewska T, He Q, Bernal Y, Rijo IV, Hedvat C, Kobos R, Curran K, Steinherz P, Jurcic J, Rosenblat T, Maslak P, Frattini M, Sadelain M. CD19-targeted T cells rapidly induce molecular remissions in adults with chemotherapy-refractory acute lymphoblastic leukemia. *Sci Transl Med* 2013; **5**: 177ra38 [PMID: 23515080 DOI: 10.1126/scitranslmed.3005930]
64 **Bestor TH**. Gene silencing as a threat to the success of gene therapy. *J Clin Invest* 2000; **105**: 409-411 [PMID: 10683368 DOI: 10.1172/JCI9459]
65 **Ivics Z**, Izsvak Z, Minter A, Hackett PB. Identification of functional domains and evolution of Tc1-like transposable elements. *Proc Natl Acad Sci USA* 1996; **93**: 5008-5013 [PMID: 8643520]
66 **Field AC**, Vink C, Gabriel R, Al-Subki R, Schmidt M, Goulden N, Stauss H, Thrasher A, Morris E, Qasim W. Comparison of lentiviral and sleeping beauty mediated αβ T cell receptor gene transfer. *PLoS One* 2013; **8**: e68201 [PMID: 23840834 DOI: 10.1371/journal.pone.0068201]
67 **Magnani C**, Turazzi N, Benedicenti F, Tettamanti S, Attianese GG, Rossi V, Montini E, Cooper L, Aiuti A, Biondi A, Biagi E. Cytokine-induced killer (CIK) cells engineered with chimeric antigen receptors (CARs) by sleeping beauty system. *Cytotherapy* 2014; **16**: S32 [DOI: 10.1016/j.jcyt.2014.01.107]
68 **Morgan RA**, Yang JC, Kitano M, Dudley ME, Laurencot CM, Rosenberg SA. Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. *Mol Ther* 2010; **18**: 843-851 [PMID: 20179677 DOI: 10.1038/mt.2010.24]
69 **Kochenderfer JN**, Dudley ME, Feldman SA, Wilson WH, Spaner DE, Maric I, Stetler-Stevenson M, Phan GQ, Hughes MS, Sherry RM, Yang JC, Kammula US, Devillier L, Carpenter R, Nathan DA, Morgan RA, Laurencot C, Rosenberg SA. B-cell depletion and remissions of malignancy along with cytokine-associated toxicity in a clinical trial of anti-CD19 chimeric-antigen-receptor-transduced T cells. *Blood* 2012; **119**: 2709-2720 [PMID: 22160384 DOI: 10.1182/blood-2011-10-384388]
70 **Bonini C**, Ferrari G, Verzeletti S, Servida P, Zappone E, Ruggieri L, Ponzoni M, Rossini S, Mavilio F, Traversari C, Bordignon C. HSV-TK gene transfer into donor lymphocytes for control of allogeneic graft-versus-leukemia. *Science* 1997; **276**: 1719-1724 [PMID: 9180086]
71 **Zhao Y**, Moon E, Carpenito C, Paulos CM, Liu X, Brennan AL, Chew A, Carroll RG, Scholler J, Levine BL, Albelda SM, June CH. Multiple injections of electroporated autologous T cells expressing a chimeric antigen receptor mediate regression of human disseminated tumor. *Cancer Res* 2010; **70**: 9053-9061 [PMID: 20926399 DOI: 10.1158/0008-5472.CAN-10-2880]
72 **Di Stasi A**, Tey SK, Dotti G, Fujita Y, Kennedy-Nasser A, Martinez C, Straathof K, Liu E, Durett AG, Grilley B, Liu H, Cruz CR, Savoldo B, Gee AP, Schindler J, Krance RA, Heslop HE, Spencer DM, Rooney CM, Brenner MK. Inducible apoptosis as a safety switch for adoptive cell therapy. *N Engl J Med* 2011; **365**: 1673-1683 [PMID: 22047558 DOI: 10.1056/NEJMoa1106152]
73 **Casucci M**, Nicolis di Robilant B, Falcone L, Camisa B, Norelli M, Genovese P, Gentner B, Gullotta F, Ponzoni M, Bernardi M, Marcatti M, Saudemont A, Bordignon C, Savoldo B, Ciceri F, Naldini L, Dotti G, Bonini C, Bondanza A. CD44v6-targeted T cells mediate potent antitumor effects against acute myeloid leukemia and multiple myeloma. *Blood* 2013; **122**: 3461-3472 [PMID: 24016461 DOI: 10.1182/blood-2013-04-493361]
74 **Brindley DA**, Davie NL, Culme-Seymour EJ, Mason C, Smith DW, Rowley JA. Peak serum: implications of serum supply for cell therapy manufacturing. *Regen Med* 2012; **7**: 7-13 [PMID: 22168489 DOI: 10.2217/rme.11.112]
75 **Ohminami H**, Yasukawa M, Kaneko S, Yakushijin Y, Abe Y, Kasahara Y, Ishida Y, Fujita S. Fas-independent and nonapoptotic cytotoxicity mediated by a human CD4(+) T-cell clone directed against an acute myelogenous leukemia-associated DEK-CAN fusion peptide. *Blood* 1999; **93**: 925-935 [PMID: 9920842]
76 **Yasukawa M**, Ohminami H, Arai J, Kasahara Y, Ishida Y, Fujita S. Granule exocytosis, and not the fas/fas ligand system, is the main pathway of cytotoxicity mediated by alloantigen-specific CD4(+) as well as CD8(+) cytotoxic T lymphocytes in humans. *Blood* 2000; **95**: 2352-2355 [PMID: 10733506]
77 **Deniger DC**, Switzer K, Mi T, Maiti S, Hurton L, Singh H, Huls H, Olivares S, Lee DA, Champlin RE, Cooper LJ. Bispecific T-cells expressing polyclonal repertoire of endogenous γδ T-cell receptors and introduced CD19-specific chimeric antigen receptor. *Mol Ther* 2013; **21**: 638-647 [PMID: 23295945 DOI: 10.1038/mt.2012.267]
78 **Muranski P**, Boni A, Antony PA, Cassard L, Irvine KR, Kaiser A, Paulos CM, Palmer DC, Touloukian CE, Ptak K, Gattinoni L, Wrzesinski C, Hinrichs CS, Kerstann KW, Feigenbaum L, Chan CC, Restifo NP. Tumor-specific Th17-polarized cells eradicate large established melanoma. *Blood* 2008; **112**: 362-373 [PMID: 18354038 DOI: 10.1182/blood-2007-11-120998]
79 **Larson SM**, Tu A, Senadheera S, Ho M, Kohn DB, de Oliveira S. Anti-CD19 chimeric antigen receptor controlled by the suicide gene HSVsr39TK in hematopoietic stem cells for immunotherapy of

B-lineage malignancies. *Blood* 2013; **122**: 1659

80 **Wang X**, Wong CW, Urak R, Mardiros A, Chang W, Taus E, Budde LE, Ostberg JR, Brown C, Forman SJ. Targeting of leukemia stem cells by interfering with niches using CD44 variant 6 chimeric antigen receptor redirected central memory T cells. *Blood* 2013; **122**: 4497

81 **Gattinoni L**, Finkelstein SE, Klebanoff CA, Antony PA, Palmer DC, Spiess PJ, Hwang LN, Yu Z, Wrzesinski C, Heimann DM, Surh CD, Rosenberg SA, Restifo NP. Removal of homeostatic cytokine sinks by lymphodepletion enhances the efficacy of adoptively transferred tumor-specific CD8+ T cells. *J Exp Med* 2005; **202**: 907-912 [PMID: 16203864 DOI: 10.1084/jem.20050732]

82 **Antony PA**, Paulos CM, Ahmadzadeh M, Akpinarli A, Palmer DC, Sato N, Kaiser A, Hinrichs CS, Klebanoff CA, Tagaya Y, Restifo NP. Interleukin-2-dependent mechanisms of tolerance and immunity in vivo. *J Immunol* 2006; **176**: 5255-5266 [PMID: 16621991]

83 **Ghiringhelli F**, Larmonier N, Schmitt E, Parcellier A, Cathelin D, Garrido C, Chauffert B, Solary E, Bonnotte B, Martin F. CD4+CD25+ regulatory T cells suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative. *Eur J Immunol* 2004; **34**: 336-344 [PMID: 14768038 DOI: 10.1002/eji.200324181]

84 **Pegram HJ**, Lee JC, Hayman EG, Imperato GH, Tedder TF, Sadelain M, Brentjens RJ. Tumor-targeted T cells modified to secrete IL-12 eradicate systemic tumors without need for prior conditioning. *Blood* 2012; **119**: 4133-4141 [PMID: 22354001 DOI: 10.1182/blood-2011-12-400044]

85 **Rapoport AP**, Stadtmauer EA, Aqui N, Vogl D, Chew A, Fang HB, Janofsky S, Yager K, Veloso E, Zheng Z, Milliron T, Westphal S, Cotte J, Huynh H, Cannon A, Yanovich S, Akpek G, Tan M, Virts K, Ruehle K, Harris C, Philip S, Vonderheide RH, Levine BL, June CH. Rapid immune recovery and graft-versus-host disease-like engraftment syndrome following adoptive transfer of Costimulated autologous T cells. *Clin Cancer Res* 2009; **15**: 4499-4507 [PMID: 19509133 DOI: 10.1158/1078-0432.CCR-09-0418]

86 **Grupp SA**, Prak EL, Boyer J, McDonald KR, Shusterman S, Thompson E, Callahan C, Jawad AF, Levine BL, June CH, Sullivan KE. Adoptive transfer of autologous T cells improves T-cell repertoire diversity and long-term B-cell function in pediatric patients with neuroblastoma. *Clin Cancer Res* 2012; **18**: 6732-6741 [PMID: 23092876 DOI: 10.1158/1078-0432.CCR-12-1432]

87 **Ritchie DS**, Neeson PJ, Khot A, Peinert S, Tai T, Tainton K, Chen K, Shin M, Wall DM, Hönemann D, Gambell P, Westerman DA, Haurat J, Westwood JA, Scott AM, Kravets L, Dickinson M, Trapani JA, Smyth MJ, Darcy PK, Kershaw MH, Prince HM. Persistence and efficacy of second generation CAR T cell against the LeY antigen in acute myeloid leukemia. *Mol Ther* 2013; **21**: 2122-2129 [PMID: 23831595 DOI: 10.1038/mt.2013.154]

88 **Pinthus JH**, Waks T, Malina V, Kaufman-Francis K, Harmelin A, Aizenberg I, Kanety H, Ramon J, Eshhar Z. Adoptive immunotherapy of prostate cancer bone lesions using redirected effector lymphocytes. *J Clin Invest* 2004; **114**: 1774-1781 [PMID: 15599402 DOI: 10.1172/JCI22284]

89 **Kershaw MH**, Wang G, Westwood JA, Pachynski RK, Tiffany HL, Marincola FM, Wang E, Young HA, Murphy PM, Hwu P. Redirecting migration of T cells to chemokine secreted from tumors by genetic modification with CXCR2. *Hum Gene Ther* 2002; **13**: 1971-1980 [PMID: 12427307 DOI: 10.1089/10430340260355374]

90 **Robinson SN**, Simmons PJ, Thomas MW, Brouard N, Javni JA, Trilok S, Shim JS, Yang H, Steiner D, Decker WK, Xing D, Shultz LD, Savoldo B, Dotti G, Bollard CM, Miller L, Champlin RE, Shpall EJ, Zweidler-McKay PA. Ex vivo fucosylation improves human cord blood engraftment in NOD-SCID IL-2Rγ(null) mice. *Exp Hematol* 2012; **40**: 445-456 [PMID: 22306295 DOI: 10.1016/j.exphem.2012.01.015]

91 **Chung EY**, Psathas JN, Yu D, Li Y, Weiss MJ, Thomas-Tikhonenko A. CD19 is a major B cell receptor-independent activator of MYC-driven B-lymphomagenesis. *J Clin Invest* 2012; **122**: 2257-2266 [PMID: 22546857 DOI: 10.1172/JCI45851]

92 **Burger JA**, Ghia P, Rosenwald A, Caligaris-Cappio F. The microenvironment in mature B-cell malignancies: a target for new treatment strategies. *Blood* 2009; **114**: 3367-3375 [PMID: 19636060 DOI: 10.1182/blood-2009-06-225326]

93 **Ramsay AG**, Johnson AJ, Lee AM, Gorgün G, Le Dieu R, Blum W, Byrd JC, Gribben JG. Chronic lymphocytic leukemia T cells show impaired immunological synapse formation that can be reversed with an immunomodulating drug. *J Clin Invest* 2008; **118**: 2427-2437 [PMID: 18551193 DOI: 10.1172/JCI35017]

94 **Davila ML**, Riviere I, Wang X, Bartido S, StefanskiJ, Qing He, Borquez-OjedaO, Taylor C, Wasielewska T, Jinrong Q, Bouhassira D, Bernal YJ, YooS, PurdonT, Halton E, Quintanilla H, Park JH, Curran KJ, Sadelain M, Brentjens RJ. Safe and effective re-induction of complete remissions in adults with relapsed B-ALL using 19-28z CAR CD19-targeted T cell therapy. *Blood* 2013; **122**: 69

95 **Cruz CR**, Micklethwaite KP, Savoldo B, Ramos CA, Lam S, Ku S, Diouf O, Liu E, Barrett AJ, Ito S, Shpall EJ, Krance RA, Kamble RT, Carrum G, Hosing CM, Gee AP, Mei Z, Grilley BJ, Heslop HE, Rooney CM, Brenner MK, Bollard CM, Dotti G. Infusion of donor-derived CD19-redirected virus-specific T cells for B-cell malignancies relapsed after allogeneic stem cell transplant: a phase 1 study. *Blood* 2013; **122**: 2965-2973 [PMID: 24030379 DOI: 10.1182/blood-2013-06-506741]

96 **Topp MS**, Gökbuget N, Zugmaier G, Degenhard E, Goebeler ME, Klinger M, Neumann SA, Horst HA, Raff T, Viardot A, Stelljes M, Schaich M, Köhne-Volland R, Brüggemann M, Ottmann OG, Burmeister T, Baeuerle PA, Nagorsen D, Schmidt M, Einsele H, Riethmüller G, Kneba M, Hoelzer D, Kufer P, Bargou RC. Long-term follow-up of hematologic relapse-free survival in a phase 2 study of blinatumomab in patients with MRD in B-lineage ALL. *Blood* 2012; **120**: 5185-5187 [PMID: 23024237 DOI: 10.1182/blood-2012-07-441030]

97 **Grupp SA**, Frey NV, Aplenc R, Barrett DM, Chew A, Kalos M, Levine BL, Litchman M, Maude SL, Rheingold SR, Shen A, Strait C, Teachey DT, Wood PA, Porter D, June CH. T cells engineered with a chimeric antigen receptor (CAR) targeting CD19 (CTL019) produce significant in vivo proliferation, complete responses and long-term persistence without GVHD in children and adults with relapsed, refractory ALL. *Blood* 2013; **122**: 67

98 **Grupp SA**, Frey NV, Aplenc R, Levine BL, Maude S, Rheingold S, Strait Barker C, Teachey D, Mahnke Y, Porter D, June CH. T cells engineered with a chimeric antigen receptor (CAR) targeting CD19 (CTL019 cells) produce significant in vivo proliferation, complete responses and long-term persistence without GVHD in children and adults with relapsed, refractory ALL. *Bone Marrow Transplant* 2014; **49**: S1 [DOI: 10.1038/bmt.2014.42]

99 **Kreitman RJ**, Pastan I. Antibody fusion proteins: anti-CD22 recombinant immunotoxin moxetumomab pasudotox. *Clin Cancer Res* 2011; **17**: 6398-6405 [PMID: 22003067 DOI: 10.1158/1078-0432.CCR-11-0487]

100 **Qin H**, Monica C, Haso W, Zhang L. Pre-clinical development of a novel chimerical antigen receptor targeting high-risk pediatric ALL over-expressing Tslpr. *Blood* 2013; **122**: 2665

101 **Kloss CC**, Condomines M, Cartellieri M, Bachmann M, Sadelain M. Combinatorial antigen recognition with balanced signaling promotes selective tumor eradication by engineered T cells. *Nat Biotechnol* 2013; **31**: 71-75 [PMID: 23242161 DOI: 10.1038/nbt.2459]

102 **Luger SM**. Treating the elderly patient with acute myelogenous leukemia. *Hematology Am Soc Hematol Educ Program* 2010; **2010**: 62-69 [PMID: 21239772 DOI: 10.1182/asheducation-2010.1.62]

103 **Peinert S**, Prince HM, Guru PM, Kershaw MH, Smyth MJ, Trapani JA, Gambell P, Harrison S, Scott AM, Smyth FE, Darcy PK, Tainton K, Neeson P, Ritchie DS, Hönemann D. Gene-modified T cells as immunotherapy for multiple myeloma and acute myeloid leukemia expressing the Lewis Y antigen. *Gene Ther* 2010; **17**: 678-686 [PMID: 20200563 DOI: 10.1038/gt.2010.21]

104 **Marin V**, Pizzitola I, Agostoni V, Attianese GM, Finney H, Lawson A, Pule M, Rousseau R, Biondi A, Biagi E. Cytokine-induced killer cells for cell therapy of acute myeloid leukemia: improvement of

their immune activity by expression of CD33-specific chimeric receptors. *Haematologica* 2010; **95**: 2144-2152 [PMID: 20713459 DOI: 10.3324/haematol.2010.026310]

105 **Thol F**, Schlenk RF. Gemtuzumab ozogamicin in acute myeloid leukemia revisited. *Expert Opin Biol Ther* 2014; **14**: 1185-1195 [PMID: 24865510 DOI: 10.1517/14712598.2014.922534]

106 **Dutour A**, Marin V, Pizzitola I, Valsesia-Wittmann S, Lee D, Yvon E, Finney H, Lawson A, Brenner M, Biondi A, Biagi E, Rousseau R. In Vitro and In Vivo Antitumor Effect of Anti-CD33 Chimeric Receptor-Expressing EBV-CTL against CD33 Acute Myeloid Leukemia. *Adv Hematol* 2012; **2012**: 683065 [PMID: 22272203 DOI: 10.1155/2012/683065]

107 **Gill S**, Tasian SK, Ruella M, Shestova O, Li Y, Porter DL, Carroll M, Danet-Desnoyers G, Scholler J, Grupp SA, June CH, Kalos M. Preclinical targeting of human acute myeloid leukemia and myeloablation using chimeric antigen receptor-modified T cells. *Blood* 2014; **123**: 2343-2354 [PMID: 24596416 DOI: 10.1182/blood-2013-09-529537]

108 **Tettamanti S**, Marin V, Pizzitola I, Magnani CF, Giordano Attianese GM, Cribioli E, Maltese F, Galimberti S, Lopez AF, Biondi A, Bonnet D, Biagi E. Targeting of acute myeloid leukaemia by cytokine-induced killer cells redirected with a novel CD123-specific chimeric antigen receptor. *Br J Haematol* 2013; **161**: 389-401 [PMID: 23432359 DOI: 10.1111/bjh.12282]

109 **Singh V**, Erb U, Zöller M. Cooperativity of CD44 and CD49d in leukemia cell homing, migration, and survival offers a means for therapeutic attack. *J Immunol* 2013; **191**: 5304-5316 [PMID: 24127558 DOI: 10.4049/jimmunol.1301543]

110 **Jiang L**, Zhao LY, He J, Zhao YF, Wu Y, Liu Y, Jiang XZ. The expression and significance of osteopontin and its receptor CD44v6 in oral squamous carcinoma. *Huaxi Kouqiang Yixue Zazhi* 2008; **26**: 248-251 [PMID: 18705503]

111 **Legras S**, Günthert U, Stauder R, Curt F, Oliferenko S, Kluin-Nelemans HC, Marie JP, Proctor S, Jasmin C, Smadja-Joffe F. A strong expression of CD44-6v correlates with shorter survival of patients with acute myeloid leukemia. *Blood* 1998; **91**: 3401-3413 [PMID: 9558399]

112 **Wing MG**, Moreau T, Greenwood J, Smith RM, Hale G, Isaacs J, Waldmann H, Lachmann PJ, Compston A. Mechanism of first-dose cytokine-release syndrome by CAMPATH 1-H: involvement of CD16 (FcgammaRIII) and CD11a/CD18 (LFA-1) on NK cells. *J Clin Invest* 1996; **98**: 2819-2826 [PMID: 8981930 DOI: 10.1172/JCI119110]

113 **Teachey DT**, Rheingold SR, Maude SL, Zugmaier G, Barrett DM, Seif AE, Nichols KE, Suppa EK, Kalos M, Berg RA, Fitzgerald JC, Aplenc R, Gore L, Grupp SA. Cytokine release syndrome after blinatumomab treatment related to abnormal macrophage activation and ameliorated with cytokine-directed therapy. *Blood* 2013; **121**: 5154-5157 [PMID: 23678006 DOI: 10.1182/blood-2013-02-485623]

114 **Lee DW**, Gardner R, Porter DL, Louis CU, Ahmed N, Jensen M, Grupp SA, Mackall CL. Current concepts in the diagnosis and management of cytokine release syndrome. *Blood* 2014; **124**: 188-195 [PMID: 24876563 DOI: 10.1182/blood-2014-05-552729]

115 **Spooren A**, Kolmus K, Laureys G, Clinckers R, De Keyser J, Haegeman G, Gerlo S. Interleukin-6, a mental cytokine. *Brain Res Rev* 2011; **67**: 157-183 [PMID: 21238488 DOI: 10.1016/j.brainresrev.2011.01.002]

116 **Lee DW**, Shah N, Stetler-Stevenson M, Sabatino M, Richards K, Delbrook C, Kochenderfer JN, Rosenberg SA, Stroncek D, Mackall CL, Wayne AS. Autologous-collected anti-CD19 chimeric antigen receptor (CAR) T cells for acute lymphocytic leukemia (ALL) and Non-Hodgkin's lymphoma (NHL) in children who have previously undergone allogeneic hematopoietic stem cell transplantation (HSCT). *Cancer Res* 2013; **73** (8 Suppl): Abstract nr LB-138 [DOI: 10.1158/1538-7445.AM2013-LB-138]

P- Reviewer: Shimoni A **S- Editor**: Gong XM **L- Editor**: A
E- Editor: Wu HL

WJSC World Journal of Stem Cells

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4252/wjsc.v7.i7.1039

World J Stem Cells 2015 August 26; 7(7): 1039-1046
ISSN 1948-0210 (online)
© 2015 Baishideng Publishing Group Inc. All rights reserved.

MINIREVIEWS

Advancement in high dose therapy and autologous stem cell rescue in lymphoma

Alessandro Isidori, Cristina Clissa, Federica Loscocco, Barbara Guiducci, Sara Barulli, Lara Malerba, Elisa Gabucci, Giuseppe Visani

Alessandro Isidori, Cristina Clissa, Federica Loscocco, Barbara Guiducci, Sara Barulli, Lara Malerba, Elisa Gabucci, Giuseppe Visani, Haematology and Haematopoietic Stem Cell Transplant Center, AORMN, 61100 Pesaro, Italy

Author contributions: Isidori A, Clissa C and Loscocco F contributed equally to this work, generated the tables and wrote the manuscript; all authors contributed to the concept, drafting, revising and final editing of this manuscript; Visani G made critical revisions related to important intellectual content of the manuscript.

Conflict-of-interest statement: The authors declare no competing financial interests.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: http://creativecommons.org/licenses/by-nc/4.0/

Correspondence to: Alessandro Isidori, MD, PhD, Haematology and Haematopoietic Stem Cell Transplant Center, Marche Nord Hospital, Via Lombroso, 61100 Pesaro, Italy. asidori@gmail.com
Telephone: +39-721-364022
Fax: +39-721-364026

Received: November 25, 2014
Peer-review started: November 26, 2014
First decision: January 8, 2015
Revised: May 18, 2015
Accepted: July 16, 2015
Article in press: July 19, 2015
Published online: August 26, 2015

Abstract

Although advanced stage aggressive non-Hodgkin's lymphomas and Hodgkin's disease are thought to be chemotherapy-responsive cancers, a considerable number of patients either relapse or never attain a remission. High-dose therapy (HDT) followed by autologous stem cell transplantation (ASCT) is often the only possibility of cure for most of these patients. However, many controversial issues still remain with respect to HDT/ASCT for lymphomas, including its role for, the optimal timing of transplantation, the best conditioning regimen and the potential use of localized radiotherapy or immunologic methods to decrease post-transplant recurrence. Recently, mainly due to the unavailability of carmustine, several novel conditioning protocols have been clinically developed, with the aim of improving the overall outcome by enhancing the anti-lymphoma effect and, at the same time, by reducing short and long-term toxicity. Furthermore, the better safety profiles of novel approaches would definitively allow patients aged more than 65-70 years to benefit from this therapeutic option. In this review, we will briefly discuss the most relevant and recent data available regarding HDT/ASCT in lymphomas.

Key words: Hodgkin lymphoma; Non-Hodgkin lymphoma; High dose therapy; Autologous stem cell transplantation; New drugs

© The Author(s) 2015. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: High-dose therapy (HDT) followed by autologous stem cell transplantation (ASCT) is considered the golden standard for the vast majority of patients with both Hodgkin and non-Hodgkin lymphoma, who either relapse or never attain a remission. However, several questions about HDT/ASCT still remain unanswered, also comprising, but not limited to, its role in newly diagnosed patients with advanced stage disease. The incorporation of novel drugs in both salvage and conditioning regimens has recently

revitalized the HDT/ASCT area, with several phase I-II trials performed during the last 5 years. This review will focus on the most recent data regarding HDT/ASCT in lymphomas.

Isidori A, Clissa C, Loscocco F, Guiducci B, Barulli S, Malerba L, Gabucci E, Visani G. Advancement in high dose therapy and autologous stem cell rescue in lymphoma. *World J Stem Cells* 2015; 7(7): 1039-1046 Available from: URL: http://www.wjgnet.com/1948-0210/full/v7/i7/1039.htm DOI: http://dx.doi.org/10.4252/wjsc.v7.i7.1039

INTRODUCTION

High-dose therapy (HDT) followed by autologous stem cell transplant (ASCT) is the therapy of choice for patients with chemosensitive, aggressive, relapsed non-Hodgkin lymphoma, basing on the results of the PARMA and CORAL study (NHL)[1,2]. Moreover, HDT/ASCT is considered the standard of care for hodgkin lymphoma (HL) patients in chemosensitive relapse[3]. Different HDT regimens followed by ASCT are able to produce rates of disease-free survival (DFS) and overall survival (OS) of approximately 30% to 70%. Despite HDT/ASCT prolonged DFS, few major drawbacks still limit the utility of this approach for a wide patient population. As an example, the introduction of Rituximab in every-day clinical practice has dramatically reduced the number of patients addressed to HDT/ASCT, specially in front-line therapy. Up to now, no regimen was demonstrated to be superior to another in randomized trials[4]. Therefore, novel strategies are urgently required. As a consequence, and due to the sudden unavailability of carmustine, the International Investigators have developed novel HDT/ASCT protocol in resistant/relapsed aggressive NHL or HD within the last 5 years, aiming to improve the outcome while reducing toxicity. In this paper, we will discuss the data emerging from few recent clinical trials testing HDT/ASCT in aggressive lymphomas.

CONDITIONING REGIMENS PRE-ASCT IN LYMPHOMAS

High dose chemotherapy and ASCT is the standard of care for patients with recurrent HL and NHL who fail immunochemotherapy upfront, improving long-term survival in 30% to 50%. Several factors impact on survival of lymphoma patients after HDT/ASCT. In this regard, the most important predictive factor is disease status at transplant (chemosensitive *vs* chemoresistant). Nevertheless, transplant related morbidity and mortality still remain relevant in influencing the outcome of the transplant procedure. Therefore, when planning an autologus transplant, the efficacy of HDT to eradicate residual disease after salvage therapy may be well balanced with toxicity to normal tissues, in order to maximize the probabilities of the procedure of being successful. Despite the efforts made to further increase the therapeutic window of new high-dose regimens, at present we do not have an evidence that clearly demonstrates the superiority of a specific HDT regimen to the others.

BEAM (carmustine, etoposide, cytarabine, melphalan) regimen is considered the regimen of choice for patients with HL and NHL submitted to ASCT, due to its acceptable safety profile and a quite high antitumor efficacy. The transplant related mortality (TRM) of the BEAM regimen is quite low, depending mainly on disease status at transplant, infectious history and prior lines of therapy. Early, non-hematological toxicities of carmustine, such as nausea, vomiting, mucositis, hepatotoxicity, diarrhea and nephrotoxicity are well known, and worldwide Clinicians know how to manage them. However, late toxicities are still a matter of concern, in particular second tumor and interstitial non-infectious pneumonitis, which have been reported in 16%-64% of patients receiving carmustine-based conditioning regimens, being fatal in approximately 9% of the patients.

Accordingly, various scientists and cooperative groups still continue in this search for the holy Grail, focusing mainly on the incorporation of novel drugs, radioimmunoconjugates, monoclonal antibodies and/or other stimuli for the immunologic system during the early pre- and post- transplantation phases.

Hodgkin lymphoma

The results of the most important trials with HDT/ASCT in HD are listed in Table 1.

Ramzi *et al*[5] reported the results of non-cryopreserved ASCT of 45 HL patients receiving an alternative CEAM regimen in which iv carmustine was substituted by oral lomustine (CCNU: 2 chloroethyl cyclohexyl nitrosourea). Forty-five relapsed/refractory HL patients underwent conditioning regimen with: lomustine 200 mg/m^2 on day-3, etoposide 1000 mg/m^2 on day-3 and day-2, cytarabine 1000 mg/m^2 on day-3 and day-2, melphalan 140 mg/m^2 on day-1. All 45 patients showed engraftment of infused stem cell. Grade 2 and grade 3 mucositis was seen in 64.5% of patients. TRM at 100 d was 2.2%. Median DFS was 20 mo (range: 4-60 mo). After a follow up of more than 2-year, the 2-year DFS in 30 evaluable patients was 77% and the 2-year OS was 84% (25/30 patients).

Czyz *et al*[6] have retrospectively evaluated the efficacy of a modified BEAM regimen followed by ASCT in 132 patients relapsed/refractory HL patients. The 10-year OS and progression free survival (PFS) were 76% and 66%, respectively. Age > 45 years, more than one salvage regimens and chemoresistant disease at transplant were all predictive for poor OS in multivariate analysis.

In 2011 Shafey *et al*[7] have retrospectively evaluated 73 patients with refractory/relapsed HL treated in a 15 year period with a double high-dose therapy consisting

Table 1 High dose therapy followed by autologous stem cell transplantation in Hodgkin lymphoma

Ref.	Year	Patients (*n*)	Status of HL	Regimen	TRM (%)	PFS (%)	OS (%)	Follow-up (mo)
Ramzi *et al*[5]	2012	45	R/R	CEAM	2.2	77	84	24
Czyz *et al*[6]	2013	132	R/R	Modified BEAM	-	66	76	68
Shafey *et al*[7]	2012	73	R/R	DICEP	1	61	80	56
Sinha *et al*[9]	2013	30	R/R	VTEPA	0	67	81	32
Di Ianni *et al*[10]	2012	58	R/R	TECA	0	72	82	60

HL: Hodgkin lymphoma; DICEP: Dose-intensive cyclophosphamide, etoposide, cisplatin; VTEPA: Vinorelbine, paclitaxel, etoposide and cisplatin; TECA: Thiotepa, etoposide and carboplatin; R/R: Relapsed/refractory; OS: Overall survival; PFS: Progression free survival.

of dose intensified cyclophosphamide, etoposide and cisplatin reinduction, followed by high-dose melphalan and ASCT. TRM was 1%. The 5-year PFS and OS rates were 61% and 80%, respectively. In multivariate analyses, response to reinduction therapy and DICEP and International Prognostic System score at relapse were the only factors independently predicting PFS and OS.

As already stated, the standard of care for refractory or relapsed HL patients is a salvage therapy followed by HDT/ASCT. However, patients whit chemoresistant disease after salvage therapy have a small probabilities of achieving a long lasting response and a long overall survival. In 2006, the Emory Univesity group tested in a phase I study, the combination of cytarabine with fixed doses of vinorelbine, paclitaxel, etoposide and cisplatin (VTEPA) as second salvage therapy in patients with resistant/relapsed lymphoma , showing an overall response rate (ORR) of 33%[8]. In 2013 the same group further examined the effectiveness of VTEPA in 30 patients with relapsed/refractory HL[9]. Among 27 evaluable patients, ORR was 70% (7 CR, 12 PR). All but 1 responding patients (66%) subsequently underwent ASCT. This therapeutic strategy (VTEPA + ASCT) produced a median PFS and OS of 28 and 38 mo from transplant, respectively.

In 2012 Di Ianni *et al*[10], reported their experience with a novel HDT regimen including thiotepa, etoposide and carboplatin (TECA) in HL patients. From March 1999 to December 2005, 58 patients with primary refractory or relapsed were enrolled in a phase II study. The conditioning regimen consisted of etoposide (250 mg/m^2 days 1-4), thiotepa (166 mg/m^2 days 2-4) and carboplatin (266 mg/m^2 days 2-4). After salvage therapy, 46 patients had chemosensitive disease (30 CR + 16 PR), whereas 12 were chemoresistant. At transplantation, 30 patients were in CR, 16 in PR and 12 showed a chemoresistance to salvage chemotherapy. TRM was 0%. The global ORR was 79.3% (37 CR, 7 PR), but 12 patients still did not respond to therapy neither after HDT/ASCT. The 5-year DFS and OS were superior for relapsed patients with respect to primary refractory ones. After 5-year of follow-up, approximately 75% of patients were alive. Even if the idea of combining thiotepa with more conventional drugs was interesting, the results of this study are in line with other trials, given the high percentage (80%) of chemosensitive patients at transplant. Hard-to-respond, chemoresistant patients (12/58, 20%) did not show any benefit from the thiotepa-containing regimen.

Non-Hodgkin lymphoma

The results of the most relevant studies with HDT/ASCT in NHL are listed in Table 2.

As for HL, the standard salvage therapy of relapsed/refractory aggressive NHL mainly relies on HDT/ASCT, hopefully in patients with chemosensitive disease. Nevertheless, reaching of long lasting DFS remains not easy.

Kim *et al*[11] reported the results of a novel NEAM regimen, administered prior to ASCT, to 69 patients with resistant/relapsed NHL. The NEAM regimen, another, novel variant of the standard BEAM, consisted of mitoxantrone (12 mg/m^2 *iv* on day-6 to day-4), etoposide (100 mg/m^2) and cytarabine (100 mg/m^2 iv every 12 h from day-6 to day-3), melphalan (single 140 mg/m^2 dose at day-2). TRM at day-100 was 2.9%. Median event free survival (EFS) was 17.9 mo, whereas estimated 2-year OS was 64.2%.

In 2012 Falzetti *et al*[12] reported the results of the TECA (thiotepa, etoposide and carboplatin) regimen administered to 45 patients with NHL at various disease stage. TRM was 4.4%. The ORR was 77.8% (30 CR, 5 PR). Ten patients (22.2%) did not respond. The mean 5-year OS was 71.1%. Patients with low (1) International prognostic index (IPI) ad diagnosis had a better ORR and 5-year OS were than for those with intermediate IPI (2 and 3).

Another strategy to increase efficacy of the conventional BEAM regimen is to add novel drugs in a new reinforced BEAM combo. In this regard, the University of Nebraska Medical Center group designed a phase I/II trial testing the safety and the efficacy of the addition of a proteasome inhibitor to standard BEAM prior to ASCT in resistant/relapsed indolent or transformed NHL (including T cell lymphomas) or mantle-cell lymphoma (MCL, only in first CR)[13]. Patients received 4 doses of escalating bortezomib (0.8, 1, 1.3, 1.5 mg/m^2) on day-11, day-8, day-5 and day-2 prior to ASCT. After the maximum tolerated dose (MTD, 1 mg/m^2) was defined, other 20 patients entered the phase II to determine a preliminary ORR, PFS and OS with this regimen. As a whole, 42 (13 + 29) patients were enrolled. Non-hematologic side effects were

Table 2 High dose therapy followed by autologous stem cell transplantation in non-Hodgkin lymphoma

Ref.	Year	Patients (*n*)	Disease	Regimen	TRM (%)	ORR (%)	OS (%)	Follow-up (mo)
Kim *et al*[11]	2012	44	Chemosensitive-NHL	NEAM	2.9	79	64	24
Falzetti *et al*[12]	2012	45	HR NHL	TT-Vp-Car	4.4	77	71	60
William *et al*[13]	2014	42	R/R NHL	V-BEAM	0	87	91	12
Visani *et al*[14]	2011	43	R/R HL and NHL	BeEAM	0	82	81	18
Visani *et al*[15]	2014	43	R/R HL and NHL	BeEAM	0	72	88	41
Isidori *et al*[16]	2014	37	R/R NHL	BeEAM	2.7	88	94	9
Musso *et al*[17]	2010	84	R/R HL and NHL	FEAM	2.4	73	88	13
Kruger *et al*[19]	2012	16	R/R NHL	RIT + BEAM	6	94	75	44
Winter *et al*[20]	2009	44	R/R NHL	Z-BEAM	2.2	77	60	33
Shimoni *et al*[21]	2012	43	R/R NHL	Z-BEAM	0	97	91	24
Briones *et al*[22]	2014	30	R/R NHL	RIT + BEAM	3.5	70	63	31

HL: Hodgkin lymphoma; RIT: Radioimmunotherapy; BeEAM: Bendamustine, etoposide, ara-C, melphalan; FEAM: Fotemustine plus etoposide, cytarabine and melphalan; NEAM: Mitoxantrone, etoposide, cytarabine, and melphalan; NHL: Non-Hodgkin lymphoma; R/R: Relapsed/refractory; OS: Overall survival; TT-Vp-Car: Thiotepa, Vepeside, carmustine; V-BEAM: Velcade plus standard BEAM.

comparable to those observed with other regimen, with the relevant exception of an increase in grade Ⅲ peripheral neuropathy, related to the use of bortezomib, and in grade Ⅲ gastrointestinal toxicity. TRM at day 100 was 0%. At 1 year after ASCT, 38 patients were evaluable for response; 32 (84%) were in CR and 1 (3%) was in PR, resulting in an impressive ORR of 87%. these results were better among patients treated in the phase Ⅱ, for whom ORR was 89% (84% CR, 5% PR). PFS was 83% at 1 year and 32% at 5 years. OS was 91% at 1 year and 67% at 5 years. The authors performed also an exploratory analysis to determine whether this regimen was more effective in a given histological pattern, finding no statistical difference. Conversely, by comparing the results of bortezomib-BEAM with standard BEAM in an historic cohort of patients matched for histology at their Institution, the authors showed an advantage in both PFS and OS at 5 years for MCL patients (57% and 72% *vs* 43% and 50%, respectively), even if not statistically significant. Even if promising, in particular in MCL patients, the bortezomib-BEAM regimen discourages, due to the lack of an evident benefit and higher than expected toxicity, its further exploration in a randomized, phase Ⅲ study.

In 2011 Visani *et al*[14] reported the efficacy of increasing doses of bendamustine (160 mg/m^2, 180 mg/m^2 and 200 mg/m^2 given on day-7 and day-6) in addition to fixed doses of etoposide, cytarabine and melphalan (BeEAM regimen) administered as preparative regimen to ASCT. Forty-three patients with resistant/relapsed HL (n = 15) and NHL (n = 28) were enrolled, 9 in the phase Ⅰ and 34 in the phase Ⅱ study. No patients experienced dose limiting toxicity. TRM at day 100 was 0%. The follow-up period at the time of publication was 18 mo, with 81% being alive and disease-free at that time. Disease type (HL *vs* NHL) and disease status at transplant (chemosensitive *vs* chemoresistant) significantly influenced DFS. Interestingly, the authors updated their experience in 2014[15], reporting a 72% PFS at 3 years, that allowed them to met the primary end-point of the study. Median PFS and OS were still not reached. Disease status at transplant (chemosensitive *vs* chemoresistant) was still a stron predictor of outcome. Conversely, disease type (HL *vs* NHL) was no longer affecting PFS nor OS[15].

By riding the same wave, Isidori *et al*[16] recently reported the preliminary data of a phase Ⅱ study to confirm the effectiveness of BeEAM as a preparative regimen for autologous stem cell transplantation in resistant/relapsed aggressive B-cell non-Hodgkin lymphoma patients. Thirty-seven patients (median age 56 years, range 19-69) with resistant/relapsed aggressive B-cell NHL were enrolled, up to now, in the study. Briefly, 27 patients had advanced stage disease (Ⅲ-Ⅳ), 12 were primary refractory and 25 had relapsed. Thirty-three patients had good performance status (WHO 0-1), and 11 patients presented with 1 or more relevant comorbidities (range: 1-5). Nineteen patients were in Ⅱ or subsequent CR after salvage therapy, whereas 16 were in PR and 2 had progressive disease. All patients engrafted, with a median time to ANC > 0.5 x 10^9/L of 10 d. TRM at day-100 was 2.7%. Eight out of 37 patients presented a fever of unknown origin (21.6%), whereas 19 patients (51%) presented a clinically documented infection. One patient died due to an incomplete hematological recovery after transplant, producing an overall transplant related mortality of 2.7%. Twenty-seven out of 37 patients are evaluable up to now for response: 22/27 (81.5%) obtained a CR, 2/27 a PR, resultin in an ORR of approximately 90%. After a median follow-up of 9 mo from transplant (range 2-24), 5/24 patients relapsed, whereas 19/24 (79.1%) are still alive, in continuous CR. The Authors concluded that the BeEAM regimen preliminary confirmed its safety and its promising efficacy in resistant-relapsed aggressive B-cell lymphomas.

In 2010 Musso *et al*[17] substituted carmustine with the chloroethylnitrosurea fotemustine (150 mg/m^2 on day-7 and day-6) in the standard BEAM (FEAM regimen). 84 resistant/relapsed HL and NHL patients were enrolled in this study. Non-hematological side effects were superimposable to those of the BEAM regimen, with 7 patients experiencing grade 4 mucositis, without any other relevant grade 4 toxicity. TRM at day-100

was 2.4%. Even if the FEAM HDT regimen showed a favorable safety profile, it is not possible to draw any conclusion regarding survival or long-term efficacy due to the short follow-up period of 17-mo sonly.

Another question that still remains unanswered is related to the relative efficacy of HDT/ASCT in NHL patient population treated with chemoimmunotherapy comprising rituximab front-line. As a fact, this is really a burning question, as all patients with B cell malignancies are treated upfront, at present, with anti-CD20 monoclonal antibody plus chemotherapy. Nevertheless, the utility of HDT/ASCT for NHL patients in first CR is still a matter of great debate.

Recently, the Southwest Oncology Group tried to answer, at least partly, this question by conducting a large, randomized trial testing HDT/ASCT as consolidation therapy, in comparison to standard chemoimmunotherapy[18]. 397 patients were enrolled, and 370/397 received five cycles of CHOP with (47%) or without (53%) rituximab. Responding patients (CR + PR) were subsequently randomized to receive 3 other cycles CHOP ± Rituximab (control group) or one additional cycle of CHOP ± Rituximab followed by ASCT (transplantation group), conditioned with standard BEAM regimen or total body irradiation (12 Gy). The primary efficacy end points were 2-year PFS and OS. Of 370 induction-eligible patients, 253 were randomly assigned to the transplantation group (125) or the control group (128). Like many of the randomized trials and several meta-analysis, this study showed an improvement in PFS for the combined high-risk and high-intermediate risk who are chemosensitive to induction therapy. However, again and again, this study was not able to demonstrate, in randomized fashion, an advantage in OS for HDT/ASCT, neither for high risk patients[18]. On the other hand, we have to keep in mind that 29% of patients who had a relapse or progression after standard therapy, were rescued with HDT/ASCT, resulting in a relevant bias for the analysis of a statistical OS benefit for HDT/ASCT over the control group. Finally, the study was not designed and powered to address subgroup-related question, and therefore any point in favor of HDT/ASCT for high risk patients is merely speculative.

The incorporation of new drugs into HDT regimen prior to ASCT in B-cell lymphomas has recently been helped by the development of radioimmunotherapy (RIT). The potential advantage of using radioimmunoconjugates, with or without chemotherapy, prior to ASCT, relies on the opportunity of delivering localized radiation therapy to the site of tumor. This allows to minimize the toxicity of total body irradiation, with the goal of decreasing relapse rate without adding toxicity to the conditioning regimen. At present, no study comparing RIT and standard radiation therapy has been done. However, few preliminary phase Ⅱ studies with RIT as a part of a preparative regimen have produce encouraging results by showing a high safety profile, a low TRM and a preliminary efficacy.

Kruger *et al*[19] enrolled 16 patients with resistant/relapsed NHL in a phase Ⅱ study testing ^{131}I-rituximab-BEAM and ASCT. A single dose of ^{131}I-rituximab was given on day-15, whereas standard BEAM started on day-6 prior to ASCT. Non hematological side effects were mild in grade, without any grade Ⅳ toxicity. All patients engrafted, with a 0% TRM at day-100. Results were encouraging, with 75% of patients being alive and disease free after a median follow-up of 44 mo from ASCT (range 4-108). Interestingly, each patient received only a limited whole body radiation of only 0.75 Gy.

Winter *et al*[20] conducted a phase Ⅰ-Ⅱ study in 44 patients with resistant/relapsed NHL, by adding yttrium-90 (^{90}Y) ibritumomab tiuxetan to standard BEAM and ASCT (Z-BEAM regimen). A significant proportion of patients (30%) entering the study had chemoresistant disease after salvage therapy. Non hematological toxicities were similar to those reported with standard BEAM. Two dose limiting toxicities occurred at 17 Gy dose level, which made 15 Gy the recommended dose for the phase Ⅱ of the study. After a median observation time of 33 mo, the estimated 3-year PFS and OS were 43% and 60%, respectively. When looking at these results, it has to keep in mind the significant proportion of chemoresistant patients, who perform extremely poor with conventional HDT regimen.

Shimoni *et al*[21] randomized 43 patients with CD20 positive aggressive B-cell lymphoma to receive either Z-BEAM (n = 22) or standard BEAM (n = 21). Ibritumomab tiuxetan was administered at 0.4 mCi/kg on day-14 prior to ASCT. Non hematological toxicities were mild and comparable within the 2 groups. TRM at day-10 was 0% in both groups. As a whole, Z-BEAM did not show a significant advantage in OS with respect to standard BEAM. However, there was a trend in 2-year PFS and OS in favor of Z-BEAM (59% and 91% *vs* 37% and 62%, respectively. The Authors speculated that Z-BEAM could be superior to standard BEAM for patients receiving frontline chemoimmuotherapy containing Rituximab. However, the sample size of the study was very small, and the statistical analysis did not allow to draw any conclusion regarding the superiority of a regimen to another.

Another study with yttrium-90 Ibritumomab tiuxetan was conducted and published by Spanish Group in 2013[22]. It was a prospective, multicenter, phase Ⅱ clinical trial which enrolled 30 patients with induction failure or refractory B-cell NHL. Patients received ^{90}Y-ibritumomab tiuxetan at a fixed dose of 0.4 mCi/kg, 14 d prior to the BEAM chemotherapy. Non hematological toxicities were similar to those reported with standard BEAM, and TRM at day-100 was 0%. Intriguingly, the vast majority of patients (25/30) underwent to HDT/ASCT with chemoresistant disease. Therefore, this regimen produce an outstanding ORR of 70%, with 60% of patients obtaining a CR; estimated 3-year PFS and OS were 61% and 63%, respectively, and the median time of observation for surviving

patients was 31 mo. The Authors enthusiastically concluded that ^{90}Y-ibritumomab tiuxetan based HDT regimen prior to ASCT results in a terrific response rate, with promising survival in a group of refractory lymphoma patients with extremely poor prognosis.

Taken together, these results are in favor of RIT-based HDT regimen prior to ASCT, given the good toxicity profile and the promising efficacy. However, none of these studies was able to demonstrate a statistical benefit for the RIT-based HDT, probably also due to the small sample size of the trials. In conclusion, a large, randomized trial comparing RIT-BEAM and standard BEAM is warranted before drawing any conclusion and before recommending the use of RIT-HDT outside from controlled clinical trials.

With regards to T-cell lymphomas, the use of HDT/ASCT is still considered an "experimental" practice. In fact, only a small amount of prospective trials evaluating the impact of HDT/ASCT as consolidation of first-line therapy have been reported up to now[23-25]. Even if the results coming from these studies suggest an improve in both DFS and OS when compared with chemo alone[23-25], an important amount of patients is not able to perform frontline transplant, principally due to disease progression during induction chemotherapy, thus underlying the need for a better induction regimen. In relapsed T-cell lymphomas, HDT/ASCT produced results similar to those obtained in relapsed, aggressive B-cell lymphomas. Reported long-term DFS approaches 30% to 50%, making HDT/ASCT a valuable option in the therapeutic armamentarium for this indication. Conversely, the use of HDT/ASCT in refractory T-cell lymphoma produce extremely poor outcome, and other strategies could be preferred for this setting of patients. In conclusion, we think that there is room for HDT/ASCT as a part of first-line treatment, especially in responding patients. On the other hand, resistant or relapsed patients may be better addressed to allogeneic transplantation or to clinical trials.

Late complications of HDT followed by ASCT in lymphomas

Late complications among HL survivors are still a matter of concern. Several papers have reported high rates of second cancers, heart disease, pulmonary fibrosis, and infections[26]. The recognition of these risks has resulted in modification of chemotherapy regimens and of radiation fields and doses[26,27]. HDT/ASCT by itself is also affected by late *sequelae* of treatment, but specific data for the HL population are limited[26,27]. Few studies identified predictors of post-transplant long-term quality of life specifically for patients with HL, who have higher rates of treatment-related late morbidity and mortality than patients with other cancer diagnoses[26,27].

The most relevant study that evaluated the risk of late morbidity and mortality, among patients with relapsed/refractory HL after HDT and ASCT was performed by the MSKCC group[28]. this study, conducted on 153 HL patients treated with HDT/ASCT between 1985 and 1998 who survived ⩾ 2 years after ASCT, demonstrated a risk ratio of second malignancy equal to 6.5 (95%CI: 3.6-10.7) when compared with the general population, but limited to 2.4 (95%CI: 1.4-4.05) when compared with patients with HL[28]. In other words, the risk of developing a second tumor after HDT/ASCT was elevated if compared with the cancer risk in the general population, but was less pronounced when compared with patients with HL in SEER registry[29].

Data on NHL are quite similar, demonstrating a higher risk of developing a second malignancy mostly for patients receiving TBI as a part of the preparative regimen to ASCT. However, to the best of our knowledge, large studies have not been conducted in this patient population.

Our guess is that, in the era of the TBI-free conditioning regimens, what really counts for the development of a second malignancies or a late effect (*e.g.,* cardiomyopathy) in lymphoma patients, is mainly the type and the dose of chemo- and radiation therapy performed before transplant, and only in a minimal extent HDT/ASCT.

CONCLUSION

High dose therapy followed by autologous stem cell transplantation has still a major role in the treatment of resistant/relapsed HL and NHL. The relevant advancements made with the incorporation of novel drugs and/or radioimmunoconjugates into preparative regimens translated in a higher PFS rate with respect to the historical standards. Furthermore, TRM and overall toxicities seem to be lower. However, data on the possible overall survival advantage given by the novel agents are still controversial, and probably only large, randomized phase Ⅲ trials could pick a winner between the plethora of new drugs recently incorporated in novel conditioning regimens. Our personal experience with Bendamustine, used both in Phase Ⅱ trials and everyday clinical practice, indicate the favorable safety profile of the BeEAM regimen, coupled with a relevant efficacy in a hard-to-treat population of resistant/relapsed lymphoma patients.

A different scenario could be represented by maintenance therapy with new drugs, such as Brentuximab Vedotin in HL or Ibrutinib or Lenalidomide in aggressive NHL, in patients chemoresistant who obtain at least a PR after HDT/ASCT. In this setting of patients, the use of a drug with a different mechanism of action and a manageable safety profile could help the physician in the path to cure of a highly resistant subpopulation of lymphoma patients.

REFERENCES

1 **Philip T**, Guglielmi C, Hagenbeek A, Somers R, Van der Lelie H, Bron D, Sonneveld P, Gisselbrecht C, Cahn JY, Harousseau JL. Autologous bone marrow transplantation as compared with salvage

chemotherapy in relapses of chemotherapy-sensitive non-Hodgkin's lymphoma. *N Engl J Med* 1995; **333**: 1540-1545 [PMID: 7477169 DOI: 10.1056/NEJM199512073332305]

2 **Gisselbrecht C**, Glass B, Mounier N, Singh Gill D, Linch DC, Trneny M, Bosly A, Ketterer N, Shpilberg O, Hagberg H, Ma D, Brière J, Moskowitz CH, Schmitz N. Salvage regimens with autologous transplantation for relapsed large B-cell lymphoma in the rituximab era. *J Clin Oncol* 2010; **28**: 4184-4190 [PMID: 20660832 DOI: 10.1200/JCO.2010.281618]

3 **Linch DC**, Winfield D, Goldstone AH, Moir D, Hancock B, McMillan A, Chopra R, Milligan D, Hudson GV. Dose intensification with autologous bone-marrow transplantation in relapsed and resistant Hodgkin's disease: results of a BNLI randomised trial. *Lancet* 1993; **341**: 1051-1054 [PMID: 8096958 DOI: 10.1016/0140-6736(93)92411-L]

4 **Oliansky DM**, Larson RA, Weisdorf D, Dillon H, Ratko TA, Wall D, McCarthy PL, Hahn T. The role of cytotoxic therapy with hematopoietic stem cell transplantation in the treatment of adult acute lymphoblastic leukemia: update of the 2006 evidence-based review. *Biol Blood Marrow Transplant* 2012; **18**: 18-36.e6 [PMID: 21803017 DOI: 10.1016/j.bbmt.2010.07.008]

5 **Ramzi M**, Mohamadian M, Vojdani R, Dehghani M, Nourani H, Zakerinia M, Haghighinejad H. Autologous noncryopreserved hematopoietic stem cell transplant with CEAM as a modified conditioning regimen in patients with Hodgkin lymphoma: a single-center experience with a new protocol. *Exp Clin Transplant* 2012; **10**: 163-167 [PMID: 22432762 DOI: 10.6002/ect.2011.0092]

6 **Czyz A**, Lojko-Dankowska A, Dytfeld D, Nowicki A, Gil L, Matuszak M, Kozlowska-Skrzypczak M, Kazmierczak M, Bembnista E, Komarnicki M. Prognostic factors and long-term outcome of autologous haematopoietic stem cell transplantation following a uniform-modified BEAM-conditioning regimen for patients with refractory or relapsed Hodgkin lymphoma: a single-center experience. *Med Oncol* 2013; **30**: 611 [PMID: 23702734 DOI: 10.1007/s12032-013-0611y]

7 **Shafey M**, Duan Q, Russel J, Duggan P, Balogh A, Stewart DA. Double high-dose therapy with dose-intensive cyclophosphamide, etoposide, cisplatin (DICEP) followed by high dose melphalan and autologous stem cell transplantation for relapsed/refractory Hodgkin lymphoma. *Leuk and Lymphoma* 2012; **53**: 596-602 [DOI: 10.3109/10428194.2011.624227]

8 **Lonial S**, Arellano M, Hutcherson D, Langston A, Flowers C, Heffner LT, Winton E, Jo Lechowicz M, Waller EK. Results of a clinical phase I dose-escalation study of cytarabine in combination with fixed-dose vinorelbine, paclitaxel, etoposide and cisplatin for the treatment of relapsed/refractory lymphoma. *Leuk Lymphoma* 2006; **47**: 2155-2162 [PMID: 17071490 DOI: 10.1080/10428190600774923]

9 **Sinha R**, Shenoy PJ, King N, Lechowicz MJ, Bumpers K, Hutcherson D, Arellano M, Langston A, Kaufman J, Heffner LT, Waller EK, Nooka A, Flowers CR, Lonial S. Vinorelbine, paclitaxel, etoposide, cisplatin, and cytarabine (VTEPA) is an effective second salvage therapy for relapsed/refractory Hodgkin lymphoma. *Clin Lymphoma Myeloma Leuk* 2013; **13**: 657-663 [PMID: 24094894 DOI: 10.1016/2013.05.007]

10 **Di Ianni M**, Ballanti S, Iodice G, Reale A, Falzetti F, Minelli O, Serio G, Martelli MF, Dammacco F, Vacca A, Ria R. High-dose thiotepa, etoposide and carboplatin as conditioning regimen for autologous stem cell transplantation in patients with high-risk Hodgkin's lymphoma. *Hematology* 2012; **17**: 23-27 [PMID: 22549444 DOI: 10.1179/102453312X13221316477534]

11 **Kim JW**, Lee HJ, Yi HG, Kim BS, Bang SM, Kim JS, Kim I, Yoon SS, Lee JS, Kim CS, Park S, Kim BK. Mitoxantrone, etoposide, cytarabine, and melphalan (NEAM) followed by autologous stem cell transplantation for patients with chemosensitive aggressive non-Hodgkin lymphoma. *Am J Hematol* 2012; **87**: 479-483 [PMID: 22388671 DOI: 10.1002/ajh.23150]

12 **Falzetti F**, Di Ianni M, Ballanti S, Iodice G, Reale A, Minelli O, Serio G, Martelli MF, Dammacco F, Vacca A, Ria R. High-dose thiotepa, etoposide and carboplatin as conditioning regimen for autologous stem cell transplantation in patients with high-risk non-Hodgkin lymphoma. *Clin Exp Med* 2012; **12**: 165-171 [PMID: 21928053 DOI: 10.1007/s10238-011-0157-2]

13 **William BM**, Allen MS, Loberiza FR, Bociek RG, Bierman PJ, Armitage JO, Vose JM. Phase I/II study of bortezomib-BEAM and autologous hematopoietic stem cell transplantation for relapsed indolent non-Hodgkin lymphoma, transformed, or mantle cell lymphoma. *Biol Blood Marrow Transplant* 2014; **20**: 536-542 [PMID: 24434781 DOI: 10.1016/j.bbmt.2014.01.004]

14 **Visani G**, Malerba L, Stefani PM, Capria S, Galieni P, Gaudio F, Specchia G, Meloni G, Gherlinzoni F, Giardini C, Falcioni S, Cuberli F, Gobbi M, Sarina B, Santoro A, Ferrara F, Rocchi M, Ocio EM, Caballero MD, Isidori A. BeEAM (bendamustine, etoposide, cytarabine, melphalan) before autologous stem cell transplantation is safe and effective for resistant/relapsed lymphoma patients. *Blood* 2011; **118**: 3419-3425 [PMID: 21816830 DOI: 10.1182/blood-2011-04-351924]

15 **Visani G**, Stefani PM, Capria S, Malerba L, Galieni P, Gaudio F, Specchia G, Meloni G, Gherlinzoni F, Gonella R, Gobbi M, Santoro A, Ferrara F, Rocchi M, Ocio EM, Caballero MD, Loscocco F, Isidori A. Bendamustine, etoposide, cytarabine, melphalan, and autologous stem cell rescue produce a 72% 3-year PFS in resistant lymphoma. *Blood* 2014; **124**: 3029-3031 [PMID: 25377565 DOI: 10.1182/blood-2014-08-596668]

16 **Isidori A**, Olivieri A, Angelucci E, Capria S, Angrilli F, Castagnari B, Vallisa D, Fattori P, mengarelli A, Patriarca F, Galieni P, Gaudio F, Scalzulli P, Tosi P, Montanari M, Baronciani D, Falorio S, Meloni G, Zaccaria A, Cascavilla N, Loscocco F, Rocchi M, Visani G. High dose therapy with bendamustine, etoposide, ara-C, melphalan (BeEAM) prior to autologous stem cell transplant is safe and effective in aggressive B-cell non Hodgkin lymphoma: an Italian phase II, multicenter study. *Haematologica* 2014; **99**: s27

17 **Musso M**, Scalone R, Marcacci G, Lanza F, Di Renzo N, Cascavilla N, Di Bartolomeo P, Crescimanno A, Perrone T, Pinto A. Fotemustine plus etoposide, cytarabine and melphalan (FEAM) as a new conditioning regimen for lymphoma patients undergoing auto-SCT: a multicenter feasibility study. *Bone Marrow Transplant* 2010; **45**: 1147-1153 [PMID: 19898504 DOI: 10.1038/bmt.2009.318]

18 **Stiff PJ**, Unger JM, Cook JR, Constine LS, Couban S, Stewart DA, Shea TC, Porcu P, Winter JN, Kahl BS, Miller TP, Tubbs RR, Marcellus D, Friedberg JW, Barton KP, Mills GM, LeBlanc M, Rimsza LM, Forman SJ, Fisher RI. Autologous transplantation as consolidation for aggressive non-Hodgkin's lymphoma. *N Engl J Med* 2013; **369**: 1681-1690 [PMID: 24171516 DOI: 10.1056/NEJMoa13101077]

19 **Kruger PC**, Cooney JP, Turner JH. Iodine-131 rituximab radioimmunotherapy with BEAM conditioning and autologous stem cell transplant salvage therapy for relapsed/refractory aggressive non-Hodgkin lymphoma. *Cancer Biother Radiopharm* 2012; **27**: 552-560 [PMID: 23062193 DOI: 10.1089/cbr.2012.1275]

20 **Winter JN**, Inwards DJ, Spies S, Wiseman G, Patton D, Erwin W, Rademaker AW, Weitner BB, Williams SF, Tallman MS, Micallef I, Mehta J, Singhal S, Evens AM, Zimmer M, Molina A, White CA, Gordon LI. Yttrium-90 ibritumomab tiuxetan doses calculated to deliver up to 15 Gy to critical organs may be safely combined with high-dose BEAM and autologous transplantation in relapsed or refractory B-cell non-Hodgkin's lymphoma. *J Clin Oncol* 2009; **27**: 1653-1659 [PMID: 19255322 DOI: 10.1200/JCO.2008.19.2245]

21 **Shimoni A**, Avivi I, Rowe JM, Yeshurun M, Levi I, Or R, Patachenko P, Avigdor A, Zwas T, Nagler A. A randomized study comparing yttrium-90 ibritumomab tiuxetan (Zevalin) and high-dose BEAM chemotherapy versus BEAM alone as the conditioning regimen before autologous stem cell transplantation in patients with aggressive lymphoma. *Cancer* 2012; **118**: 4706-4714 [PMID: 22252613 DOI: 10.1002/cncr.27418]

22 **Briones J**, Novelli S, García-Marco JA, Tomás JF, Bernal T, Grande C, Canales MA, Torres A, Moraleda JM, Panizo C, Jarque I, Palmero F, Hernández M, González-Barca E, López D, Caballero D. Autologous stem cell transplantation after conditioning with yttrium-90 ibritumomab tiuxetan plus BEAM in refractory non-Hodgkin diffuse large B-cell lymphoma: results of a prospective,

multicenter, phase II clinical trial. *Haematologica* 2014; **99**: 505-510 [PMID: 24162789 DOI: 10.3324/haematol.2013.093450]
23 **Corradini P**, Tarella C, Zallio F, Dodero A, Zanni M, Valagussa P, Gianni AM, Rambaldi A, Barbui T, Cortelazzo S. Long-term follow-up of patients with peripheral T-cell lymphomas treated up-front with high-dose chemotherapy followed by autologous stem cell transplantation. *Leukemia* 2006; **20**: 1533-1538 [PMID: 16871285 DOI: 10.1038/sj.leu.2404306]
24 **Reimer P**, Rüdiger T, Geissinger E, Weissinger F, Nerl C, Schmitz N, Engert A, Einsele H, Müller-Hermelink HK, Wilhelm M. Autologous stem-cell transplantation as first-line therapy in peripheral T-cell lymphomas: results of a prospective multicenter study. *J Clin Oncol* 2009; **27**: 106-113 [PMID: 19029417 DOI: 10.1200/JCO.2008.17.4870]
25 **d'Amore F**, Relander T, Lauritzsen GF, Jantunen E, Hagberg H, Anderson H, Holte H, Österborg A, Merup M, Brown P, Kuittinen O, Erlanson M, Østenstad B, Fagerli UM, Gadeberg OV, Sundström C, Delabie J, Ralfkiaer E, Vornanen M, Toldbod HE. Up-front autologous stem-cell transplantation in peripheral T-cell lymphoma: NLG-T-01. *J Clin Oncol* 2012; **30**: 3093-3099 [PMID: 22851556 DOI: 10.1200/JCO.2011.40.2719]
26 **Fernandez HF**, Escalón MP, Pereira D, Lazarus HM. Autotransplant conditioning regimens for aggressive lymphoma: are we on the right road? *Bone Marrow Transplant* 2007; **40**: 505-513 [PMID: 17589535 DOI: 10.1038/sj.bmt.175744]
27 **Hodgson DC**. Late effects in the era of modern therapy for Hodgkin lymphoma. *Hematology Am Soc Hematol Educ Program* 2011; **2011**: 323-329 [PMID: 22160053 DOI: 10.1182/asheducation-2011.1.323]
28 **Isidori A**, Piccaluga PP, Loscocco F, Guiducci B, Barulli S, Ricciardi T, Picardi P, Visani G. High-dose therapy followed by stem cell transplantation in Hodgkin's lymphoma: past and future. *Expert Rev Hematol* 2013; **6**: 451-464 [PMID: 23991931 DOI: 10.1586/17474086.2013.814451]
29 **Goodman KA**, Riedel E, Serrano V, Gulati S, Moskowitz CH, Yahalom J. Long-term effects of high-dose chemotherapy and radiation for relapsed and refractory Hodgkin's lymphoma. *J Clin Oncol* 2008; **26**: 5240-5247 [PMID: 18809615 DOI: 10.1200/JCO.2007.15.5507]

P- Reviewer: Jun Y, Kiselev SL **S- Editor**: Tian YL
L- Editor: A **E- Editor**: Wu HL

WJSC World Journal of Stem Cells

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4252/wjsc.v7.i7.1047

World J Stem Cells 2015 August 26; 7(7): 1047-1053
ISSN 1948-0210 (online)
© 2015 Baishideng Publishing Group Inc. All rights reserved.

MINIREVIEWS

Stem cells: Sources, and regenerative therapies in dental research and practice

Lobna Abdel Aziz Aly

Lobna Abdel Aziz Aly, Oral and Maxillofacial Surgery, Faculty of Dentistry, Future University, Cairo 11759, Egypt

Author contributions: Aly LAA solely contributed to this work.

Conflict-of-interest statement: None.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: http://creativecommons.org/licenses/by-nc/4.0/

Correspondence to: Lobna Abdel Aziz Aly, Associate Professor, Oral and Maxillofacial Surgery, Faculty of Dentistry, Future University, 90th st, New Cairo, Cairo 11759, Egypt. lobna72@gmail.com
Telephone: +2-2-26701496

Received: July 27, 2014
Peer-review started: July 27, 2014
First decision: August 14, 2014
Revised: June 21, 2015
Accepted: July 7, 2015
Article in press: July 9, 2015
Published online: August 26, 2015

Abstract

Stem cells are considered to be among the principle scientific breakthroughs of the twentieth century for the future of medicine, and considered to be an important weapon to fight against diseases, particularly those that have resisted the efforts of science for a long time. Human dental tissues have limited potentials to regenerate but the discovery of dental stem cells have developed new and surprising scenario in regenerative dentistry. Stem cell treatments are one example of the possibility using adult cells sourced from patients' own bodies' means that it can be expected that in the near future such treatments may become routine at dental practices. The hope is that it will become possible to regenerate bone and dental tissues including the periodontal ligament, dental pulp and enamel, and that the creation of new teeth may also become feasible. In view of this possibility of achieving restoration with regenerative medicine, it can be considered that a new era of dentistry is beginning. Thus the aim of this review is to give dental professionals a brief overview of different stem cells sources and the latest findings and their implications for improving oral health and treating certain conditions of the human mouth and face.

Key words: Stem cells; Regeneration; Dental research

© The Author(s) 2015. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Stem cells are considered to be among the principle scientific breakthroughs of the 20th century. Human dental tissue has limited potentials to regenerate but the discovery of dental stem cells have developed new and surprising scenario in regenerative dentistry. The hope is that it will become possible to regenerate bone and dental tissues and that the creation of new teeth may become feasible. Thus our review gives dental professionals a brief overview of different stem cells sources and the latest findings and their implications for improving oral health and treating certain conditions of the human mouth and face.

Aly LAA. Stem cells: Sources, and regenerative therapies in dental research and practice. *World J Stem Cells* 2015; 7(7): 1047-1053 Available from: URL: http://www.wjgnet.com/1948-0210/full/v7/i7/1047.htm DOI: http://dx.doi.org/10.4252/wjsc.v7.i7.1047

INTRODUCTION

Stem cell therapies could lead to novel cures and palliative treatments. Stem cell research has clinical implications considering cell repair, replacement or regeneration to improve organ function. Thus future studies will require the collaboration of researchers from different specialties, including stem cell biology, material science, and of course, basic and clinical dentistry.

The loss of hard or soft dental tissue not only harms the patient psychologically, but also causes atrophy of the tooth supporting tissues. Regeneration may define as restoring a deficient part by complete regeneration of its architecture and function. Dramatic tooth extraction, sever periodontitis, implant dehiscence defects, tumor, or congenital anomalies may lead to alveolar bone deficiencies[1]. Alveolar bone defect regeneration techniques have many advances using minimally morbid techniques enhanced the success and patient acceptance[2,3]. Successful regeneration techniques of the craniofacial bone deficiencies, are used to optimize therapeutic approaches to bone regeneration[4].

Autogenous bone graft remains the preferred reconstructive method but have some limitations such as increased operative time for bone graft harvesting, donor site morbidity, graft resorption, and limited availability. Various types of bone graft have become available to overcome these limitations[5,6]. There have been recent interest in the development of new grafting materials using allogeneic, xenogeneic and synthetic bioimplants for reconstructive bony procedures, and have been used as alternative for autogenous bone grafts[7-9]. A new combination of protein therapy, gene therapy and cell therapy and tissue engineering had been successfully developed and considered to be a more efficient and safer therapeutic system for bone and soft tissue regeneration[10].

Stem cells are nonspecific cells with powerful self-regeneration properties and they are capable of organizing other cell types in the body. They are also play an important role as those undifferentiated cells have precursor properties, capable of forming many different cell types and have the property of unlimited self-renewal. Within dentistry, the hope is to regenerate both bone and dental tissues, including the periodontal ligament, dental pulp and enamel, so that new teeth could even be created, even in the presence of caries, pulpitis and periapical diseases, which are the primary causes of tooth loss[11].

"Stem cells" are precursor cells that can give rise to multiple tissue types. Totipotent stem cells are cells that can give rise to a fully functional organism as well as to every cell type of the body. *Pluripotent* stem cells are capable of giving rise to virtually any tissue type, but not to a functioning organism. Multipotent stem cells are more differentiated cells that can give rise only to a limited number of tissues. For example, a specific type of multipotent stem cell called a mesenchymal stem cell has been shown to produce bone, muscle, cartilage, fat, and other connective tissues[12,13].

The effectiveness of stem cells can be attributed to many factors such as: Stem cells can be expanded *ex vivo*, thus a small number of them can be sufficient to heal large defects or to treat diseases. Stem cells in the presence of vasculature may elaborate and organize tissues *in vivo*. Also they may play an important role in regulating local and systemic immune reactions of the host favoring tissue regeneration.

STEM CELL TYPES AND SOURCES

Stem cells have important criteria of self-renewable and the ability to differentiate into at least two different types of cell. Types of stem cells are determined based on their source and differ in regard to the types of cells into which they differentiate[14,15].

Embryonic stem cells, they are pluripotent cells that can differentiate into any other type of cell[14].

Adult stem cells, also called somatic stem cells, lack the potency of their embryonic counterparts, but have been used successfully to treat diseases. They can be harvested from an individual and, be used to regenerate tissue by autologous or allogeneic transplant[14].

Induced pluripotent stem cells - adult stem cells having the potential to serve as the source of a large number of autologous stem cells, but the main draw-backs are their capacity for proliferation and concomitant potential for carcinogenicity[16].

Bone marrow-derived mesenchymal stem cells can self-replicate and have been differentiated into osteoblasts, chondrocytes, myoblasts, adipocytes and other cell types. MSCs are often viewed as a yardstick of adult stem cells (Figure 1)[17].

Adipose-derived stem cells (AS) are typically isolated from lipectomy or liposuction aspirates. AS have been differentiated into adipocytes, chondrocytes, myocytes, neuronal and osteoblast lineages. They have many advantages over other adult stem cell populations, as adipose tissue is available in large number and become easily accessible[18].

Dental stem cells - recently these cells have been found in various dental tissues, such as Stem Cells from Human Exfoliated Deciduous teeth[19], dental Pulp Stem Cells[20], Periodontal Ligament Stem Cells (PDLSCs)[21,22], while in dental papilla of wisdom teeth Stem Cells from Apical Papilla is present[23] and researchers termed stem cell found in dental follicles of developing wisdom teeth as Dental Follicle Precursor Cells[24-26].

There are some advantages of dental stem cells: (1) they are readily accessible and provide an easy and least invasive way to obtain them; (2) stem cell banking is a reasonable and simple alternative to harvesting stem cells[27]; (3) they show good interaction with scaffolds[28]; and (4) hhey have a high proliferative and multidifferentiation potential[29-31].

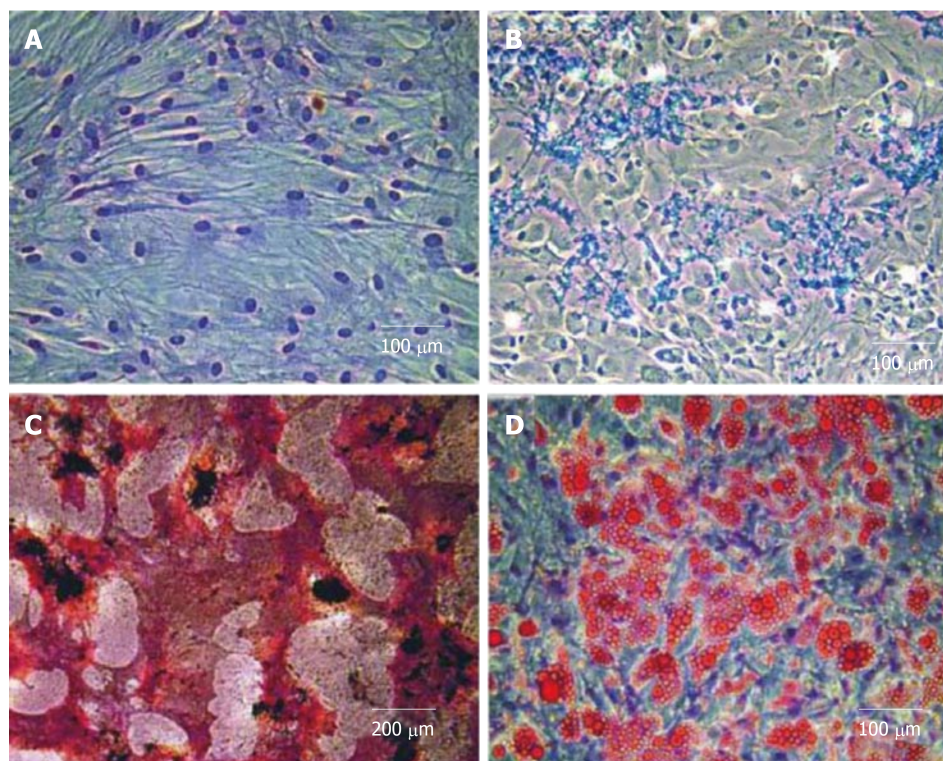


Figure 1 Mesenchymal stem cells are viewed as a yardstick of adult stem cells. A: Human mesenchymal stem cells (MSCs) isolated from anonymous adult human bone marrow donor after culture expansion (H and E staining); B: Chondrocytes derived from human mesenchymal stem cells showing positive staining to Alcian blue. Additional molecular and genetic markers can be used to further characterize MSC-derived chondrocytes; C: Osteoblasts derived from human mesenchymal stem cells showing positive von Kossa staining for calcium deposition (black) and active alkaline phosphatase enzyme (red). Additional molecular and genetic markers can be used to further characterize MSC-derived chondrocytes; D: Adipocytes derived from human mesenchymal stem cells showing positive Oil Red-O staining of intracellular lipids. Additional molecular and genetic markers can be used to further characterize MSC-derived chondrocytes[16].

ROLE OF MESENCHYMAL STEM CELLS IN TISSUE REGENERATION

By chemotaxis Mesenchymal Stem Cells can migrate to tissues showing inflammation and injury in the organism[32]. They can differentiate into different cell types, able to secrete a variety of cytokines, showing anti-inflammatory activity and create an anabolic microenvironment. Moreover, they play a role in regeneration of injured tissues by various means, as they directly differentiate into tissue-specific cells and thus substitute damaged or lost cells. On the other hand, they indirectly influence tissue regeneration by secretion of soluble factors. Thirdly, they are able to modulate the inflammatory response. So, they can effectively promote vascularization, cell proliferation, differentiation and modulate an inflammatory process[33,34].

CHALLENGES IN STEM CELL BIOLOGY

Notable increase in the applications of craniofacial tissue engineering, among them is: (1) isolation of stem cells from several craniofacial tissues and their clinical therapeutic applications in the craniofacial structures; (2) several prototypes of the human-shaped temporomandibular joint condyle have been engineered with integrated cartilage and bone layers from a single population of mesenchymal stem cells; (3) cell-based or non-cell-based tissue engineering of periodontium elements, including the periodontal ligament and cementum; (4) the application of stem cells, growth factors, and/or biomaterials in craniofacial bone engineering; and (5) adipose stem cells, with its efficient applications in facial soft and hard tissue reconstructive surgeries[35].

Tissue engineering of the temporomandibular joint

Current approaches for replacing degenerated mandibular condyles suffer from deficiencies such as donor site morbidity, immune-rejection, implant wear and tear, and pathogen transmission. So, advances in the management of temporomandibular joint degenerative diseases have been established: (1) to promote matrix synthesis and tissue maturation of stem cell-derived chondrogenic and osteogenic cells (Figure 2)[36]; (2) to enhance the mechanical properties of a tissue engineered mandibular condyle for ultimate in situ implantation into the human temporomandibular joint (Figure 3)[37]; and (3) to facilitate the remodeling potential of a tissue-engineered mandibular condyle.

Periodontal tissue engineering[35]

The modulation of the exuberant host response to microbial contamination that plagues the periodontal wound is considered as the main challenge. It clarified

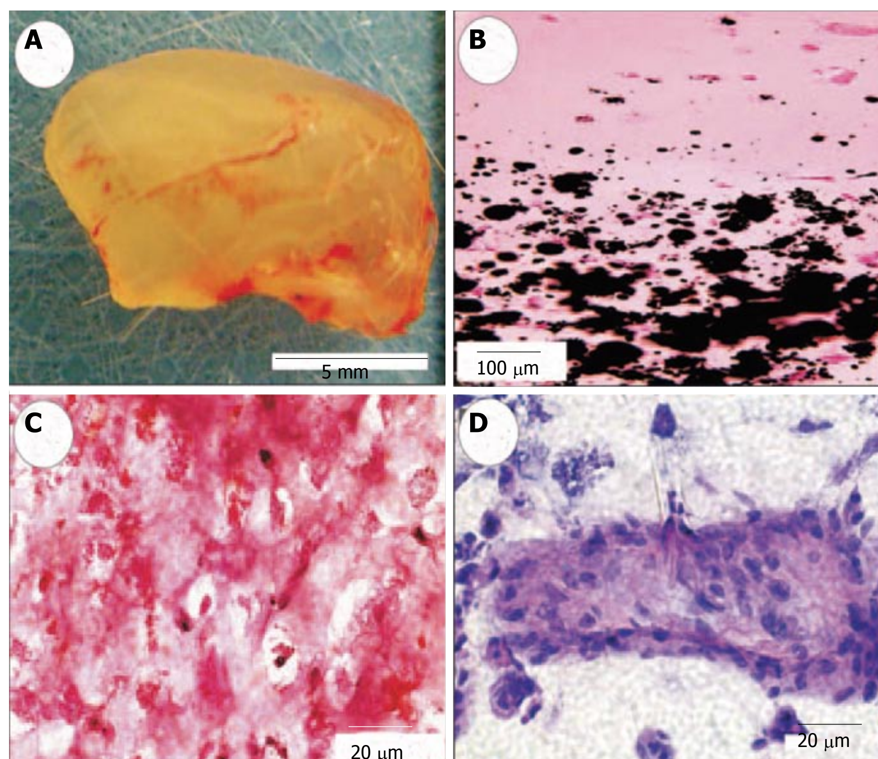


Figure 2 Engineered neogenesis of human-shaped mandibular condyle from mesenchymal stem cells. A: Harvested osteochondral construct retained the shape and dimension of the cadaver human mandibular condyle after *in vivo* implantation; B: Von Kossa-stained section showing the interface between stratified chondral and osseous layers. Multiple mineralization nodules are present in the osseous layer (lower half of the photomicrograph), but absent in the chondral layer; C: Positive safranin O staining of the chondrogenic layer indicates the synthesis of abundant glycosaminoglycans; D: H and E-stained section of the osteogenic layer showing a representative osseous island-like structure consisting of MSC-differentiated osteoblast-like cells on the surface and in the center. Reproduced with permission from Biomedical Engineering Society[36].

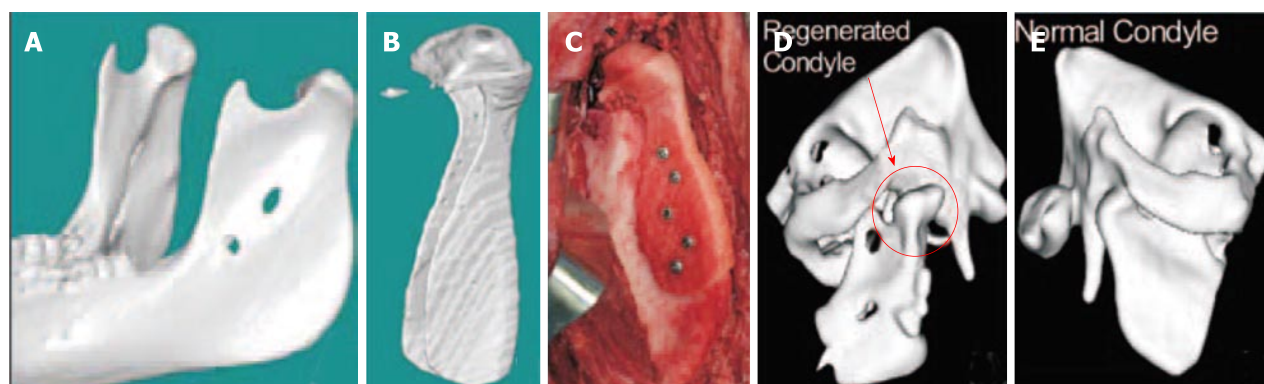


Figure 3 Design and engineering of minipig mandibular condyle. A: Original computed tomography scan of minipig mandible; B: Image-based design of condyle scaffold; C: Ppolycaprolactone degradable polymer scaffold fabricated with selective laser sintering attached to the ramus; D: Regrowth of condyle following 3 months' implantation (new condyle shown in red circle); E: Comparison with normal condyle from contralateral side in Yucatan minipig[37].

the interactions of multiple cell lineages including cementogenic cells, fibroblasts, and osteogenic cells. Regeneration of severe periodontal defects necessitates the attraction of endogenous periodontal tissue forming cells by growth factors.

As regard the periodontal ligament (PDL) function, a recent report identified stem cells in human PDL (PDLSCs) and found that PDLSCs implanted into nude mice generated cementum/PDL-like structures that resemble the native PDL as a thin layer of cementum that interfaced with dense collagen fibers, similar to Sharpey's fibers (Figure 4)[38,39]. Thus, the PDLSCs have the potential for forming periodontal structures, including the cementum and PDL.

Challenges in improving stem cell-based researches

Tissue engineered bone with customized shape and dimensions have the potential for the biological replacement of craniofacial bones, and segmental defects in the appendicular bones.

Stem cell therapy encompasses new technologies and therapies and posses many challenges in stem cell

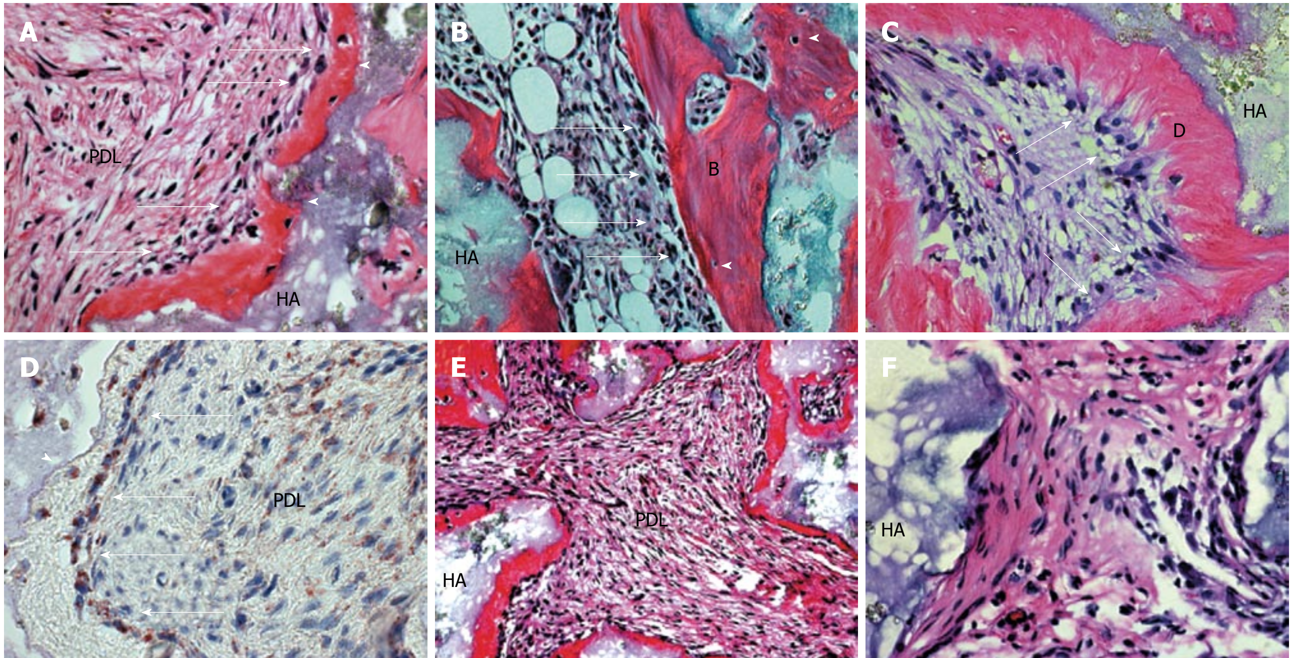


Figure 4 Generation of cementum-like and PDL-like structures *in vivo* by PDLSCs. A: After 8 wk of transplantation, PDLSCs differentiated into cementoblast-like cells (arrows) that formed a cementum-like structure (C) on the surface of the hydroxyapatite tricalcium phosphate (HA) carrier; cementocyte-like cells (arrowhead) and PDL-like tissue (PDL) were also generated; B: BMSSC transplant was used as control to show the formation of a bone/marrow structure containing osteoblasts (arrows), osteocytes (arrowhead), and elements of bone (B) and haemopoietic marrow (HP); C: DPSC transplant was also used as a control to show a dentin/pulp-like structure containing odontoblasts (arrows) and dentin like (D) and pulp-like (Pulp) tissue; D: Immunohistochemical staining showed that PDLSCs generated cementum-like structure (C) and differentiated into cementoblast-like cells (arrows) and cementocyte-like cells (arrowhead) that stained positive for human-specific mitochondria antibody. Part of the PDL-like tissue (PDL) also stained positive for human specific mitochondria antibody (within dashed line); E: Of 13 selected strains of single-colony derived PDLSC, only eight (61%) generated cementum/PDL-like structures *in vivo* as shown at lower magnification (approximately 20). New cementum-like structure (C) formed adjacent to the surfaces of the carrier (HA) and associated with PDL-like tissue (PDL); F: The othser five strains did not generate mineralised or PDL-like tissues *in vivo*[38].

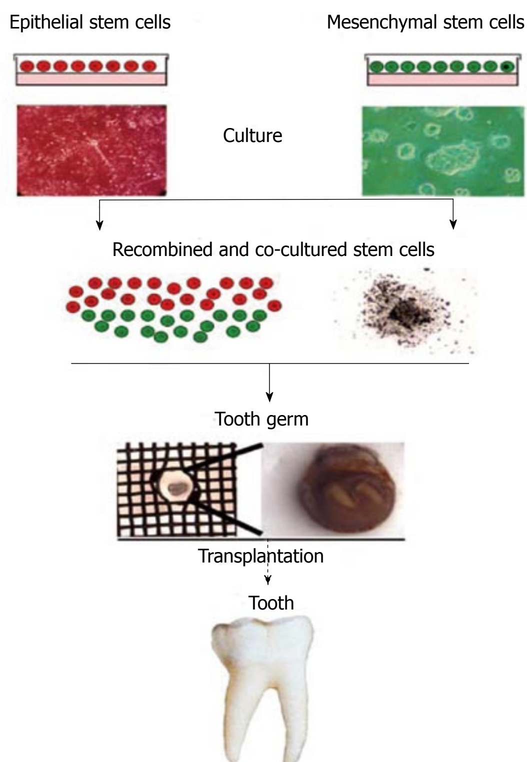


Figure 5 Use of stem cells for tooth formation *in vitro* and *ex vivo*. A tooth germ can be created in vitro after co-culture of isolated epithelial and mesenchymal stem cells. This germ could be implanted into the alveolar bone and finally develop into a fully functional tooth[40].

research, such as: (1) biological challenges: Despite biological evidence showing that regeneration can occur in humans, complete and predictable regeneration still remains an elusive clinical goal (especially in advanced periodontal defects); (2) technical challenges: The technical challenges in stem cell therapy are associated with cell manipulations, scaffold materials and delivery systems; and (3) clinical challenges: Clinical challenges in stem cell-based periodontal therapy relate to immune rejection after administration, oncogenic properties of stem cells and functional integration of transplanted tissues into the host.

Stem cells for tooth engineering

Building a tooth logically requires the association/cooperation of odontogenic mesenchymal and epithelial cells. The recombination of dissociated dental epithelial and mesenchymal tissues leads to tooth formation both *in vitro* and *in vivo* (Figure 5). The bioengineered teeth have been produced in ectopic sites and with missing some essential elements such as the complete root and periodontal tissues that allow correct anchoring into the alveolar bone. Recently, a new approach has been proposed for growing teeth in the mouse mandible[40].

CONCLUSION

For dental applications, stem cell therapy and tissue

engineering are gold solution for bone and soft tissue regeneration and provide hope of future application in humans within the next few years. Tissue engineering modalities will provide numerous clinical dental applications, including improved treatments for intraosseous periodontal defects, enhanced maxillary and mandibular grafting procedures, perhaps more biological methods to repair teeth after carious damage and possibly even re-growing lost teeth. This review highlights the sources and the regenerative therapies in the clinical practice of dentistry in the future.

REFERENCES

1 **Petrungaro PS**, Amar S. Localized ridge augmentation with allogenic block grafts prior to implant placement: case reports and histologic evaluations. *Implant Dent* 2005; **14**: 139-148 [PMID: 15968185 DOI: 10.1097/01.id.0000163805.98577.ab]

2 **Froum SJ**, Gomez C, Breault MR. Current concepts of periodontal regeneration. A review of the literature. *N Y State Dent J* 2002; **68**: 14-22 [PMID: 12442729]

3 **Raghoebar GM**, Louwerse C, Kalk WW, Vissink A. Morbidity of chin bone harvesting. *Clin Oral Implants Res* 2001; **12**: 503-507 [PMID: 11564111 DOI: 10.1034/j.1600-0501.2001.120511.x]

4 **Polimeni G**, Albandar JM, Wikesjö UM. Prognostic factors for alveolar regeneration: osteogenic potential of resident bone. *J Clin Periodontol* 2004; **31**: 840-844 [PMID: 15367186 DOI: 10.1111/j.1600-051X.2004.00590.x]

5 **Manson PN**, Crawley WA, Hoopes JE. Frontal cranioplasty: risk factors and choice of cranial vault reconstructive material. *Plast Reconstr Surg* 1986; **77**: 888-904 [PMID: 3520618 DOI: 10.1097/00006534-198606000-00003]

6 **Kübler N**, Michel C, Zöller J, Bill J, Mühling J, Reuther J. Repair of human skull defects using osteoinductive bone alloimplants. *J Craniomaxillofac Surg* 1995; **23**: 337-346 [PMID: 8839327 DOI: 10.1016/S1010-5182(05)80128-3]

7 **Sàndor GK**, Kainulainen VT, Queiroz JO, Carmichael RP, Oikarinen KS. Preservation of ridge dimensions following grafting with coral granules of 48 post-traumatic and post-extraction dento-alveolar defects. *Dent Traumatol* 2003; **19**: 221-227 [PMID: 12848717 DOI: 10.1034/j.1600-9657.2003.00164.x]

8 **Meijer GJ**, de Bruijn JD, Koole R, van Blitterswijk CA. Cell-based bone tissue engineering. *PLoS Med* 2007; **4**: e9 [PMID: 17311467 DOI: 10.1371/journal.pmed.0040009]

9 **Taga ML**, Granjeiro JM, Cestari TM, Taga R. Healing of critical-size cranial defects in guinea pigs using a bovine bone-derived resorbable membrane. *Int J Oral Maxillofac Implants* 2008; **23**: 427-436 [PMID: 18700364]

10 **Shanti RM**, Li WJ, Nesti LJ, Wang X, Tuan RS. Adult mesenchymal stem cells: biological properties, characteristics, and applications in maxillofacial surgery. *J Oral Maxillofac Surg* 2007; **65**: 1640-1647 [PMID: 17656295 DOI: 10.1016/j.joms.2007.04.008]

11 **Javazon EH**, Beggs KJ, Flake AW. Mesenchymal stem cells: paradoxes of passaging. *Exp Hematol* 2004; **32**: 414-425 [PMID: 15145209 DOI: 10.1016/j.exphem.2004.02.004]

12 **Thomson JA**, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. *Science* 1998; **282**: 1145-1147 [PMID: 9804556]

13 **Aflatoonian B**, Moore H. Human primordial germ cells and embryonic germ cells, and their use in cell therapy. *Curr Opin Biotechnol* 2005; **16**: 530-535 [PMID: 16154336]

14 **Marion NW**, Mao JJ. Mesenchymal stem cells and tissue engineering. *Methods Enzymol* 2006; **420**: 339-361 [PMID: 17161705 DOI: 10.1016/S0076-6879(06)20016-8]

15 **Lyahyai J**, Mediano DR, Ranera B, Sanz A, Remacha AR, Bolea R, Zaragoza P, Rodellar C, Martín-Burriel I. Isolation and characterization of ovine mesenchymal stem cells derived from peripheral blood. *BMC Vet Res* 2012; **8**: 169 [PMID: 22999337 DOI: 10.1186/1746-6148-8-169]

16 **Alhadlaq A**, Mao JJ. Mesenchymal stem cells: isolation and therapeutics. *Stem Cells Dev* 2004; **13**: 436-448 [PMID: 15345137 DOI: 10.1089/scd.2004.13.436]

17 **Schäffler A**, Büchler C. Concise review: adipose tissue-derived stromal cells--basic and clinical implications for novel cell-based therapies. *Stem Cells* 2007; **25**: 818-827 [PMID: 17420225 DOI: 10.1634/stemcells.2006-0589]

18 **Miura M**, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG, Shi S. SHED: stem cells from human exfoliated deciduous teeth. *Proc Natl Acad Sci USA* 2003; **100**: 5807-5812 [PMID: 12716973]

19 **Gronthos S**, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. *Proc Natl Acad Sci USA* 2000; **97**: 13625-13630 [PMID: 11087820 DOI: 10.1073/pnas.240309797]

20 **Morsczeck C**, Moehl C, Götz W, Heredia A, Schäffer TE, Eckstein N, Sippel C, Hoffmann KH. In vitro differentiation of human dental follicle cells with dexamethasone and insulin. *Cell Biol Int* 2005; **29**: 567-575 [PMID: 15951208 DOI: 10.1016/j.cellbi.2005.03.020]

21 **Morsczeck C**. Gene expression of runx2, Osterix, c-fos, DLX-3, DLX-5, and MSX-2 in dental follicle cells during osteogenic differentiation in vitro. *Calcif Tissue Int* 2006; **78**: 98-102 [PMID: 16467978 DOI: 10.1007/s00223-005-0146-0]

22 **Seo BM**, Miura M, Sonoyama W, Coppe C, Stanyon R, Shi S. Recovery of stem cells from cryopreserved periodontal ligament. *J Dent Res* 2005; **84**: 907-912 [PMID: 16183789 DOI: 10.1177/154405910508401007]

23 **Sonoyama W**, Liu Y, Yamaza T, Tuan RS, Wang S, Shi S, Huang GT. Characterization of the apical papilla and its residing stem cells from human immature permanent teeth: a pilot study. *J Endod* 2008; **34**: 166-171 [PMID: 18215674 DOI: 10.1016/j.joen.2007.11.021]

24 **Morsczeck C**, Götz W, Schierholz J, Zeilhofer F, Kühn U, Möhl C, Sippel C, Hoffmann KH. Isolation of precursor cells (PCs) from human dental follicle of wisdom teeth. *Matrix Biol* 2005; **24**: 155-165 [PMID: 15890265]

25 **Tamaki Y**, Nakahara T, Ishikawa H, Sato S. In vitro analysis of mesenchymal stem cells derived from human teeth and bone marrow. *Odontology* 2013; **101**: 121-132 [PMID: 22772774]

26 **Pan K**, Sun Q, Zhang J, Ge S, Li S, Zhao Y, Yang P. Multilineage differentiation of dental follicle cells and the roles of Runx2 over-expression in enhancing osteoblast/cementoblast-related gene expression in dental follicle cells. *Cell Prolif* 2010; **43**: 219-228 [PMID: 20546240 DOI: 10.1111/j.1365-2184.2010.00670.x]

27 **Sunil P**, Manikandhan R, Muthu M, Abraham S. Stem cell therapy in oral and maxillofacial region: An overview. *J Oral Maxillofac Pathol* 2012; **16**: 58-63 [PMID: 22434942 DOI: 10.4103/0973-029X.92975]

28 **Thirumala S**, Goebel WS, Woods EJ. Clinical grade adult stem cell banking. *Organogenesis* 2009; **5**: 143-154 [PMID: 20046678 DOI: 10.4161/org.5.3.9811]

29 **Kémoun P**, Laurencin-Dalicieux S, Rue J, Farges JC, Gennero I, Conte-Auriol F, Briand-Mesange F, Gadelorge M, Arzate H, Narayanan AS, Brunel G, Salles JP. Human dental follicle cells acquire cementoblast features under stimulation by BMP-2/-7 and enamel matrix derivatives (EMD) in vitro. *Cell Tissue Res* 2007; **329**: 283-294 [PMID: 17443352]

30 **Bakopoulou A**, Leyhausen G, Volk J, Tsiftsoglou A, Garefis P, Koidis P, Geurtsen W. Comparative analysis of in vitro osteo/odontogenic differentiation potential of human dental pulp stem cells (DPSCs) and stem cells from the apical papilla (SCAP). *Arch Oral Biol* 2011; **56**: 709-721 [PMID: 21227403 DOI: 10.1016/j.archoralbio.2010.12.008]

31 **Honda MJ**, Imaizumi M, Tsuchiya S, Morsczeck C. Dental follicle stem cells and tissue engineering. *J Oral Sci* 2010; **52**: 541-552 [PMID: 21206155 DOI: 10.2334/josnusd.52.541]

32 **Wang L**, Li Y, Chen X, Chen J, Gautam SC, Xu Y, Chopp M. MCP-1, MIP-1, IL-8 and ischemic cerebral tissue enhance human bone marrow stromal cell migration in interface culture. *Hematology* 2002; **7**: 113-117 [PMID: 12186702 DOI: 10.1080/1024533029002858 8]

33 **Porada CD**, Almeida-Porada G. Mesenchymal stem cells as therapeutics and vehicles for gene and drug delivery. *Adv Drug Deliv Rev* 2010; **62**: 1156-1166 [PMID: 20828588 DOI: 10.1016/j.addr.2010.08.010]

34 **El-Menoufy H**, Aly LA, Aziz MT, Atta HM, Roshdy NK, Rashed LA, Sabry D. The role of bone marrow-derived mesenchymal stem cells in treating formocresol induced oral ulcers in dogs. *J Oral Pathol Med* 2010; **39**: 281-289 [PMID: 19804505]

35 **Aly LA**, El-Menoufy H, Sadeq HS, Ragae A, Sabry D. Efficiency of systemic versus intralesional bone marrow-derived stem cells in regeneration of oral mucosa after induction of formocresol induced ulcers in dogs. *Dent Res J* (Isfahan) 2014; **11**: 212-221 [PMID: 24932192]

36 **Alhadlaq A**, Mao JJ. Tissue-engineered neogenesis of human-shaped mandibular condyle from rat mesenchymal stem cells. *J Dent Res* 2003; **82**: 951-956 [PMID: 14630893 DOI: 10.1177/154405910308201203]

37 **Mao JJ**, Giannobile WV, Helms JA, Hollister SJ, Krebsbach PH, Longaker MT, Shi S. Craniofacial tissue engineering by stem cells. *J Dent Res* 2006; **85**: 966-979 [PMID: 17062735 DOI: 10.1177/154405910608501101]

38 **Seo BM**, Miura M, Gronthos S, Bartold PM, Batouli S, Brahim J, Young M, Robey PG, Wang CY, Shi S. Investigation of multipotent postnatal stem cells from human periodontal ligament. *Lancet* 2004; **364**: 149-155 [PMID: 15246727 DOI: 10.1016/S0140-6736(04)16627-0]

39 **Shi S**, Gronthos S, Chen S, Reddi A, Counter CM, Robey PG, Wang CY. Bone formation by human postnatal bone marrow stromal stem cells is enhanced by telomerase expression. *Nat Biotechnol* 2002; **20**: 587-591 [PMID: 12042862 DOI: 10.1038/nbt0602-587]

40 **Nakao K**, Morita R, Saji Y, Ishida K, Tomita Y, Ogawa M, Saitoh M, Tomooka Y, Tsuji T. The development of a bioengineered organ germ method. *Nat Methods* 2007; **4**: 227-230 [PMID: 17322892 DOI: 10.1038/nmeth1012]

P- Reviewer: Chen S, Ferreira MM **S- Editor**: Tian YL
L- Editor: A **E- Editor**: Wu HL

WJSC

World Journal of Stem Cells

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4252/wjsc.v7.i7.1054

World J Stem Cells 2015 August 26; 7(7): 1054-1063
ISSN 1948-0210 (online)
© 2015 Baishideng Publishing Group Inc. All rights reserved.

MINIREVIEWS

Rat embryonic stem cells create new era in development of genetically manipulated rat models

Kazushi Kawaharada, Masaki Kawamata, Takahiro Ochiya

Kazushi Kawaharada, Research and Development Division, DS Pharma Biomedical Co. Ltd., Osaka 564-0053, Japan

Masaki Kawamata, Takahiro Ochiya, Division of Molecular and Cellular Medicine, National Cancer Center Research Institute, Tokyo 104-0045, Japan

Author contributions: Kawaharada K, Kawamata M and Ochiya T contributed to this paper.

Conflict-of-interest statement: Kazushi Kawaharada is an employee of DS Pharma Biomedical Co., Ltd. The other authors declared no conflict of interest.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: http://creativecommons.org/licenses/by-nc/4.0/

Correspondence to: Dr. Takahiro Ochiya, Division of Molecular and Cellular Medicine, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan. tochiya@ncc.go.jp
Telephone: +81-3-35422511
Fax: +81-3-55650727

Received: August 22, 2014
Peer-review started: August 23, 2014
First decision: September 28, 2014
Revised: May 29, 2015
Accepted: July 16, 2015
Article in press: July 18, 2015
Published online: August 26, 2015

Abstract

Embryonic stem (ES) cells are isolated from the inner cell mass of a blastocyst, and are used for the generation of gene-modified animals. In mice, the transplantation of gene-modified ES cells into recipient blastocysts leads to the creation of gene-targeted mice such as knock-in and knock-out mice; these gene-targeted mice contribute greatly to scientific development. Although the rat is considered a useful laboratory animal alongside the mouse, fewer gene-modified rats have been produced due to the lack of robust establishment methods for rat ES cells. A new method for establishing rat ES cells using signaling inhibitors was reported in 2008. By considering the characteristics of rat ES cells, recent research has made progress in improving conditions for the stable culture of rat ES cells in order to generate gene-modified rats efficiently. In this review, we summarize several advanced methods to maintain rat ES cells and generate gene-targeted rats.

Key words: Embryonic stem cells; Transgenic rat

© The Author(s) 2015. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: Rat embryonic stem (ES) cells are thought to be an essential tool for creating transgenic rats. Since the method for establishing rat ES cells using signaling inhibitors was reported, numerous approaches have been made to propagate rat ES cells efficiently. Additionally, recent investigations have demonstrated the usefulness of the signal inhibitors for microinjection. In this review, we summarize the several advanced methods to maintain rat ES cells and generate gene-targeted rats.

Kawaharada K, Kawamata M, Ochiya T. Rat embryonic stem cells create new era in development of genetically manipulated rat models. *World J Stem Cells* 2015; 7(7): 1054-1063 Available from: URL: http://www.wjgnet.com/1948-0210/full/v7/i7/1054.htm DOI: http://dx.doi.org/10.4252/wjsc.v7.i7.1054

INTRODUCTION

Rattus norvegicus, is widely used as a laboratory animal in many kinds of scientific field, such as biochemical, biomedical, and pharmacological studies. In 1828, albino mutants were brought into laboratories for physiological studies such as fasting studies. Since the nineteenth century, over 700 rat strains including inbred and outbred strains have been developed, and have been used for a multitude of studies[1,2]. Rats offer some advantages over mice in some transplantation, behavior, and pharmacokinetic studies, because the larger size and greater intelligence of rats compared to mice enables ease of surgical operation, a large-volume of blood sampling, and assessment of high-level learning[3,4].

In addition, it has been suggested that rats are a useful model for physiological studies owing to the heart rate of rats being closer than that of mice to the human heart rate[2]. For these reasons, the publications involving rats outnumbered those involving mice for many years. However, far fewer scientific procedures have used transgenic rats than have used mice[5]. One of the reasons for this disparity has been a lack of reliable methods to establish rat embryonic stem (ES) cells. Genetically modified mice have been routinely created using mouse ES cells with gene-manipulation technologies. These genetically modified mice have contributed greatly to scientific development since the first establishment of mouse ES cell technology in 1981[6]. Meanwhile in rats, advancement of one of the gene-targeting technology using zinc-finger nucleases (ZFNs) allowed to generate the first knock-out rats in 2009[7,8]. Microinjection of ZFNs into the pronuclei of rat embryos leads to the creation of knock-out rats. ZFNs are engineered proteins with DNA-binding and nuclease activity, which facilitates the targeted editing of genomes by creating double-strand breaks in the DNA at specified locations and promoting non-homologous end-joining. Moreover, a robust method to establish rat ES cells was reported in 2008[9,10], and the generation of knock-out rats was achieved using rat ES cell-based technology in 2010[11].

ESTBLISHMENT, CHARACTERIZATION, AND MAINTENANCE OF RAT EMBRYONIC STEM CELLS

Attempts for establishment of rat embryonic stem cells

Rat ES cells are isolated from the inner cell mass (ICM) of a blastocyst. Figure 1 shows a procedure for the establishment of rat ES cells (our unpublished data). The defining properties of ES cells are following; they are derived from an ICM with pluripotency; they have a stable, normal karyotype *in vitro*; they can be propagated indefinitely in theory without differentiation; they can differentiate spontaneously into multiple cell types representative of all three embryonic germ layers, both in teratomas after grafting and *in vitro* under appropriate conditions; and they can give rise to any cell type in the body, including germ cells, when injected into host blastocysts[12]. Many researchers attempted to establish rat ES cells by using the same conditions as those of mouse ES cells, namely leukemia inhibitory factor (LIF) in combination with bone morphogenetic protein (BMP) or fetal bovine serum (FBS). The rat blastocyst-derived cells cultured under these conditions expressed various embryonic stem cell specific markers such as stage-specific embryonic antigen- Ⅰ (SSEA-1), Oct4, and alkaline phosphatase, whereas the chimeric rats or teratocarcinoma derived from the rat blastocyst-derived cells were not confirmed[13-15].

Establishment of rat embryonic stem cells using small molecules

In 2008, a robust and efficient method to establish mouse ES cells was reported by Ying *et al*[16], which are also applicable to establish rat ES cells. To maintain the pluripotent state of mouse ES cells, LIF activates the Janus kinase/signal transducer and activator of transcription signaling pathway, while BMP and serum activate the Sma and Mad Related Family signaling pathway[17]. Differentiation of mouse ES cells is induced *via* activation of mitogen-activated protein kinase/ extracellular signal-related kinase (ERK) kinase (MEK) pathway by the autocrine stimulation of fibroblast growth factor-4 (FGF4)[18]. Ying *et al*[16] revealed that LIF, serum and BMP affect the downstream of ERK, and demonstrated that the inhibition of MEK and the FGF receptor (FGFR) maintains the pluripotency of mouse ES cells under serum-free conditions. Moreover, the self-renewal capacity of mouse ES cells was promoted by an additional inhibition of glycogen synthase kinase 3 (GSK3), as the GSK3 pathway is involved in the maintenance of ES cells in the undifferentiated state *via* β-catenin/Wnt signaling[19-25]. These findings revealed that two types of culture conditions, a combination of the MEK inhibitor and GSK3 inhibitor (2i condition), and a combination of the MEK inhibitor, FGFR inhibitor, and GSK3 inhibitor (3i condition), enable not only the maintenance of mouse ES cells but also the "robust" and "effective" establishment of rat ES cells displaying all the defining properties of ES cells[9,10,26-31]. The morphology of rat ES cells is rounded and loosely attached on feeder cells, unlike mouse ES cells; however, the rat ES cells express the same undifferentiated markers as mouse ES cells: Oct4, Nanog, Sox2, Rex1, FGF4, and SSEA1. Kawamata *et al*[26] succeeded in the establishment of rat ES cells even in FBS-containing medium using Rho-associated, coiled-coil containing protein kinase (ROCK) inhibitor and activin receptor-like kinase (ALK5) inhibitor in addition to the MEK inhibitor and GSK3 inhibitor, which cells showed the defining properties such as pluripotent markers, embryonic body formation, and normal karyotype (Figure 2; our unpublished data). Table 1 shows various attempts to establish rat ES cells, and the properties of the resultant cells.

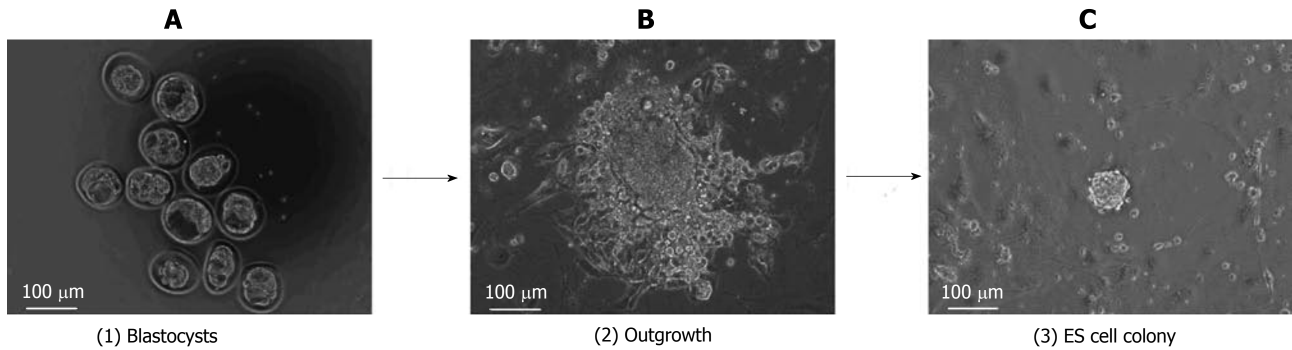
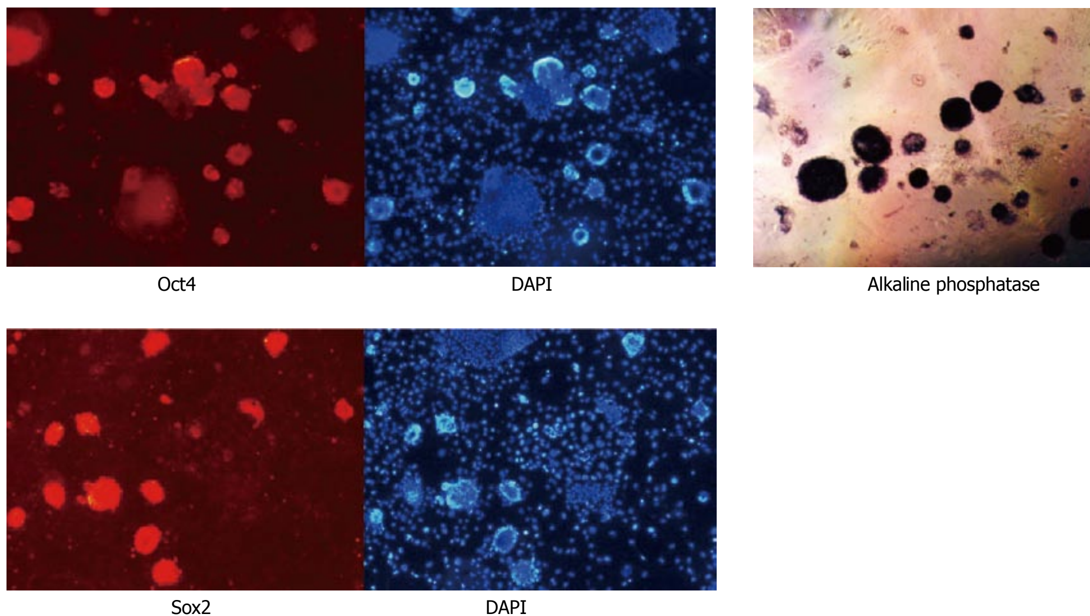
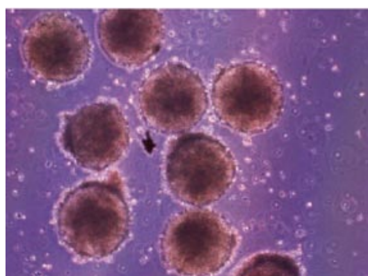


Figure 1 Establishment of rat embryonic stem cells. A: E4.5 Blastocysts are isolated from Sprague-Dawley rats, and the blastocysts dissolved zona pellucida are put on mouse embryonic fibroblasts (MEF) feeders; B: After 7-10 d, the outgrowth formed from the blastocysts are dispersed, and transferred on MEF feeders; C: Approximately 7 d after culture, rat embryonic stem (ES) cells are appeared (our unpublished data).





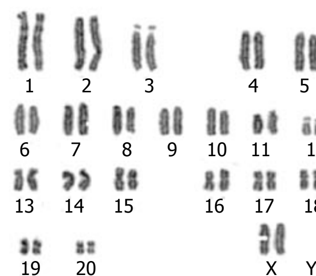


Figure 2 Properties of the rat embryonic stem cells. The rat embryonic stem cells are established using the combination of four inhibitors and serum: mitogen-associated protein kinase/extracellular signal-related kinase kinase (MEK) inhibitor, glycogen synthase kinase 3 inhibitor, activin receptor-like kinase (ALK5) inhibitor, Rho-associated, coiled-coil containing protein kinase inhibitor and FBS. A: Expression of the pluripotent markers Oct4 and Sox2, and alkaline phosphatase staining; B: Embryonic body formation; C: Karyotype analysis by G-band staining (our unpublished data). DAPI: 4',6 -diamidino-2-phenylindole; FBS: Fetal bovine serum.

Improvement of rat embryonic stem cell culture efficiency

Numerous approaches have been made to efficiently propagate rat ES cells. Soon after the establishment of rat ES cells[9,10], rat induced pluripotent stem (iPS) cells were established using the 2i culture conditions[32,33]. The additional inhibition of ALK5 allowed to propagate rat iPS cells as a homogeneous population, with less spontaneously differentiated colonies as compared to the 2i condition without ALK5 inhibition[32]. Furthermore, the inhibition of ROCK prevents from apoptosis in human ES/iPS cells and enhances their attachment on

Table 1 Summary of components for the establishment of rat embryonic stem cells and the properties of the resultant cells

Ref.	Additives	Pluripotent markers	Karyotype stability	EB/teratocarcinoma	Germline transmission
Iannacconne *et al*[13]	FBS	AP	Unconfirmed	Failed	Failed
	LIF	SSEA1			
Takahama *et al*[14]	FBS	AP	Unconfirmed	Unconfirmed	Unconfirmed
	LIF	Oct3/4			
		SSEA1			
Vassilieva *et al*[15]	FBS	AP	Unconfirmed	Unconfirmed	Unconfirmed
	LIF	Oct3/4			
		SSEA1			
Buehr *et al*[9]	(A) N2B27		Confirmed	Confirmed	Confirmed
	FGFRi	AP			
	MEKi	Oct3/4			
	GSK3i	SSEA1			
	(B)	Sox2			
	N2B27				
	MEKi	Nanog			
	GSK3i	Fgf4			
	LIF	Rex1			
Li *et al*[10]	(A) N2B27		Confirmed	Confirmed	Confirmed
	MEKi	AP			
	GSK3i	Oct3/4			
	(B)	SSEA1			
	N2B27				
	MEKi	Sox2			
	GSK3i	Nanog			
	LIF				
Kawamata *et al*[26]	FBS	AP	Confirmed	Confirmed	Confirmed
	MEKi	Oct3/4			
	GSK3i	Sox2			
	ALK5i	Nanog			
	ROCKi	Rex1			

EB: Embryo culture media; FBS: Fetal bovine serum; LIF: Leukemia inhibitory factor; MEK: Mitogen-activated protein kinase/ERK kinase; GSK3: Glycogen synthase kinase 3; FGFR: Fibroblast growth factor-receptor; ALK5: Activin receptor-like kinase; ROCK: Rho-associated, coiled-coil containing protein kinase; AP: Alkaline phosphatase.

feeder cells after enzymatic dissociation[34,35]. These studies suggested that the addition of the ALK5 inhibitor and ROCK inhibitor to either the 2i or 3i condition promotes the efficient culture of rat ES cells. Kawamata *et al*[26] confirmed the effect of the ROCK inhibitor on the self-renewal of rat ES cells by using Oct4-Venous-transgenic rat ES cells, in which cells were derived from the transgenic rats expressing the fluorescent Venus protein under the Oct4 promoter/enhancer. In a condition containing all the four inhibitors (the ROCK inhibitor Y-27632, the MEK inhibitor PD0325901, the ALK5 inhibitor A-83-01, and the GSK3 inhibitor CHIR99021) referred to as YPAC, a great number of colonies expressing the pluripotent markers Oct4 and alkaline phosphatase were appeared after single-cell enzymatic dissociation. In contrast, the rat ES cell colonies maintained withdrawal of the ROCK inhibitor from the YPAC condition expressed the pluripotent markers, but the colonies were sparse under the three-inhibitor condition compared to the YPAC condition[26]. Kawamata *et al*[26] concluded that the ROCK inhibitor promotes the attachment of rat ES cells on feeder cells, which leads to efficient culture expansion. Li *et al*[36] compared the 2i condition with the YPAC condition, and concluded that rat ES cells could expand approximately twice more under the YPAC condition than under the 2i condition, while maintaining the undifferentiated state. A combination of the serum and the 2i condition was also attempted for the culture of rat ES cells, and as a result, the addition of the serum enhanced the attachment of rat ES cells to feeder cells[29]. However, the addition of serum triggers differentiation in the cultured ES cells, and may introduce unexpected side effects due to unknown factors within the serum[37]. Actually, the expression level of a trophectoderm marker Cdx2 was three times higher in the rat ES cells cultured in the serum with YPAC than in the cells cultured in the serum-free 2i condition[38].

Regulation of Cdx2 expression in rat embryonic stem cells

Cdx2 is the marker of trophectoderm cells, which is not expressed in mouse ES cells; hence, some studies attempted to find culture condition to repress the expression of Cdx2[39,40]. It was reported that the expression of Cdx2 was related to the concentration of the GSK3 inhibitor. The low concentration of the GSK3 inhibitor 1.5 μmol/L was found to maintain pluripotency and reduce Cdx2 expression; however, the higher concentration of the GSK3 inhibitor 3 μmol/L, which has

generally been used to establish and maintain rat ES cells, promotes the expression of Cdx2[41]. In addition, a decrease in Cdx2 expression was reported by maintaining rat ES cells on Matrigel® or in suspension[29].

Maintenance of chromosomal states in rat embryonic stem cells

One of the defining properties of ES cells is the retention of a normal karyotype after prolonged culture. However, the rat ES cells cultured under the 2i condition retained normal karyotypes fewer than 40%, which were lower than that found in mouse ES cells[42]. It is known that the low germline-competence of mouse ES cells results from abnormal karyotypes[43], and hence, the instability of karyotypes in rat ES cells would make germline transmission difficult. It is reported that the combination of the serum and YPAC improves the karyotype stability, which retains normal karyotypes with over 70%[26]. The effect of YPAC on karyotype stability was clarified by Li *et al*[36] by the successful generation of germline-competent chimeric rats by using the highly passaged rat ES cells maintained under the serum-free YPAC condition, while the rat ES cells maintained under the 2i condition failed to contribute germline. These results suggest that the inhibition of ROCK and/or ALK5 in addition to the 2i condition can stabilize the karyotypes of rat ES cells.

DIFFERENTIATION OF RAT EMBRYONIC STEM CELLS INTO FUNCTIONAL CELLS

Only a few studies have reported a stable induction method to differentiate rat ES cells into functional cells *in vitro*. Cao *et al*[44] supplied a protocol to differentiate rat ES cells into cardiomyocytes, and showed that the functional properties of the differentiated cells were similar to those of rat fetal cardiomyocytes and mouse ES cell-derived cardiomyocytes. However, the efficiency of the cardiac differentiation of rat ES cells was approximately 40%; this was lower than that of mouse ES cells, which were differentiated with over 80% efficiency[45]. Normally, the first step for *in vitro* differentiation of mouse ES cells is to induce cell aggregation into embryonic bodies (EBs), which can be accomplished in a relatively simple procedure using a differentiation medium containing serum. However, apoptosis was observed in rat ES cells during the formation of EBs in the differentiation medium[26,44]. On the other hand, Peng *et al*[46] succeeded in efficient differentiation of neural precursors from rat ES cells. They demonstrated that the ROCK inhibitor facilitates the neural differentiation and the GSK3 inhibitor maintains the survivability of the differentiated cells, and high-efficiency neural precursor differentiation (90%) was achieved using the two inhibitors. For the survival of neural precursors differentiated from mouse ES cells, it is sufficient only in the ROCK inhibitor[47]. Thus, optimized differentiation media for rat ES cells are necessary to induce rat ES cells into functional cells efficiently. These media may facilitate not only *in vitro* studies but also *in vivo* studies such as the transplantation of rat ES cell-derived differentiated cells or grafts to animal models, which *in vivo* studies were not reported even though rats are more relevant to humans than mice.

GENERATION OF KNOCK-IN AND KNOCK-OUT RATS USING RAT EMBRYONIC STEM CELLS

Gene-targeting to rat embryonic stem cells

Gene targeting to rat ES cells has been explored in some previous studies. Tong *et al*[11] and Yamamoto *et al*[48] reported that the efficiency of homologous recombination was 1.12%-3.70% and 0.9%, respectively, while Meek *et al*[28] reported that the efficiency was similar to that of mouse and human ES cells. Moreover, the gene-targeted rat ES cells express the pluripotent markers to the same extent as the parent ES cells, and retain the ability to differentiate into all three germ layers[28]. It was noted that serum was effective at minimizing the damage caused when electroporation was performed[49]. In fact, Yamamoto *et al*[48] used 1% serum during their electroporation procedure. Such efforts are not necessary for the rat ES cells maintained in the condition developed by Kawamata *et al*[26], because the culture condition already contains 20% FBS.

Embryonic stem cell maintenance for creating knock-out rats

Soon after the generation of transgenic rats using rat ES cells[26,50], the first study of targeted gene disruption in rats *via* homologous recombination in rat ES cells were reported by Tong *et al*[11]. Figure 3 shows a process of generating genetically-modified rats. Tong *et al*[11] succeeded in generating germline chimeric rats by selecting the colonies that formed a small, round, compact shape, and loose attachment on feeder cells, not the large or strongly adherent colonies. This finding provides a guideline to choose rat ES cell colonies for microinjection. Tong *et al*[11] found that over 65% of the large and strongly adherent colonies were polyploid, and concluded that such chromosomal abnormalities caused the failure of germline transmission, as the same as the case in mouse ES cells. Hence, the likelihood of successful germline transmission could be increased by the selection of small, loosely adherent rat ES cell colonies for the purpose of microinjection.

An unstable state of rat ES cells in recipient blastocysts after microinjection was also considered as a possible cause for failure to produce germline-competent chimeric rats. In order to study the fate of rat ES cells in recipient blastocysts, Kawamata *et al*[26] delivered CAG-AmCyan1-transgenic rat ES cells into recipient

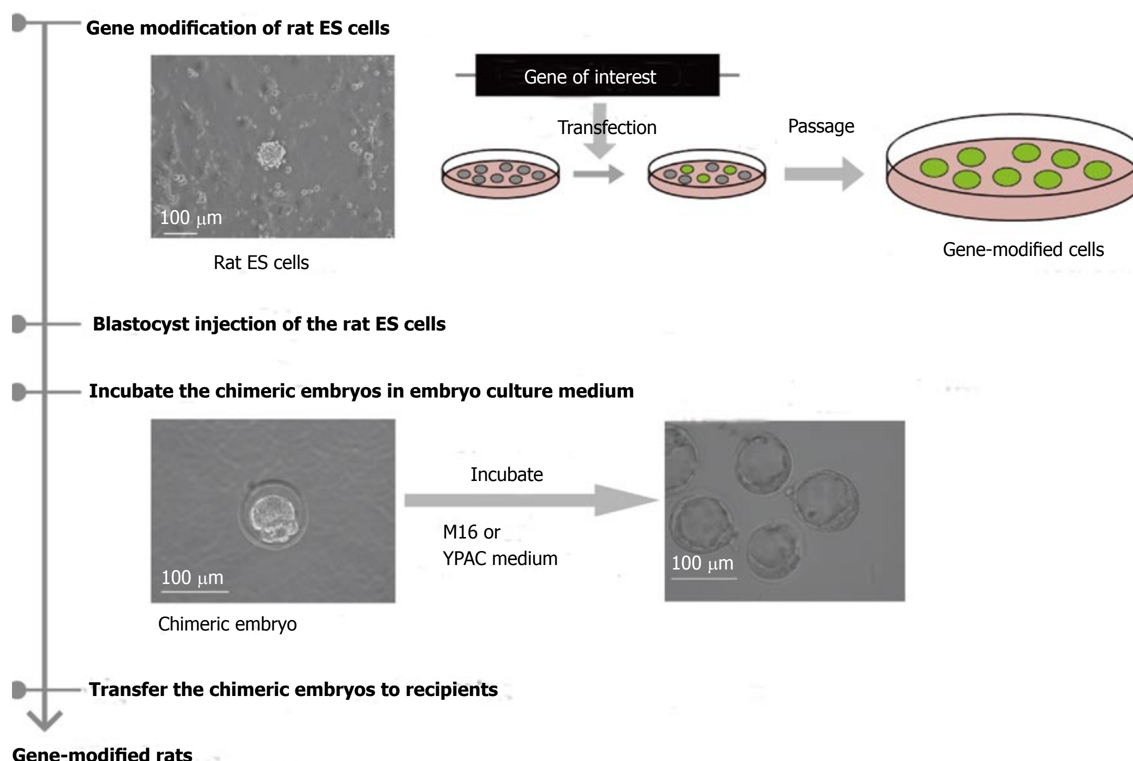


Figure 3 Generation of embryonic stem cell-derived gene-modified rats. First, a gene of interest is introduced into rat embryonic stem (ES) cells. Next, the gene-targeted rat ES cells are injected into blastocysts and are incubated in embryo culture medium or YPAC medium. Then, the chimeric rat embryos are transferred into recipient rats. After that, gene-modified rats will be generated following breeding of the chimeric rats obtained from chimeric embryos.

blastocysts by microinjection. In the absence of YPAC, few cyan-positive cells were present in the blastocysts after 30 h incubation, whereas cyan-positive rat ES cells remained on the surface of the ICM when YPAC was used in both the injection and incubation media. It is reasonable that the addition of the ROCK inhibitor not only suppresses apoptosis, but also promotes adherence to the ICM. Some embryo culture media such as M2, M16, and KSOM have been generally used for the microinjection of mouse ES cells. Similarly, the use of the embryo culture media is also proposed for microinjection of rat ES cells[51]. Specifically, embryos are washed in M2 medium after collection from pregnant rats, and the chimeric embryos are incubated in M16 medium after microinjection to allow recovery. However, the use of the YPAC medium might offer a superior microinjection method to the conventional methods.

Embryonic stem cell-based knock-out rats and their phenotypes

Two kinds of gene-targeted rats - p53-knock-out rats and protease-activated receptor-2 (Par-2) knock-out rats - have been developed using rat ES cell technology, by three groups[11,48,52]. The p53-knock-out rats and p53-knock-out mice revealed differing phenotypes: only 4% of female rats homozygous for the p53 knock-out could survive, whereas the female mice homozygous for the p53 knock-out were normally present at weaning[52]; the major tumor type of the p53-knock-out rats is hemangiosarcoma, while that of the p53-knock-out mice is lymphoma[53]; and developmental abnormalities associated with neural tube defects occurred more frequently in the female p53-knock-out rats than in those female mice[54]. Such sexual distortion associated with the neural tube defects also cause in human[55,56].

A phenotype of Par-2-knock-out rats were reported by Yamamoto *et al*[48]. The Par-2-knock-out rats showed a lack of responsiveness to PAR-2 receptor activating peptides, which clearly indicated the deficiency of Par-2 protein.

Rat models in studying human diseases

The transgenic rat model of Huntington's disease reflects more typical adult patient pathologies in comparison to the transgenic mice[57]. The transgenic rat model of Alzheimer's disease, harboring mutant human transgenes for amyloid precursor protein and presenilin 1, manifests a complete repertoire of Alzheimer's disease pathological features and demonstrates a markedly greater abundance of soluble oligomeric amyloid-β peptides than mice harboring the same human transgenes[58]. Moreover, the usefulness of transgenic rat models was shown in autism and fragile X syndrome researches. Transgenic rat models lacking disease-associated genes such as fragile X mental retardation 1 gene and neuroligin-3, and these mutations display traits that may be analogous to the characteristics of in their respective human diseases[59].

Although gene knock-out strategies are a promising

Table 2 List of knock-out rats generated using new gene-targeted technologies and rat embryonic stem cell-based technologies

Ref.	Cell type	Technology	Target
Ménoret *et al*[69]	Embryo	Meganuclease	Rag1
Ménoret *et al*[70]	Embryo	ZFN	Immunoglobulin heavy chain
Zschemisch *et al*[71]	Embryo	ZFN	Rag1
Geurts *et al*[7]	Embryo	ZFN	GFP
	Embryo	ZFN	IgM
	Embryo	ZFN	Rab38
Mashimo *et al*[8]	Embryo	ZFN	IL-2 receptor gamma
Moreno *et al*[72]	Embryo	ZFN	Renin
Chu *et al*[73]	Embryo	ZFN	Mdr1
Vaira *et al*[74]	Embryo	ZFN	Reptin
Tesson *et al*[66]	Embryo	TALEN	IgM
Ferguson *et al*[67]	Embryo	TALEN	Tlr4
Yamamoto *et al*[48]	ES cells	Homologous Recombination	Par2
Tong *et al*[11]	ES cells	Homologous Recombination	p53
Kawamata *et al*[52]	ES cells	ZFN	p53

ES: Embryonic stem; ZFN: Zinc-finger nuclease; TALEN: Transcription activator-like effector nuclease.

tool to clarify the mechanism of human diseases such as those mentioned above, some human disease-related gene knock-outs are developmentally lethal. This problem can often be overcome through the use of conditional-knock-out animals, which enable site-specific and inducible gene deletion. Brown *et al*[60] first generated the conditional-knock-out rats *via* ZFN-mediated technologies. The system is based on Cre/loxP recombination system, and a target gene is modified by the insertion of two flanking loxP sites, enabling excision of the flanked (floxed) gene segment through Cre-mediated recombination. Conditional-knock-out mice are obtained by mating the floxed mouse line with a Cre-transgenic line, such that the target gene in the progeny becomes inactivated *in vivo* within the expression domain of Cre[61,62]. To create the conditional-knock-out rats, Brown *et al*[60] mated the two strains: floxed allele-harboring rats and Cre allele-harboring rats, generated *via* pronuclear co-injection of a pair of ZFNs along with each donor plasmid.

PERSPECTIVES

Rats are used as the first-preferred animal species for *in vivo* tests of chemicals. In the meantime, *in vitro* alternative methods reflecting a response to chemicals have been demanded due to concerns about animal welfare. The use of the *in vitro* alternatives has many advantages: a decrease in the number of animals used, a reduced cost of animal maintenance, a smaller quantity of chemical needed for testing, and a shortening of the time essential to prepare for and conduct experiments[63-65]. Rat ES cells could provide innovative *in vitro* screening models, as rat ES cells have an infinite proliferative capacity and can be continuously supplied.

The generation of transgenic rats have been supported by not only rat ES cell-based gene-modification technology but also other new tools for gene modification such as ZFNs, transcription activator-like effector nuclease and clustered regularly interspaced short palindromic repeat[66-68]. Table 2 shows the knock-out rats generated by using of rat ES cells and new gene-modification technologies. However, the new technologies carry a risk of off-target effects by site-specific nuclease activity, and require screening to detect targeted events. In contrast, using ES cell-based technologies allows researchers to easily approach the generation of genetically modified rats with the knowledge obtained from experience in generating genetically modified mice. Therefore, rat ES cell-based gene targeting is still an essential tool for gene modifications.

CONCLUSION

Exploring small molecules for the optimum culture conditions for rat ES cells opened up avenues for the generation of genetically modified rats. Although ES cell-based knock-out/knock-in rats have not been widely generated due to the karyotype instability of rat ES cells, further exploration of new combinations of small molecules and growth factors will facilitate germline transmission of genetically modified rat ES cell clones. Additionally, human ES cells with a naïve state could be created by above effort[75]. As the phenotypes of gene-knock-out animals can be different among species, rats represent a valuable tool in which phenotypes can be generated and compared to those of mice with relative ease. Studies using a combination of both mouse and rat disease models will provide beneficial information to clarify the mechanisms of human disease, leading to the development of new drugs. We hope that many researchers again choose to utilize rat models for research, and establish a new platform for basic and clinical applications using gene modification technology.

ACKNOWLEDGMENTS

The authors would like to express the deepest appreciation to Yoshihiro Sumita and Tadayoshi Ueda whose comments and suggestions were of inestimable value for this manuscript. The authors also thanks to Aya Yoshimura (National Institute of Neuroscience) for technical advice, Luc Gailhouste (National Cancer Center Research Institute) for comments on this manuscript and Shigeyuki Ota, Lao Xintian, Makiko Morita, Hiroko Koreishi, Kenryo Furushima and Yasuyuki Tsutsumishita for their sincere encouragement.

REFERENCES

1 **Voigt B**, Serikawa T. Pluripotent stem cells and other technologies will eventually open the door for straightforward gene targeting in the rat. *Dis Model Mech* 2009; **2**: 341-343 [PMID: 19553695 DOI:

10.1242/dmm.002824]
2 **Hedrich JH**. History, Strains and Models. The Laboratory Rat, 2000: 3-16
3 **Jacob HJ**, Kwitek AE. Rat genetics: attaching physiology and pharmacology to the genome. *Nat Rev Genet* 2002; **3**: 33-42 [PMID: 11823789 DOI: 10.1038/nrg702]
4 **Abbott A**. Laboratory animals: the Renaissance rat. *Nature* 2004; **428**: 464-466 [PMID: 15057803 DOI: 10.1038/428464a]
5 **Zhao S**, Liu E, Chu Y, Zheng H, Kitajima S, Morimoto M. Numbers of Publications Related to Laboratory Animals. *Scand J Lab Anim Sci* 2007; **34**: 81-86
6 **Evans MJ**, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. *Nature* 1981; **292**: 154-156 [PMID: 7242681 DOI: 10.1038/292154a0]
7 **Geurts AM**, Cost GJ, Freyvert Y, Zeitler B, Miller JC, Choi VM, Jenkins SS, Wood A, Cui X, Meng X, Vincent A, Lam S, Michalkiewicz M, Schilling R, Foeckler J, Kalloway S, Weiler H, Ménoret S, Anegon I, Davis GD, Zhang L, Rebar EJ, Gregory PD, Urnov FD, Jacob HJ, Buelow R. Knockout rats via embryo microinjection of zinc-finger nucleases. *Science* 2009; **325**: 433 [PMID: 19628861 DOI: 10.1126/science.1172447]
8 **Mashimo T**, Takizawa A, Voigt B, Yoshimi K, Hiai H, Kuramoto T, Serikawa T. Generation of knockout rats with X-linked severe combined immunodeficiency (X-SCID) using zinc-finger nucleases. *PLoS One* 2010; **5**: e8870 [PMID: 20111598 DOI: 10.1371/journal.pone.0008870]
9 **Buehr M**, Meek S, Blair K, Yang J, Ure J, Silva J, McLay R, Hall J, Ying QL, Smith A. Capture of authentic embryonic stem cells from rat blastocysts. *Cell* 2008; **135**: 1287-1298 [PMID: 19109897 DOI: 10.1016/j.cell.2008.12.007]
10 **Li P**, Tong C, Mehrian-Shai R, Jia L, Wu N, Yan Y, Maxson RE, Schulze EN, Song H, Hsieh CL, Pera MF, Ying QL. Germline competent embryonic stem cells derived from rat blastocysts. *Cell* 2008; **135**: 1299-1310 [PMID: 19109898 DOI: 10.1016/j.cell.2008.12.006]
11 **Tong C**, Li P, Wu NL, Yan Y, Ying QL. Production of p53 gene knockout rats by homologous recombination in embryonic stem cells. *Nature* 2010; **467**: 211-213 [PMID: 20703227 DOI: 10.1038/nature09368]
12 **Pera MF**, Reubinoff B, Trounson A. Human embryonic stem cells. *J Cell Sci* 2000; **113** (Pt 1): 5-10 [PMID: 10591620]
13 **Iannaccone PM**. Rat pluripotent embryonic stem cells and method of obtaining and using same. International Application No.: PCT/US1994/009787
14 **Takahama Y**, Ochiya T, Sasaki H, Baba-Toriyama H, Konishi H, Nakano H, Terada M. Molecular cloning and functional analysis of cDNA encoding a rat leukemia inhibitory factor: towards generation of pluripotent rat embryonic stem cells. *Oncogene* 1998; **16**: 3189-3196 [PMID: 9671398 DOI: 10.1038/sj.onc.1201826]
15 **Vassilieva S**, Guan K, Pich U, Wobus AM. Establishment of SSEA-1- and Oct-4-expressing rat embryonic stem-like cell lines and effects of cytokines of the IL-6 family on clonal growth. *Exp Cell Res* 2000; **258**: 361-373 [PMID: 10896787 DOI: 10.1006/excr.2000.4940]
16 **Ying QL**, Wray J, Nichols J, Batlle-Morera L, Doble B, Woodgett J, Cohen P, Smith A. The ground state of embryonic stem cell self-renewal. *Nature* 2008; **453**: 519-523 [PMID: 18497825 DOI: 10.1038/nature06968]
17 **Ying QL**, Nichols J, Chambers I, Smith A. BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. *Cell* 2003; **115**: 281-292 [PMID: 14636556 DOI: 10.1016/S0092-8674(03)00847-X]
18 **Kunath T**, Saba-El-Leil MK, Almousailleakh M, Wray J, Meloche S, Smith A. FGF stimulation of the Erk1/2 signalling cascade triggers transition of pluripotent embryonic stem cells from self-renewal to lineage commitment. *Development* 2007; **134**: 2895-2902 [PMID: 17660198 DOI: 10.1242/dev.02880]
19 **Doble BW**, Woodgett JR. GSK-3: tricks of the trade for a multi-tasking kinase. *J Cell Sci* 2003; **116**: 1175-1186 [PMID: 12615961 DOI: 10.1242/jcs.00384]
20 **Jope RS**, Johnson GV. The glamour and gloom of glycogen synthase kinase-3. *Trends Biochem Sci* 2004; **29**: 95-102 [PMID: 15102436 DOI: 10.1016/j.tibs.2003.12.004]
21 **Sato N**, Meijer L, Skaltsounis L, Greengard P, Brivanlou AH. Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. *Nat Med* 2004; **10**: 55-63 [PMID: 14702635 DOI: 10.1038/nm979]
22 **Wu D**, Pan W. GSK3: a multifaceted kinase in Wnt signaling. *Trends Biochem Sci* 2010; **35**: 161-168 [PMID: 19884009 DOI: 10.1016/j.tibs.2009.10.002]
23 **Lyashenko N**, Winter M, Migliorini D, Biechele T, Moon RT, Hartmann C. Differential requirement for the dual functions of β-catenin in embryonic stem cell self-renewal and germ layer formation. *Nat Cell Biol* 2011; **13**: 753-761 [PMID: 21685890 DOI: 10.1038/ncb2260]
24 **Wray J**, Kalkan T, Gomez-Lopez S, Eckardt D, Cook A, Kemler R, Smith A. Inhibition of glycogen synthase kinase-3 alleviates Tcf3 repression of the pluripotency network and increases embryonic stem cell resistance to differentiation. *Nat Cell Biol* 2011; **13**: 838-845 [PMID: 21685889 DOI: 10.1038/ncb2267]
25 **Martello G**, Sugimoto T, Diamanti E, Joshi A, Hannah R, Ohtsuka S, Göttgens B, Niwa H, Smith A. Esrrb is a pivotal target of the Gsk3/Tcf3 axis regulating embryonic stem cell self-renewal. *Cell Stem Cell* 2012; **11**: 491-504 [PMID: 23040478 DOI: 10.1016/j.stem.2012.06.008]
26 **Kawamata M**, Ochiya T. Generation of genetically modified rats from embryonic stem cells. *Proc Natl Acad Sci USA* 2010; **107**: 14223-14228 [PMID: 20660726 DOI: 10.1073/pnas.1009582107]
27 **Hirabayashi M**, Kato M, Kobayashi T, Sanbo M, Yagi T, Hochi S, Nakauchi H. Establishment of rat embryonic stem cell lines that can participate in germline chimerae at high efficiency. *Mol Reprod Dev* 2010; **77**: 94 [PMID: 19899136 DOI: 10.1002/mrd.21123]
28 **Meek S**, Buehr M, Sutherland L, Thomson A, Mullins JJ, Smith AJ, Burdon T. Efficient gene targeting by homologous recombination in rat embryonic stem cells. *PLoS One* 2010; **5**: e14225 [PMID: 21151976 DOI: 10.1371/journal.pone.0014225]
29 **Hong J**, He H, Weiss ML. Derivation and characterization of embryonic stem cells lines derived from transgenic Fischer 344 and Dark Agouti rats. *Stem Cells Dev* 2012; **21**: 1571-1586 [PMID: 21995453 DOI: 10.1089/scd.2011.0370]
30 **Men H**, Bauer BA, Bryda EC. Germline transmission of a novel rat embryonic stem cell line derived from transgenic rats. *Stem Cells Dev* 2012; **21**: 2606-2612 [PMID: 22455749 DOI: 10.1089/scd.2012.0040]
31 **Men H**, Bryda EC. Derivation of a germline competent transgenic Fischer 344 embryonic stem cell line. *PLoS One* 2013; **8**: e56518 [PMID: 23437152 DOI: 10.1371/journal.pone.0056518]
32 **Li W**, Wei W, Zhu S, Zhu J, Shi Y, Lin T, Hao E, Hayek A, Deng H, Ding S. Generation of rat and human induced pluripotent stem cells by combining genetic reprogramming and chemical inhibitors. *Cell Stem Cell* 2009; **4**: 16-19 [PMID: 19097958 DOI: 10.1016/j.stem.2008.11.014]
33 **Hamanaka S**, Yamaguchi T, Kobayashi T, Kato-Itoh M, Yamazaki S, Sato H, Umino A, Wakiyama Y, Arai M, Sanbo M, Hirabayashi M, Nakauchi H. Generation of germline-competent rat induced pluripotent stem cells. *PLoS One* 2011; **6**: e22008 [PMID: 21789202 DOI: 10.1371/journal.pone.0022008]
34 **Ishizaki T**, Uehata M, Tamechika I, Keel J, Nonomura K, Maekawa M, Narumiya S. Pharmacological properties of Y-27632, a specific inhibitor of rho-associated kinases. *Mol Pharmacol* 2000; **57**: 976-983 [PMID: 10779382]
35 **Watanabe K**, Ueno M, Kamiya D, Nishiyama A, Matsumura M, Wataya T, Takahashi JB, Nishikawa S, Nishikawa S, Muguruma K, Sasai Y. A ROCK inhibitor permits survival of dissociated human embryonic stem cells. *Nat Biotechnol* 2007; **25**: 681-686 [PMID: 17529971 DOI: 10.1038/nbt1310]
36 **Li T**, Zhao XY, Teng F, Li X, Jiang M, Li W, Wang X, Wang J, Liu L, Liu Z, Wang L, Zhou Q. Derivation of germline competent rat embryonic stem cells from DA rats. *J Genet Genomics* 2012; **39**:

603-606 [PMID: 23177148 DOI: 10.1016/j.jgg.2012.06.006]
37 **Tong C**, Huang G, Ashton C, Li P, Ying QL. Generating gene knockout rats by homologous recombination in embryonic stem cells. *Nat Protoc* 2011; **6**: 827-844 [PMID: 21637202 DOI: 10.1038/nprot.2011.338]
38 **Hong J**, He H, Bui P, Ryba-White B, Rumi MA, Soares MJ, Dutta D, Paul S, Kawamata M, Ochiya T, Ying QL, Rajanahalli P, Weiss ML. A focused microarray for screening rat embryonic stem cell lines. *Stem Cells Dev* 2013; **22**: 431-443 [PMID: 22889370 DOI: 10.1089/scd.2012.0279]
39 **Ralston A**, Rossant J. Genetic regulation of stem cell origins in the mouse embryo. *Clin Genet* 2005; **68**: 106-112 [PMID: 15996204 DOI: 10.1111/j.1399-0004.2005.00478.x]
40 **Sasaki H**. Mechanisms of trophectoderm fate specification in preimplantation mouse development. *Dev Growth Differ* 2010; **52**: 263-273 [PMID: 20100249 DOI: 10.1111/j.1440-169X.2009.01158.x]
41 **Chen Y**, Blair K, Smith A. Robust self-renewal of rat embryonic stem cells requires fine-tuning of glycogen synthase kinase-3 inhibition. *Stem Cell Reports* 2013; **1**: 209-217 [PMID: 24319657 DOI: 10.1016/j.stemcr.2013.07.003]
42 **Blair K**, Leitch HG, Mansfield W, Dumeau CÉ, Humphreys P, Smith AG. Culture parameters for stable expansion, genetic modification and germline transmission of rat pluripotent stem cells. *Biol Open* 2012; **1**: 58-65 [PMID: 23213369 DOI: 10.1242/bio.2011029]
43 **Liu X**, Wu H, Loring J, Hormuzdi S, Disteche CM, Bornstein P, Jaenisch R. Trisomy eight in ES cells is a common potential problem in gene targeting and interferes with germ line transmission. *Dev Dyn* 1997; **209**: 85-91 [PMID: 9142498]
44 **Cao N**, Liao J, Liu Z, Zhu W, Wang J, Liu L, Yu L, Xu P, Cui C, Xiao L, Yang HT. In vitro differentiation of rat embryonic stem cells into functional cardiomyocytes. *Cell Res* 2011; **21**: 1316-1331 [PMID: 21423272 DOI: 10.1038/cr.2011.48]
45 **Boheler KR**, Czyz J, Tweedie D, Yang HT, Anisimov SV, Wobus AM. Differentiation of pluripotent embryonic stem cells into cardiomyocytes. *Circ Res* 2002; **91**: 189-201 [PMID: 12169644 DOI: 10.1161/01.RES.0000027865.61704.32]
46 **Peng X**, Gao H, Wang Y, Yang B, Liu T, Sun Y, Jin H, Jiang L, Li L, Wu M, Qian Q. Conversion of rat embryonic stem cells into neural precursors in chemical-defined medium. *Biochem Biophys Res Commun* 2013; **431**: 783-787 [PMID: 23321306 DOI: 10.1016/j.bbrc.2013.01.023]
47 **Koyanagi M**, Takahashi J, Arakawa Y, Doi D, Fukuda H, Hayashi H, Narumiya S, Hashimoto N. Inhibition of the Rho/ROCK pathway reduces apoptosis during transplantation of embryonic stem cell-derived neural precursors. *J Neurosci Res* 2008; **86**: 270-280 [PMID: 17828770 DOI: 10.1002/jnr.21502]
48 **Yamamoto S**, Nakata M, Sasada R, Ooshima Y, Yano T, Shinozawa T, Tsukimi Y, Takeyama M, Matsumoto Y, Hashimoto T. Derivation of rat embryonic stem cells and generation of protease-activated receptor-2 knockout rats. *Transgenic Res* 2012; **21**: 743-755 [PMID: 22002084 DOI: 10.1007/s11248-011-9564-0]
49 **Bahnson AB**, Boggs SS. Addition of serum to electroporated cells enhances survival and transfection efficiency. *Biochem Biophys Res Commun* 1990; **171**: 752-757 [PMID: 2403361 DOI: 10.1016/0006-291X(90)91210-J]
50 **Hirabayashi M**, Kato M, Sanbo M, Kobayashi T, Hochi S, Nakauchi H. Rat transgenesis via embryonic stem cells electroporated with the Kusabira-orange gene. *Mol Reprod Dev* 2010; **77**: 474 [PMID: 20422708 DOI: 10.1002/mrd.21181]
51 **Li P**, Schulze EN, Tong C, Ying QL. Rat embryonic stem cell derivation and propagation. Advanced protocols for animal transgenesis (Springer Protocols Handbooks). Berlin: Springer, 2011: 457-475
52 **Kawamata M**, Ochiya T. Two distinct knockout approaches highlight a critical role for p53 in rat development. *Sci Rep* 2012; **2**: 945 [PMID: 23230510 DOI: 10.1038/srep00945]
53 **Huang G**, Tong C, Kumbhani DS, Ashton C, Yan H, Ying QL. Beyond knockout rats: new insights into finer genome manipulation in rats. *Cell Cycle* 2011; **10**: 1059-1066 [PMID: 21383544 DOI: 10.4161/cc.10.7.15233]
54 **Armstrong JF**, Kaufman MH, Harrison DJ, Clarke AR. High-frequency developmental abnormalities in p53-deficient mice. *Curr Biol* 1995; **5**: 931-936 [PMID: 7583151 DOI: 10.1016/S0960-9822(95)00183-7]
55 **Copp AJ**, Brook FA, Estibeiro JP, Shum AS, Cockroft DL. The embryonic development of mammalian neural tube defects. *Prog Neurobiol* 1990; **35**: 363-403 [PMID: 2263736 DOI: 10.1016/0301-0082(90)90037-H]
56 **Chen X**, Watkins R, Delot E, Reliene R, Schiestl RH, Burgoyne PS, Arnold AP. Sex difference in neural tube defects in p53-null mice is caused by differences in the complement of X not Y genes. *Dev Neurobiol* 2008; **68**: 265-273 [PMID: 18004765 DOI: 10.1002/dneu.20581]
57 **von Hörsten S**, Schmitt I, Nguyen HP, Holzmann C, Schmidt T, Walther T, Bader M, Pabst R, Kobbe P, Krotova J, Stiller D, Kask A, Vaarmann A, Rathke-Hartlieb S, Schulz JB, Grasshoff U, Bauer I, Vieira-Saecker AM, Paul M, Jones L, Lindenberg KS, Landwehrmeyer B, Bauer A, Li XJ, Riess O. Transgenic rat model of Huntington's disease. *Hum Mol Genet* 2003; **12**: 617-624 [PMID: 12620967 DOI: 10.1093/hmg/ddg075]
58 **Cohen RM**, Rezai-Zadeh K, Weitz TM, Rentsendorj A, Gate D, Spivak I, Bholat Y, Vasilevko V, Glabe CG, Breunig JJ, Rakic P, Davtyan H, Agadjanyan MG, Kepe V, Barrio JR, Bannykh S, Szekely CA, Pechnick RN, Town T. A transgenic Alzheimer rat with plaques, tau pathology, behavioral impairment, oligomeric aβ, and frank neuronal loss. *J Neurosci* 2013; **33**: 6245-6256 [PMID: 23575824 DOI: 10.1523/JNEUROSCI.3672-12.2013]
59 **Hamilton SM**, Green JR, Veeraragavan S, Yuva L, McCoy A, Wu Y, Warren J, Little L, Ji D, Cui X, Weinstein E, Paylor R. Fmr1 and Nlgn3 knockout rats: novel tools for investigating autism spectrum disorders. *Behav Neurosci* 2014; **128**: 103-109 [PMID: 24773431 DOI: 10.1037/a0035988]
60 **Brown AJ**, Fisher DA, Kouranova E, McCoy A, Forbes K, Wu Y, Henry R, Ji D, Chambers A, Warren J, Shu W, Weinstein EJ, Cui X. Whole-rat conditional gene knockout via genome editing. *Nat Methods* 2013; **10**: 638-640 [PMID: 23749298 DOI: 10.1038/nmeth.2516]
61 **Friedel RH**, Wurst W, Wefers B, Kühn R. Generating conditional knockout mice. *Methods Mol Biol* 2011; **693**: 205-231 [PMID: 21080282 DOI: 10.1007/978-1-60761-974-1_12]
62 **Sauer B**. Functional expression of the cre-lox site-specific recombination system in the yeast Saccharomyces cerevisiae. *Mol Cell Biol* 1987; **7**: 2087-2096 [PMID: 3037344 DOI: 10.1128/MCB.7.6.2087]
63 **DelRaso NJ**. In vitro methodologies for enhanced toxicity testing. *Toxicol Lett* 1993; **68**: 91-99 [PMID: 8516778 DOI: 10.1016/0378-4274(93)90122-E]
64 **LeCluyse EL**, Bullock PL, Parkinson A. Strategies for restoration and maintenance of normal hepatic structure and function in long-term cultures of rat hepatocytes. *Adv Drug Deliver Rev* 1996; **22**: 133-186 [DOI: 10.1016/S0169-409X(96)00418-8]
65 **Soldatow VY**, Lecluyse EL, Griffith LG, Rusyn I. In vitro models for liver toxicity testing. *Toxicol Res* (Camb) 2013; **2**: 23-39 [PMID: 23495363 DOI: 10.1039/C2TX20051A]
66 **Tesson L**, Usal C, Ménoret S, Leung E, Niles BJ, Remy S, Santiago Y, Vincent AI, Meng X, Zhang L, Gregory PD, Anegon I, Cost GJ. Knockout rats generated by embryo microinjection of TALENs. *Nat Biotechnol* 2011; **29**: 695-696 [PMID: 21822240 DOI: 10.1038/nbt.1940]
67 **Ferguson C**, McKay M, Harris RA, Homanics GE. Toll-like receptor 4 (Tlr4) knockout rats produced by transcriptional activator-like effector nuclease (TALEN)-mediated gene inactivation. *Alcohol* 2013; **47**: 595-599 [PMID: 24199847 DOI: 10.1016/j.alcohol.2013.09.043]
68 **Shan Q**, Wang Y, Li J, Zhang Y, Chen K, Liang Z, Zhang K, Liu J, Xi JJ, Qiu JL, Gao C. Targeted genome modification of crop plants using a CRISPR-Cas system. *Nat Biotechnol* 2013; **31**: 686-688 [PMID: 23929338 DOI: 10.1038/nbt.2650]
69 **Ménoret S**, Fontanière S, Jantz D, Tesson L, Thinard R, Rémy S, Usal C, Ouisse LH, Fraichard A, Anegon I. Generation of Rag1-knockout immunodeficient rats and mice using engineered meganucleases. *FASEB J* 2013; **27**: 703-711 [PMID: 23150522

DOI: 10.1096/fj.12-219907]
70 **Ménoret S**, Iscache AL, Tesson L, Rémy S, Usal C, Osborn MJ, Cost GJ, Brüggemann M, Buelow R, Anegon I. Characterization of immunoglobulin heavy chain knockout rats. *Eur J Immunol* 2010; **40**: 2932-2941 [PMID: 21038471 DOI: 10.1002/eji.201040939]
71 **Zschemisch NH**, Glage S, Wedekind D, Weinstein EJ, Cui X, Dorsch M, Hedrich HJ. Zinc-finger nuclease mediated disruption of Rag1 in the LEW/Ztm rat. *BMC Immunol* 2012; **13**: 60 [PMID: 23136839 DOI: 10.1186/1471-2172-13-60]
72 **Moreno C**, Hoffman M, Stodola TJ, Didier DN, Lazar J, Geurts AM, North PE, Jacob HJ, Greene AS. Creation and characterization of a renin knockout rat. *Hypertension* 2011; **57**: 614-619 [PMID: 21242461 DOI: 10.1161/HYPERTENSIONAHA.110.163840]
73 **Chu X**, Zhang Z, Yabut J, Horwitz S, Levorse J, Li XQ, Zhu L, Lederman H, Ortiga R, Strauss J, Li X, Owens KA, Dragovic J, Vogt T, Evers R, Shin MK. Characterization of multidrug resistance 1a/P-glycoprotein knockout rats generated by zinc finger nucleases. *Mol Pharmacol* 2012; **81**: 220-227 [PMID: 22049154 DOI: 10.1124/mol.111.074179]
74 **Vaira S**, Yang C, McCoy A, Keys K, Xue S, Weinstein EJ, Novack DV, Cui X. Creation and preliminary characterization of a leptin knockout rat. *Endocrinology* 2012; **153**: 5622-5628 [PMID: 22948215 DOI: 10.1210/en.2012-1462]
75 **Gafni O**, Weinberger L, Mansour AA, Manor YS, Chomsky E, Ben-Yosef D, Kalma Y, Viukov S, Maza I, Zviran A, Rais Y, Shipony Z, Mukamel Z, Krupalnik V, Zerbib M, Geula S, Caspi I, Schneir D, Shwartz T, Gilad S, Amann-Zalcenstein D, Benjamin S, Amit I, Tanay A, Massarwa R, Novershtern N, Hanna JH. Derivation of novel human ground state naive pluripotent stem cells. *Nature* 2013; **504**: 282-286 [PMID: 24172903 DOI: 10.1038/nature12745]

P- Reviewer: Feng ZL, Sidhu KS **S- Editor**: Gong XM
L- Editor: A **E- Editor**: Wu HL

WJSC World Journal of Stem Cells

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.4252/wjsc.v7.i7.1064

World J Stem Cells 2015 August 26; 7(7): 1064-1077
ISSN 1948-0210 (online)
© 2015 Baishideng Publishing Group Inc. All rights reserved.

ORIGINAL ARTICLE

Basic Study

Simplified three-dimensional culture system for long-term expansion of embryonic stem cells

Christina McKee, Mick Perez-Cruet, Ferman Chavez, G Rasul Chaudhry

Christina McKee, Mick Perez-Cruet, Ferman Chavez, G Rasul Chaudhry, OU-WB Institute for Stem Cell and Regenerative Medicine, Oakland University, Rochester, MI 48309, United States

Christina McKee, G Rasul Chaudhry, Department of Biological Sciences, Oakland University, Rochester, MI 48309, United States

Mick Perez-Cruet, Beaumont Health System, Royal Oak, MI 48073, United States

Ferman Chavez, Department of Chemistry, Oakland University, Rochester, MI 48309, United States

Author contributions: McKee C preformed the majority of experiments, and helped write the manuscript; Perez-Cruet M and Chavez F provided resources; Chavez F synthesized scaffold material; Perez-Cruet M and Chavez F were involved in the editing of the manuscript; Chaudhry GR designed and guided the study, and helped write the manuscript.

Supported by Oakland University and Oakland University-William Beaumont Institute for Stem Cell and Regenerative Medicine (OU-WB ISCRM).

Institutional review board statement: This research is conducted with cell lines purchased from ATCC and has been approved by the Oakland University Institutional Biosafety Committee (IBC protocol number: 1814).

Institutional animal care and use committee: All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of Oakland University (IACUC protocol number: 14033).

Conflict-of-interest statement: None.

Data sharing statement: None.

Open-Access: This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: http://creativecommons.org/licenses/by-nc/4.0/

Correspondence to: Dr. G Rasul Chaudhry, Department of Biological Sciences, Oakland University, 2200 North Squirrel Road, Rochester, MI 48309,
United States. chaudhry@oakland.edu
Telephone: +1-248-3703350
Fax: +1-248-3703586

Received: February 21, 2015
Peer-review started: February 22, 2015
First decision: April 27, 2015
Revised: May 27, 2015
Accepted: June 18, 2015
Article in press: June 19, 2015
Published online: August 26, 2015

Abstract

AIM: To devise a simplified and efficient method for long-term culture and maintenance of embryonic stem cells requiring less frequent passaging.

METHODS: Mouse embryonic stem cells (ESCs) labeled with enhanced yellow fluorescent protein were cultured in three-dimensional (3-D) self-assembling scaffolds and compared with traditional two-dimentional (2-D) culture techniques requiring mouse embryonic fibroblast feeder layers or leukemia inhibitory factor. 3-D scaffolds encapsulating ESCs were prepared by mixing ESCs with polyethylene glycol tetra-acrylate (PEG-4-Acr) and thiol-functionalized dextran (Dex-SH). Distribution of ESCs in 3-D was monitored by confocal microscopy. Viability and proliferation of encapsulated cells during long-term culture were determined by propidium iodide as well as direct cell counts and PrestoBlue (PB) assays. Genetic expression of pluripotency markers (Oct4, Nanog, Klf4, and Sox2) in ESCs grown under 2-D and 3-D culture

conditions was examined by quantitative real-time polymerase chain reaction. Protein expression of selected stemness markers was determined by two different methods, immunofluorescence staining (Oct4 and Nanog) and western blot analysis (Oct4, Nanog, and Klf4). Pluripotency of 3-D scaffold grown ESCs was analyzed by *in vivo* teratoma assay and *in vitro* differentiation *via* embryoid bodies into cells of all three germ layers.

RESULTS: Self-assembling scaffolds encapsulating ESCs for 3-D culture without the loss of cell viability were prepared by mixing PEG-4-Acr and Dex-SH (1:1 v/v) to a final concentration of 5% (w/v). Scaffold integrity was dependent on the degree of thiol substitution of Dex-SH and cell concentration. Scaffolds prepared using Dex-SH with 7.5% and 33% thiol substitution and incubated in culture medium maintained their integrity for 11 and 13 d without cells and 22 ± 5 d and 37 ± 5 d with cells, respectively. ESCs formed compact colonies, which progressively increased in size over time due to cell proliferation as determined by confocal microscopy and PB staining. 3-D scaffold cultured ESCs expressed significantly higher levels ($P < 0.01$) of Oct4, Nanog, and Kl4, showing a 2.8, 3.0 and 1.8 fold increase, respectively, in comparison to 2-D grown cells. A similar increase in the protein expression levels of Oct4, Nanog, and Klf4 was observed in 3-D grown ESCs. However, when 3-D cultured ESCs were subsequently passaged in 2-D culture conditions, the level of these pluripotent markers was reduced to normal levels. 3-D grown ESCs produced teratomas and yielded cells of all three germ layers, expressing brachyury (mesoderm), NCAM (ectoderm), and GATA4 (endoderm) markers. Furthermore, these cells differentiated into osteogenic, chondrogenic, myogenic, and neural lineages expressing Col1, Col2, Myog, and Nestin, respectively.

CONCLUSION: This novel 3-D culture system demonstrated long-term maintenance of mouse ESCs without the routine passaging and manipulation necessary for traditional 2-D cell propagation.

Key words: Three-dimensional culture; Pluripotency; Embryonic stem cells; Self-assembling scaffold; Hydrogel

© The Author(s) 2015. Published by Baishideng Publishing Group Inc. All rights reserved.

Core tip: The pluripotent nature of embryonic stem cells (ESCs) makes them an ideal source for cell-based therapeutics and regenerative medicine. Efficient and reproducible expansion of ESCs *ex vivo* is critical for high quality cells for translational applications. However, propagation of ESCs is technically challenging, and often leads to differentiation due to inefficient two-dimensional culture techniques *in vitro*. To mimic the three-dimensional microenvironment *in vivo*, self-assembling scaffolds made from thiol-functionalized dextran and polyethylene glycol tetra-acrylate were designed to encapsulate and propagate mouse ESCs. This culture system is simple, robust, efficient and reproducible, permitting long-term maintenance of ESCs without routine passaging and manipulation.

McKee C, Perez-Cruet M, Chavez F, Chaudhry GR. Simplified three-dimensional culture system for long-term expansion of embryonic stem cells. *World J Stem Cells* 2015; 7(7): 1064-1077 Available from: URL: http://www.wjgnet.com/1948-0210/full/v7/i7/1064.htm DOI: http://dx.doi.org/10.4252/wjsc.v7.i7.1064

INTRODUCTION

The pluripotent state of embryonic stem cells (ESCs) allows their use in a wide array of translational and clinical applications[1]. Mouse ESCs have been used to investigate developmental and diseases processes, toxicology, cell-based therapeutics and regenerative medicine[2,3]. Research performed using animal models[4,5] and more recently human clinical trials[6,7] have shown promising potential for ESCs in cell therapies, repair of damaged tissues and organs, and *in vitro* disease modeling. However, these applications require routine and efficient expansion of pluripotent ESCs and controlled differentiation to obtain a homogenous population of cells. The pluripotency of ESCs is controlled by an intrinsic regulatory network[8] and extrinsic factors including the microenvironment, organization and composition of the extracellular matrix (ECM), cell-cell signaling, and the temporal and spatial gradient of soluble factors[9-12]. The complex relationship between stem cell fate and their native microenvironment results in a large discrepancy between *in vivo* and *in vitro* culture conditions effecting the quality of cultured cells[13].

Conventionally, ESCs are grown in two-dimensional (2-D) plastic culture plates on mouse embryonic fibroblast (MEF) feeder layers or ECM components (such as gelatin and Matrigel)[14]. Mouse ESCs can be maintained in their pluripotent state by the addition of soluble cytokines, such as leukemia inhibitory factor (LIF), to the culture media[11,15]. However, reliance on MEF feeder layer, cytokines, and/or growth factors complicates maintenance of ESCs due to the potential transmission of xenogeneic pathogens and the fluctuation of lot-to-lot quality[9]. Furthermore, the distribution of soluble factors in 2-D culture lacks the spatial gradient observed in three-dimensional (3-D) microenvironments, which can alter cell growth and fate determination[16]. Studies have shown that the ECM composition and organization send mechanical signals for cell differentiation and the culture of ESCs in 2-D culture can signal differentiation into specific cell lineages[17]. For these reasons, the maintenance of the self-renewing state of pluripotent ESCs and induced-pluripotent stem cells remains a challenge[18]. In addition to strict culture media and growth conditions, ESCs require regular passaging (every 2 to 3 d).

Consequently, culturing of ESCs is laborious, expensive and requires a high level of expertise[19].

In order to overcome the problems associated with 2-D culture, we hypothesized that 3-D culture may better mimic the *in vivo* environment supporting the growth and maintenance of ESC pluripotency. 3-D growth of ESCs can be facilitated by hydrogel scaffolds, composed of hydrophilic polymer networks, which emulate the fully hydrated native ECM and natural soft tissue[20]. Hydrogel constructs incorporating drugs, cytokines, and growth factors have been shown to promote proliferation, directed differentiation, and integration of cells to regenerate target tissue[21-24]. Recently, ESCs were cultured in 3-D hydrogel scaffolds but required routine passaging, much like 2-D cultures[19,25].

Studies have utilized dextran-based hydrogels to promote neovascularization and differentiation of ESCs into endothelial cells[24,26]. Whereas, thiol-functionalized dextran (Dex-SH) was combined with PEG functionalized with tetra-acrylate (PEG-4-Acr) to form chemically cross-linked hydrogels by a Michael-type addition for differentiation of chondrogenic progenitors[27]. In this report, we developed a 3-D culture system for propagation and maintenance of mouse ESCs utilizing Dex-SH and PEG-4-Acr. Cells grown in the 3-D scaffolds proliferated for extended periods of time, and exhibited ESC characteristics including self-renewal and pluripotency. Interestingly, ESCs grown in 3-D scaffolds had upregulated expression of pluripotency genes. This novel culture system is efficient, reproducible and less cumbersome for long-term maintenance of ESCs without the routine passaging and manipulations associated with 2-D culture. These studies should help facilitate development of methods for expansion of high quality and homogenous populations of human ESCs, which are critically important for regenerative medicine and therapeutic applications.

MATERIALS AND METHODS

Maintenance and growth of ESCs

The mouse ESC line 7AC5, labeled with enhanced yellow fluorescent protein (EYFP/GFP), (ATCC, Manassas, VA) was cultured on irradiated MEF feeder layer with ESC medium containing high glucose Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Aleken Biologicals, Nashville, TX), 0.1 mmol/L 2-mercaptoethanol (Sigma, St. Louis, MO), 0.1 mmol/L nonessential amino acids (Invitrogen), 1 mM sodium pyruvate (Sigma), and 1000 U/mL of leukemia inhibitory factor (LIF; Chemicon International, Temecula, CA) as previously described[15].

Preparation of self-assembling scaffolds

Dextran (25 kDa, M_W/M_N = 1.30, Sigma) was functionalized with pendant SH groups at differing degrees of thiol substitution ranging from 4% to 34%, using a published method[27]. Dex-SH was characterized by ^{1}HNMR spectroscopy using a 400 MHz Bruker Avance II spectrometer (Bruker, Billerica, MA).

Hydrogel scaffolds were formed by mixing Dex-SH with PEG-4-Acr (20 kDa, Creative PEGWorks Winston Salem, NC) *via* a Michael addition reaction, as previously described[22]. For this study, scaffolds were prepared with final polymer concentrations of 5% w/v. The molar ratio of thiol to acrylate groups used was 1:1.

Encapsulation of ESCs in self-assembling scaffolds

To encapsulate cells, Dex-SH and PEG-4-Acr were dissolved separately in culture medium and mixed with various concentrations (1×10^4 to 4×10^6 cells/mL) of ESCs, which were harvested at 70% confluency. Unless otherwise stated, ESCs were encapsulated at a concentration of 2×10^6 cells/mL. The resulting mixture was transferred to either a well of a 96-well plate or 1cc syringe for polymerization to produce fixed or floating scaffolds, respectively. Floating scaffolds were transferred from the syringes to 24-well plates. ESCs encapsulated in 3-D scaffolds were incubated the ESC medium and changed every 3 to 4 d or as needed. Cell growth was monitored by phase-contrast and confocal microscopy and analyzed by NIS Elements AR software (Nikon Instruments Inc., Melville, NY).

Swelling test

The degradation rate of floating scaffolds was determined by swelling tests performed under physiological conditions (37 ℃). The initial dry weight (W_i) of the floating scaffolds was measured before incubation in ESC medium. At regular intervals, the scaffolds were removed from the medium to record the swollen weight (W_s) for analysis. The swelling ratio was defined as the difference between W_s and W_i divided by W_i[28,29]. The degradation time was determined by the time required to completely dissolve the hydrogel scaffolds of each condition prepared in triplicate.

Cell viability and proliferation assays

Cell viability was determined qualitatively by propidium iodide (PI, 1 mg/mL) (Fisher Scientific, Pittsburgh, PA) staining in triplicate experiments, and was visualized using fluorescent microscopy.

The quantitative analysis of cell growth in the scaffolds was determined by direct microscopic count using hemocytometer and PrestoBlue (PB) assays (Invitrogen), following the manufacturer's instructions. The scaffolds were incubated in PB solution for 4 h, before measuring the absorption of the solution at 570 nm and normalized to the reference wavelength of 600 nm using the Epoch microplate reader (BioTek, Winooski, VT). PB, a resazurin-based solution, was reduced proportional to the number of metabolically active cells to fluorescent resorufin.

Teratoma formation assay

For teratoma formation, ESCs were harvested following trypsin treatment, washed and re-suspended in PBS, and mixed with an equal volume of Matrigel (BD

Table 1 Mouse primer sets used in quantitative real time polymerase chain reaction

Target-gene	Forward (5'- 3')	Reverse (5'-3')	Product size (bp)
Oct4	TCTGTTCCCGTCACTGCTCT	TGTCTACCTCCCTTGCCTTG	96
Nanog	GCAAGCGGTGGCAGAAAAAC	GCAATGGATGCTGGGATACTCA	92
Klf4	AAGCCAAAGAGGGGAAGAAG	CAGTGGTAAGGTTTCTCGCC	146
Sox2	CGAACTGGAGAAGGGGAGAG	AAGCGTTAATTTGGATGGGA	165
Col1	GCAGGTTCACCTACTCTGTC	CTTGCCCCATTCATTTGTCT	62
Col2	ACCCCCAGGTGCTAATGG	AACACCTTTGGGACCATCTTT	76
Myog	CCTAAAGTGGAGATCCTGCG	GTGGGAGTTGCATTCACTGG	147
Nestin	AGACAGTGAGGCAGATGAG	CTCTCAGCTGTGGTGGTGAA	224
Brachyury (T)	CACACCACTGACGCACAC	GAGGCTATGAGGAGGCTTTG	132
FGF5	GCTCAATGATCAGAAGGAGGA	TCAGCTGGTCTTGAATGAGG	175
GATA4	GATGGGACGGGACACTACCTG	ACCTGCTGGCGTCTTAGATTT	309
Gapdh	GCACAGTCAAGGCCGAGAAT	GCCTTCTCCATGGTGGTGAA	151
β-Actin	AGCCATGTACGTAGCCATCC	CTCTCAGCTGTGGTGGTGAA	228

Biosciences, San Jose, CA)[30]. Cells (1×10^6) were subcutaneously injected (20 μL) using a Hamilton syringe into 4-wk-old male immune-compromised SCID (severe combined immunodeficient)-beige mice (Fox Chase SCID Beige, Charles River, Wilmington, MA). Animals were anesthetized by inhalation of isoflurane gas for the injection of cells, and monitored daily. All efforts were made to minimize discomfort. After teratoma formation (3 to 4 wk), the animals were humanely euthanized by CO_2 overdose. Teratoma tissue was explanted, and flash frozen in liquid nitrogen for isolation of RNA using the RNeasy Midi kit (Qiagen, Germantown, MD)[31]. Teratoma assays were performed in triplicate. All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of Oakland University (IACUC protocol number: 14033).

Differentiation of ESCs

To differentiate ESCs, embryoid bodies (EBs) were prepared by the hanging drop method[32]. EBs were cultured in ESC growth medium for differentiation into a myogenic phenotype. EBs treated with 10^{-7} mol/L trans-retinoic acid (RA) were cultured in β-glycerol phosphate (10 mmol/L; Sigma), and ascorbic acid (50 μg/mL; Sigma) for osteogenic differentiation[32]; TGF-β (10 ng/mL; Sigma), insulin (10 μg/mL; Sigma) and ascorbic acid (50 μg/mL; Sigma) for chondrogenic differentiation[15]; and neurobasal medium (Invitrogen) supplemented with B-27 (10 μL/mL; Invitrogen), L-glutamine (0.5 mmol/L; Sigma), penicillin/streptomycin (1 μL/mL; Sigma) and bFGF (5 ng/mL) for neural differentiation[33]. Cell morphology was monitored by light microscopy on a daily basis. Osteogenic cells were analyzed for calcium deposition by von Kossa staining[32]. Proteoglycans produced by chondrogenic derivatives were visualized by alcian blue staining[15]. Analysis of lineage specific markers was performed as described below.

Expression of genetic markers

Gene expression studies were performed using quantitative real time polymerase chain reaction (qRT-PCR). RNA was isolated from cells using the RNeasy Mini kit (Qiagen). ESCs grown in 3-D scaffolds were flash frozen with liquid nitrogen, ground into a fine powder using a mortar and pestle, and homogenized using the QIAshredder column (Qiagen)[34]. RNA was purified by treating with RNase-free DNase (Promega, Madison, WI) and cDNA was synthesized with the iScript kit (BioRad, Hercules, CA). PCR reactions were performed in a 10 μL reaction volume using the BioRad CFX90 Real-Time PCR system and SsoAdvanced SYBR Green Supermix. The specific PCR conditions used were as follows: polymerase activation 3 min at 95 ℃, 40 cycles of denaturation, 15 s at 95 ℃; annealing, 20 s at 60 ℃; and melt curve, 5 s/step at 60 ℃-95 ℃. The markers used in this study represent pluripotency, all three germ layers, as well as osteogenic, chondral, myogenic, and neural cell lineages. Primers (IDT Technologies, Coralville, IA) are listed in the supplemental material (Table 1). All reactions were prepared in triplicate and normalized to reference genes, Gapdh and β-Actin.

Immunofluorescence staining

Cells were fixed in 4% paraformaldehyde for 10 min, washed with PBS, permeabilized with 0.5% Triton X-100 (Sigma) for 10 min, and then blocked with 2% BSA (Sigma) for 1 h at room temperature. Fixed cells were treated with primary antibodies (1:100 diluted in blocking buffer), Oct4 (ab19857, Abcam Inc., Cambridge, MA), Nanog (sc-33760, Santa Cruz Biotechnology, Santa Cruz, CA), brachyury (sc-20109, Santa Cruz), NCAM (sc-10735, Santa Cruz), and GATA4 (sc-25310) overnight at 4 ℃. Primary antibody treated cells were washed, and then stained with 1:200 diluted secondary antibodies, anti-rabbit Alexa Fluor 568 (A-11011, Molecular Probes, Eugene, OR) or Cy3-labeled anti-mouse IgG (072-01-18-06, KPL, Gaithersburg, MD, United States). Nuclei were counterstained with 1 mg/mL of 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Molecular Probes). Images were acquired using confocal microscopy.

Western blot assay

Cells were lysed in RIPA buffer with protease inhibitors (1 mmol/L PMSF) (Fisher Scientific), centrifuged at

12000 rpm for 20 min at 4 ℃, and the supernatants were collected. Protein concentrations were determined by the Pierce 660 nm protein assay (Fisher Scientific,) using the NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE), with BSA as a standard. Equal amounts of protein (10 μg) of 2-D and 3-D scaffold grown cells were resolved on 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (BioRad). Membranes were incubated for 30 min at room temperature in blocking buffer [5% non-fat dry milk in PBS containing 0.1% Tween-20 (PBST)]. The blocked membranes were then probed with 1:200 diluted primary antibodies overnight at 4 ℃ against Oct4 (Abcam), Nanog (Santa Cruz), Klf4 (ab21949, Abcam) and β-Actin (sc-130656, Santa Cruz). After washing with PBST, membranes were incubated with 1:10000 diluted horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature (Santa Cruz). Proteins on the membranes were detected by using an ECL chemiluminescence kit (BioRad) and by exposing the membranes to X-ray film. Finally, protein bands were analyzed using ImageJ (NIH, Bethesda, MA), normalized to β-Actin and expressed in arbitrary densitometric units.

Statistical analysis

All quantitative data were expressed as mean ± SE. One-way ANOVA analysis was performed on natural log transformed data, and analyzed for unequal variances. Post hoc tests used for multiple comparisons include Games-Howell analysis for PB analysis as well as Tukey and Dunnett's test for relative gene expression in qRT-PCR studies. Results with a P value less than 0.05 were considered to be significant ($^aP < 0.05$ and $^bP < 0.01$). All analyses were performed using SPSS version 11.5 (SPSS Inc, United States). The statistical methods of this study were reviewed by Dr. Harvey Qu from the Department of Statistics at Oakland University.

RESULTS

Viability and growth of ESCs in the 3-D scaffolds

The viability of 3-D grown ESCs was not affected by encapsulation or the process of scaffold self-assembly. Cell growth increased over a period of 3 wk, as evident by the increase in the intensity as well as the number of EYFP/GFP expressing cells in fixed scaffolds (Figure 1A-B). PI staining of the scaffolds 1 d after culturing showed that a majority of the cells were viable cells (green) and contained only a small fraction (< 5%) of dead cells (yellow). Presence of dead cells could be due to damage resulting from trypsinization of ESCs used for encapsulation. When trypsinized ESCs were tested by trypan blue staining, a similar fraction of dead cells (about 10%) was observed, indicating that encapsulation did not cause cell death. However, it is interesting to note that unlike in 2-D culture, encapsulated dead cells did not lyse during 3-D culture. We speculate that the scaffold microenvironment limited proteolytic activity. In our preliminary studies, the colony size of viable cells was restricted due to the limited swelling of fixed scaffolds (Figure 1A and B). We reasoned that if the scaffolds were allowed to swell freely, ESC growth and colony size would increase. Indeed, when ESCs were cultured in the floating scaffolds, cell growth and colony size increased in a time dependent manner (Figure 1C and D). Growth of encapsulated cells in floating scaffolds was confirmed by direct cell counts as shown in Figure 1E. After a lag period, ESCs grew at a generation rate of 36 h. A similar growth pattern was observed using PB staining. The results depicted in Figure 1F showed a continuous and significant increase in proliferation at 2, 3 and 4 wk of culture following a period of acclimatization. The highest increase in cell growth was observed between week 3 and 4, following which, the scaffolds degraded rapidly. Evidently, cell growth and colony size increased concomitant with reduction in scaffold integrity. Conceivably, scaffolds that retain integrity beyond 4 wk would continue to support the undifferentiated growth of ESCs.

Table 2 Effects of thiol substitutions of Dex-SH on the rate of scaffold degradation

Degree of thiol substitution of Dex-SH	Time in degradation (d)
4%	10 ± 2
6%	13 ± 4
7.5%	22 ± 5
12%	28 ± 6
30%	35 ± 5
33%	37 ± 5
34%	37 ± 5

Effect of composition and cell concentration on the rate of degradation of scaffolds

The polymer concentration and MW of Dex-SH and PEG-4-Acr, as well as the degree of thiol substitution of Dex-SH influenced the polymerization and degradation rate of scaffolds (Table 2), similar to previous reports[23,27]. After preliminary evaluation, we selected Dex-SH (MW = 25 kDa) with 7.5% and 33% thiol substitution and PEG-4-Acr (MW = 20 kDa) at 5% w/v for further studies for propagating cells for various culture periods; these preparations yielded scaffolds that degraded without cells in 11 and 13 d and degraded with cells in 22 ± 5 d and 37 ± 5 d, respectively.

Scaffolds prepared using 7.5% thiol substitution of Dex-SH and various cell concentrations, ranging from 1 × 10^4 cells/mL to 10 × 10^6 cells/mL, exhibited different swelling profiles, although the initial swelling capacity of the scaffolds was similar. Scaffolds containing various cell concentrations (0, 2 × 10^6 cells/mL or 4 × 10^6 cells/mL) as shown in Figure 2A, exhibited comparable swelling ratios until day 9; after which, control scaffolds without cells degraded rapidly, followed by scaffolds prepared with 4 × 10^6 cells/mL and finally scaffolds with 2 × 10^6 cells/mL. Scaffolds with the higher number of cells (4

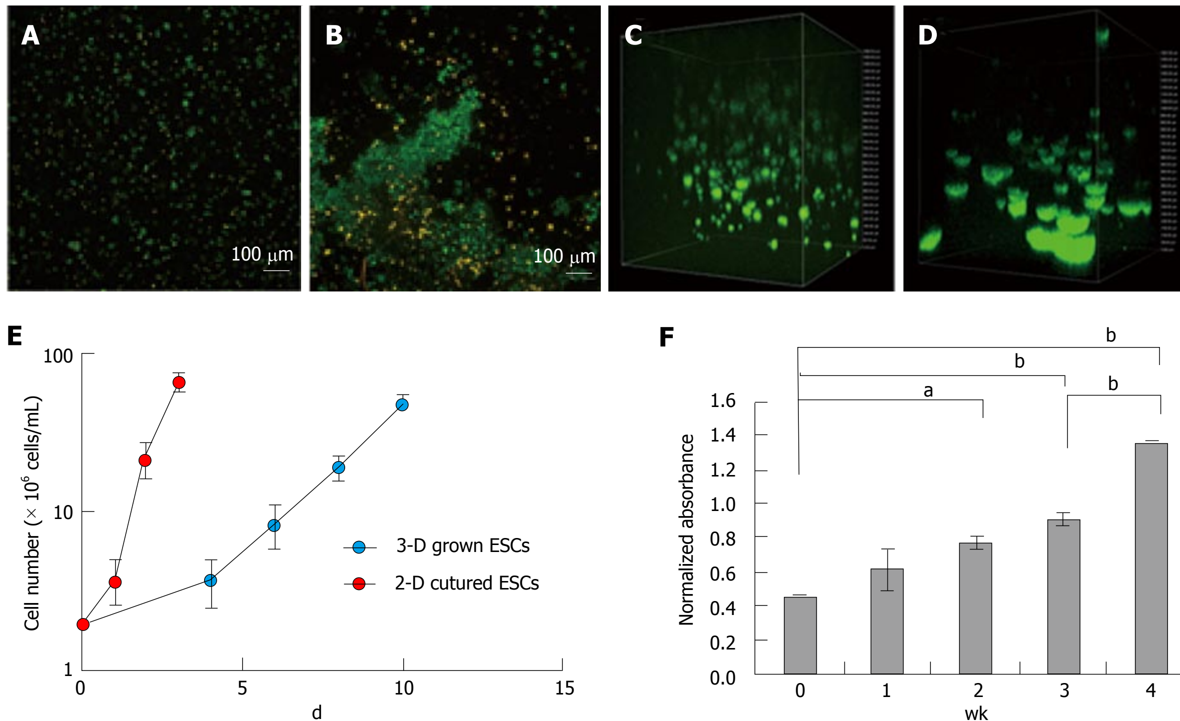


Figure 1 Cell viability and growth of embryonic stem cells encapsulated in self-assembling three-dimensional scaffolds. Enhanced yellow fluorescent protein-labeled ESCs were encapsulated (2 × 10^6 cells/mL) in fixed and floating scaffolds prepared using Dex-SH with 7.5% thiol substitution and PEG-4-Acr. Encapsulated cells were cultured in ESC medium and periodically cell growth was visualized using fluorescent microscopy. A and B: Live (green) and dead (yellow) cells in fixed scaffolds stained with PI at day 1 and week 3, respectively. While the viable cells increased with time, the number of dead cells remained constant. Scale bars = 100 μm; C and D: Three-dimensional (3-D) confocal images of floating scaffolds representing ESC growth at day 3 and week 3, respectively; E: Growth of ESCs encapsulated in 3-D scaffolds was assayed by direct count using hemocytometer. Cells seeded in 3-D scaffolds (at 2 × 10^6 cells/mL) were incubated in ESC medium and counted at various time intervals. Data presented as cell number (x 10^6 cells/mL) ± SE (n = 3) and plotted against the days of incubation; F: Quantitative determination of ESC proliferation in floating scaffolds at week 0, 1, 2, 3, and 4 of culture after staining with PB as analyzed by microplate reader. The results were expressed as the normalized absorbance ± SE (n = 3), with a significant increase in cell number, compared to initial ESC viability at time 0. $^aP < 0.05$ and $^bP < 0.01$ using one-way ANOVA with the non-equal variance hypothesis and Games-Howell multiple comparisons test. ESCs: Embryonic stem cells; Dex-SH: Thiol functionalized dextran; PEG-4-Acr: Polyethylene glycol tetra-acrylate.

Table 3 Cell concentration for optimal growth of embryonic stem cells under 2-D and 3-D culture conditions

Culture conditions	Cell concentration (cells/mL)	Cell concentration (cells/cm^2)
2-D Culture	---	3 × 10^4 to 5 × 10^4[33]
3-D Culture	1 × 10^4	1 × 10^3

3-D: Three-dimensional; 2-D: Two-dimensional.

× 10^6 cells/mL) swelled to 2-fold of their initial weight and started to degrade on day 19 of culture. Whereas, scaffolds with a lower number of cells (2 × 10^6 cells/mL) swelled to nearly 3 times their initial weight and started to degrade on day 27 of culture. Furthermore, scaffolds prepared with the lower cell concentration (2 × 10^6 cells/mL) resulted in rapid clonal growth (Figure 2B; B1, B2) as compared to the higher cell concentration (4 × 10^6 cells/mL) (Figure 2B; B3, B4). Quantitative analysis of colony size showed a 2-fold increase in the mean diameter of colonies in scaffolds prepared with lower numbers of cells (Figure 2C). The scaffold supported the growth of encapsulated ESCs at concentrations as low as 1 × 10^4 cells/mL (or 10^3/cm^2), suggesting that low concentration had no effect on the cell viability (Figure 2D). Maintenance of ESCs at low concentrations is more advantageous in 3-D culture compared to 2-D culture (Table 3), since low seeding densities in 2-D culture results in poor growth, decreased viability and differentiation of ESCs.

Expression of pluripotency markers in the 3-D scaffold cultured ESCs

To confirm whether 3-D grown ESCs maintained pluripotency, expression of selected ESC-specific markers was analyzed using qRT-PCR. The results depicted in Figure 3 indicate that expression of Oct4 and Nanog increased 2.8 and 3.0 fold, respectively in 3-D scaffold (prepared using 33% Dex-SH) grown ESCs as compared to cells grown under 2-D culture conditions. The expression of these markers significantly and successively increased in 3-D grown ESCs during 1, 2 and 3 wk. The expression of Klf4 also increased significantly (1.8 fold) above the level of 2-D grown ESCs but only during the second week of cell growth; whereas, the expression of Sox2 was not affected by 3-D culture. Concomitant with the start of degradation of the scaffolds at 6 wk of culture, expression of pluripotency markers gradually decreased to the levels of 2-D grown ESCs.

Immunofluorescence analysis of 3-D grown ESCs

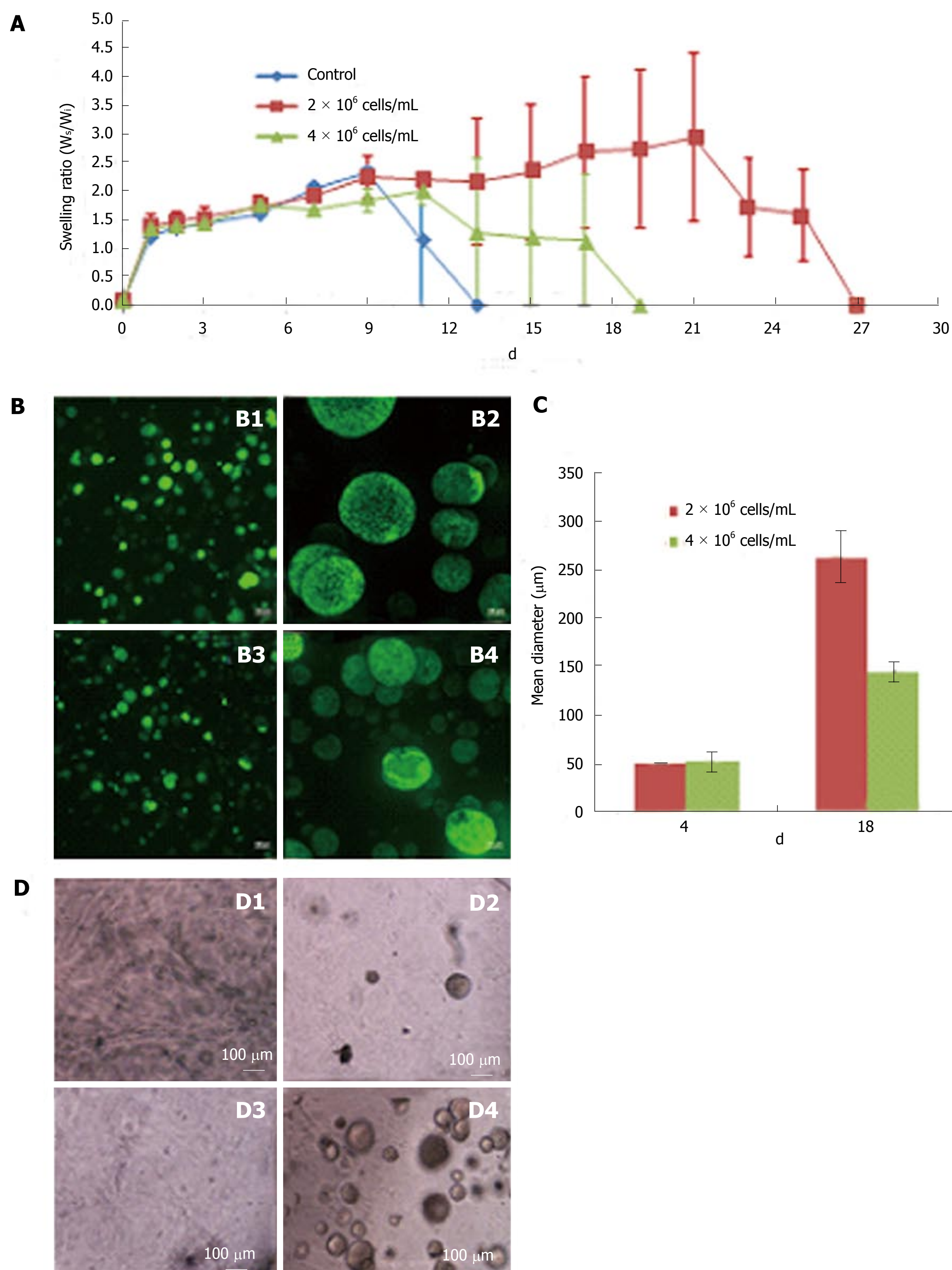


Figure 2 Effect of cell concentration on the rate of scaffold degradation and embryonic stem cells colony size. A: Swelling tests of floating scaffolds prepared with 7.5% thiol substitution were used to compare the rate of degradation of scaffolds prepared with various cell concentrations (0, 2 × 10^6 cells/mL and 4 × 10^6 cells/mL). Scaffolds with and without ESCs were incubated in ESC medium and weighed periodically. Data expressed as the mean swelling ratio (W_s/W_i) ± SE (n = 3); B: Representative composite confocal images of ESCs grown in floating scaffolds encapsulating 2 × 10^6 cells/mL (B1 and B2) and 4 × 10^6 cells/mL (B3 and B4) at day-4 and day-18, respectively. Scale bars = 100 μm; C: Quantification of ESC colony size in floating scaffolds encapsulating 2 × 10^6 cells/mL or 4 × 10^6 cells/mL at day-4 and day-18 of culture. Cell colony size was expressed as mean diameter (μm) ± SE (n = 3); D: Growth of low concentrations of ESCs in 3-D scaffolds. Light micrographs of the scaffolds encapsulating 1 × 10^4 cells/mL at day-0 and day-7 (D1 and D2, respectively) and 1 × 10^5 cells/mL at day-0 and day-7 (D3 and D4 respectively). Scale bars = 100 μm. ESCs: Embryonic stem cells; 3-D: Three-dimensional.

showed upregulation of protein levels of Oct4 and Nanog as evident by the increase in the fluorescent intensity of cells stained with the respective antibodies (Figure 4A). Furthermore, expression of these proteins

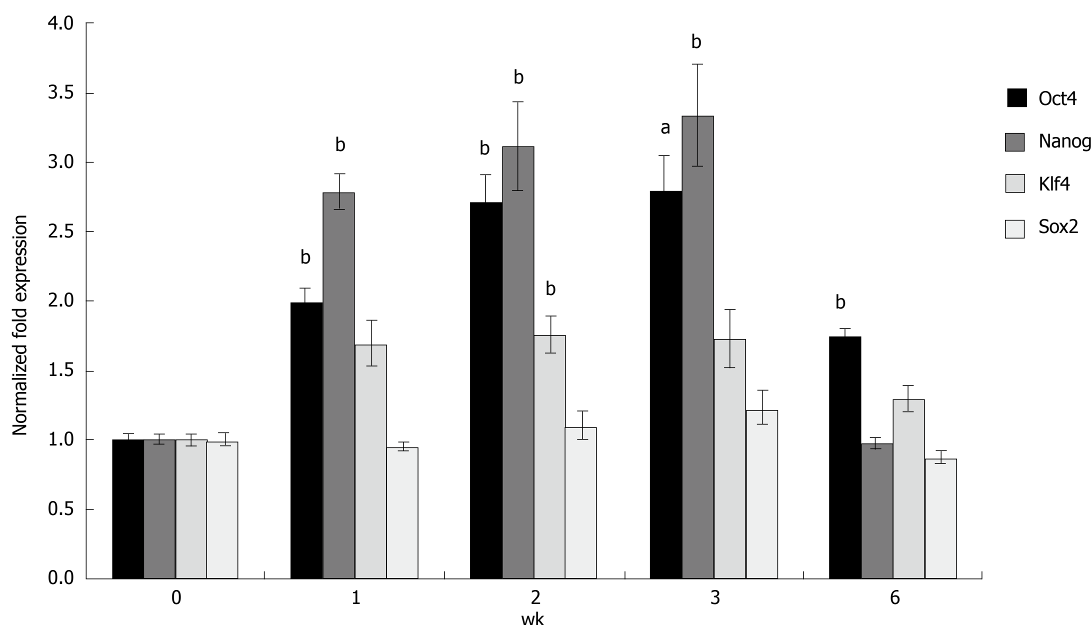


Figure 3 Expression of pluripotent markers in three-dimensional grown embryonic stem cells. ESCs in scaffolds prepared with Dex-SH with 33% thiol substitution were cultured and analyzed for the expression of selected markers, Oct4, Nanog, Klf4, and Sox2 using qRT-PCR at week 0, 1, 2, 3, and 6. Results of experiments were expressed as the fold expression ± SE (n = 3) normalized to reference genes Gapdh and β-Actin, where a significant (^aP < 0.05 and ^bP < 0.01) increase of pluripotent marker expression was compared to initial 2-D grown ESCs (day 0), using one-way ANOVA with the non-equal variance hypothesis and Dunnett's multiple comparisons test. Results depicted showed upregulation of Oct4, Nanog, and Klf4, but Sox2 was maintained at levels similar to the control. Expression of Oct4 and Nanog was successively and significantly higher at week 1, 2 and 3 until the onset of degradation (week 6), at which point their expression was decreased to initial levels. ESCs: Embryonic stem cells; Dex-SH: Thiol funtionalized dextran; qRT-PCR: Quantitative real time polymerase chain reaction; 2-D: Two-dimensional.

was analyzed by western blot and results are shown in Figure 4B. Similarly, levels of Oct4, Nanog and Klf4 were visibly increased in the 3-D grown cells when compared with the control. Quantitative analysis of the western blot confirmed the increase in Oct4, Nanog and Klf4 (1.9, 3.9 and 1.3 fold, respectively) compared to the control (Figure 4C); this was well correlated with the observed transcription levels of these genes.

Maintenance of pluripotency of 3-D grown ESCs was further investigated by subculturing under 2-D culture conditions (Figure 5A, A1-A3). They displayed typical compact colony morphology (even upon passaging five times) in 2-D culture and were indistinguishable from the initial ESCs seeded and grown in 3-D scaffolds. Expression of pluripotent genes in ESCs first grown in 3-D scaffolds for 2 wk and then subcultured under 2-D conditions were analyzed. The results depicted in Figure 5B showed that the expression of Oct4, Nanog, and Klf4 returned to levels comparable to traditionally 2-D propagated ESCs (Figure 5B).

Pluripotency of 3-D scaffold grown ESCs was further validated by *in vivo* teratoma assays using immune-compromised mice. The animals injected with 1×10^6 cells formed teratomas within 4 wk (Figure 6A and B). Analysis of explanted teratoma tissue showed expression of Brachyury, FGF5, and GATA4, suggesting differentiation of injected ESCs into cell lineages of all three germ layers (Figure 6C). Conceivably, the 3-D scaffold provided a microenvironment for upregulation of pluripotent genes, maintenance of pluripotency and self-renewal of ESCs.

Differentiation of the 3-D scaffolds grown ESCs into various cell lineages

Differentiation potential of the 3-D grown ESCs was also studied *via* EB formation. The EBs from 3-D grown ESCs were allowed to spontaneously differentiate into all three germ layers, mesoderm, ectoderm, and endoderm and expressed specific protein markers such as Brachyury, NCAM, and GATA4 (Figure 7A). Furthermore, 3-D grown ESCs were induced to differentiate into various cell lineages, including osteogenic, chondrogenic, myogenic, and neural cell types. The results of differentiation depicted osteogenic derivatives that had the cobblestone appearance of osteoblast cells and were positive for calcium deposition as determined by von Kossa staining (Figure 7B, B1). Chondrogenic derivatives of 3-D grown ESCs were analyzed by alcian blue staining showing positive proteoglycan production (Figure 7B, B2). Light micrographs of myogenic derivatives of 3-D grown ESCs had spindle shaped morphology (Figure 7B, B3), while neural derivatives of ESCs displayed neurofilaments (Figure 7B, B4). Further analysis of the 3-D grown ESC derivatives revealed expression of cell-specific markers (Figure 7C) as determined by qRT-PCR; osteogenic, chondrogenic, myogenic, and neural derivatives expressed Collagen type 1 (Col1), Collagen type 2 (Col2), Myogenin (Myog), and Nestin, respectively. The expression of these cell-specific markers signified that 3-D grown ESCs maintained differentiation potential.

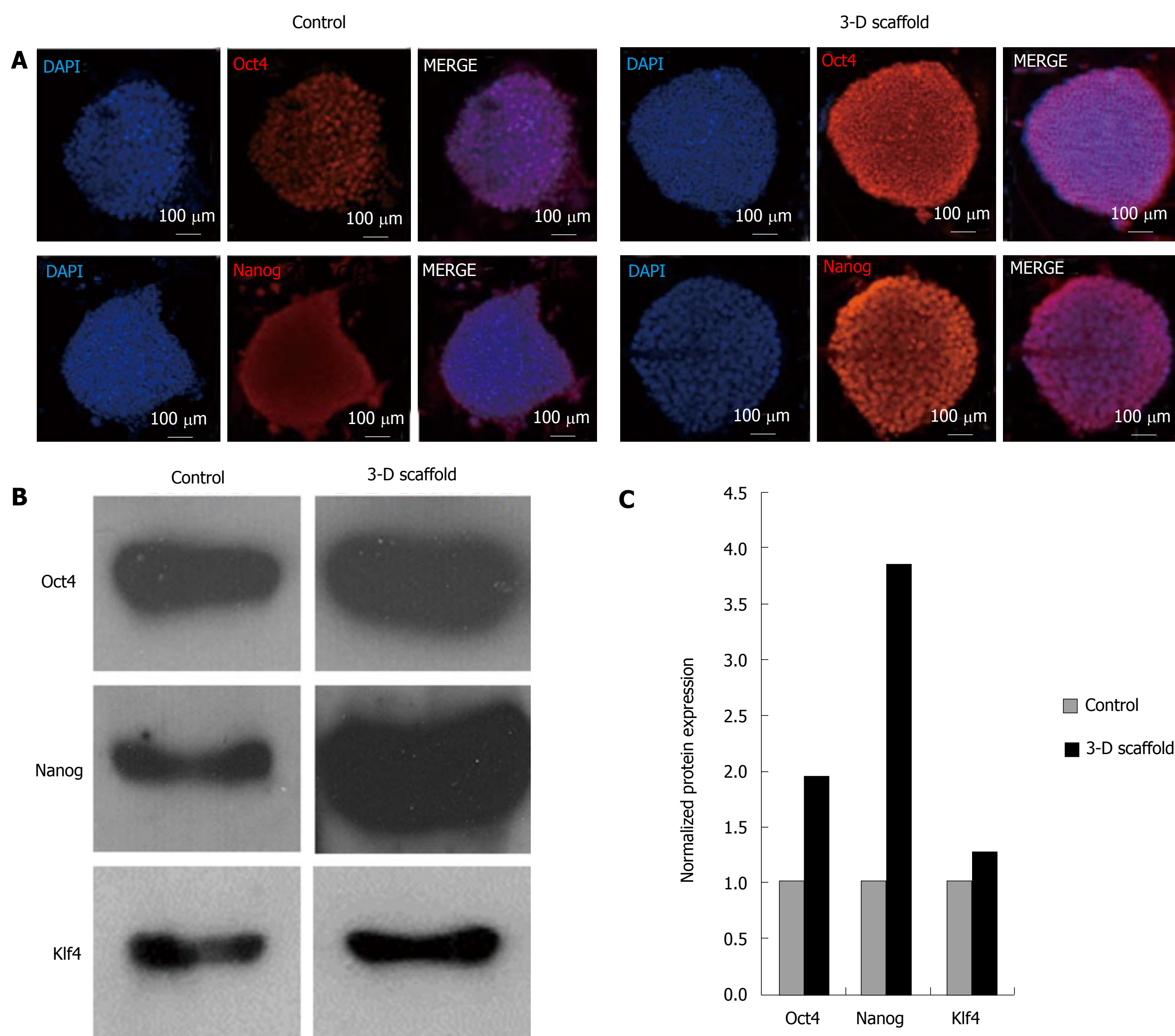


Figure 4 Protein expression of pluripotency markers in embryonic stem cells grown in two-dimensional and three-dimensional cultures. ESCs grown in two-dimensional (2-D) and in 3-D floating scaffolds (prepared with 33% thiol substitution) were analyzed by immunofluorescence staining and western blotting. A: Cells grown in 2-D culture and 3-D scaffolds for 2 wk were cultured on coverslips for 2 d and stained with primary antibodies, Oct4 and Nanog, treated with Alexa Fluor 568 conjugated secondary antibodies, and counterstained with DAPI. Confocal images depicted an increase in Oct4 and Nanog protein expression in 3-D scaffold grown ESCs compared to 2-D cultured cells; B: Western blot analysis showed increased expressions of Oct4, Nanog, and Klf4 proteins in ESCs grown in the 3-D scaffold for 2 wk compared to initial 2-D grown ESCs (day 0). Cell were lysed in RIPA buffer and aliquots containing an equal amount of protein (10 μg) were subjected to 12% polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and probed with antibodies against Oct4, Nanog, and Klf4; C: Quantitative analysis of western blots (normalized to β-actin levels) showed that Oct4, Nanog and Klf4 protein levels in 3-D grown cells were increased by 1.9, 3.9 and 1.3 fold, respectively, as compared to 2-D cultured controls. Representative results are shown. ESCs: Embryonic stem cells. DAPI: 4',6-diamidino-2-phenylindole dihydrochloride.

DISCUSSION

In this report, we described an alternative to traditional 2-D culturing of ESCs without the need for a MEF feeder layer and routine passaging. This method was developed utilizing two components, Dex-SH and PEG-4-Acr, for self-assembling 3-D hydrogel scaffolds. Evidently, this hydrogel scaffold better emulated the fully hydrated native 3-D microenvironment and supported the pluripotent growth of ESCs. This method eliminates laborious and time-consuming manipulations, which often results in the loss of ESC lines due to contamination of cultures or differentiation of cells.

The polymerization of Dex-SH and PEG-4-Acr, *via* a Michael addition reaction, facilitated the self-assembly of the 3-D scaffolds and encapsulation of ESCs. Polymerization was dependent upon several factors including concentration and ratio of polymers, degree of thiol substitution of Dex-SH, and cell concentration. Optimal conditions for scaffold polymerization, cell encapsulation, and cell viability were achieved by varying these parameters. The results showed that higher the degree of thiol substitution of Dex-SH, the faster the polymerization of hydrogel. Consistent with a previous report[27], the ratio and amount of Dex-SH and PEG-4-Acr also affected the formation and swelling properties of the hydrogel scaffolds. In one study, 10% w/v polymer concentrations were used to prepare Dex-

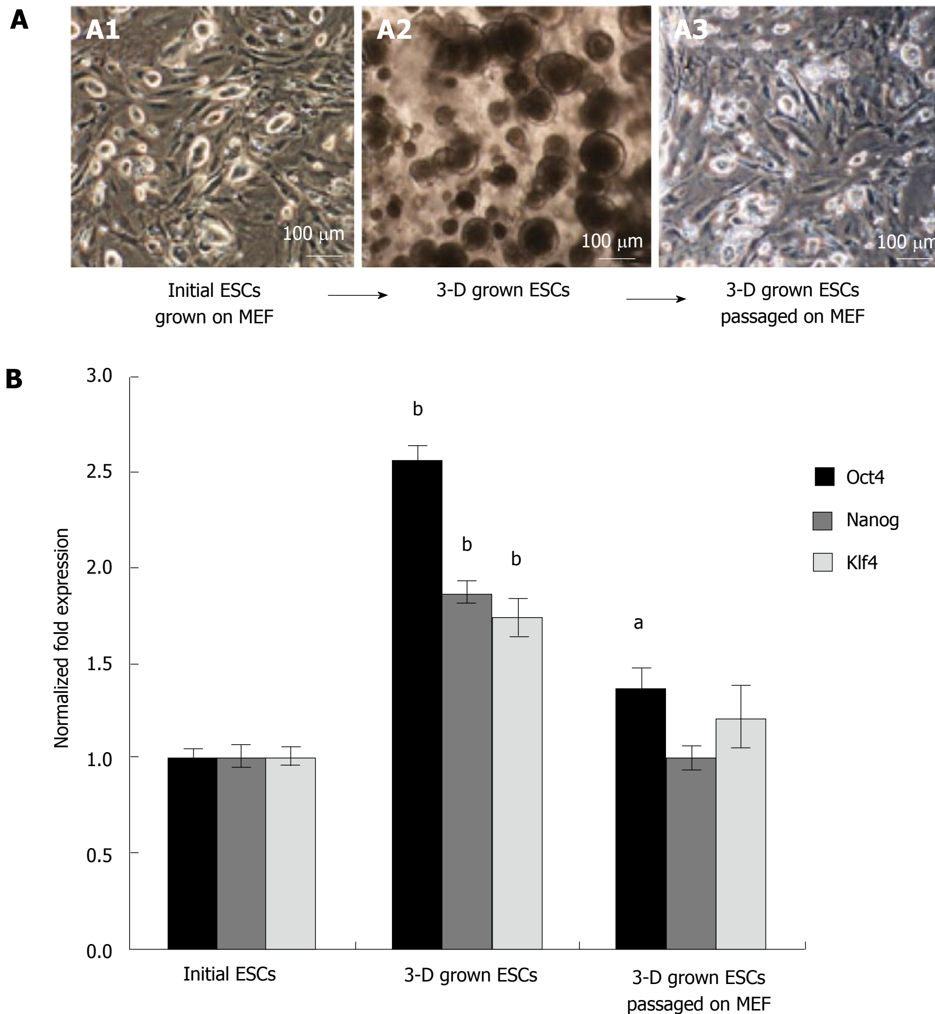


Figure 5 Three-dimensional grown embryonic stem cells were subcultured under two-dimensional culture conditions expressed normal levels of pluripotent markers. A: Light micrographs depicting cell morphology of the initial ESCs used for encapsulation (A1), cultured in three-dimensional (3-D) scaffolds for 2 wk (A2), and subsequently passaged 5 times in 2-D culture (A3); B: Expression of Oct4, Nanog, and Klf4 in ESCs grown in 2-D culture, 3-D grown ESCs, and 3-D grown ESCs, which were subsequently passaged 5 times in 2-D culture as determined by qRT-PCR. Results were expressed as the fold expression ± SE (n = 3) normalized to reference genes Gapdh and β-Actin where a significant (^aP < 0.05 and ^bP < 0.01) increase of marker expression was compared to initial 2-D grown ESCs, using one-way ANOVA and Tukey multiple comparisons test. ESCs: Embryonic stem cells; MEF: Mouse embryonic fibroblasts; qRT-PCR: Quantitative real time polymerase chain reaction; ANOVA: Analysis of variance.

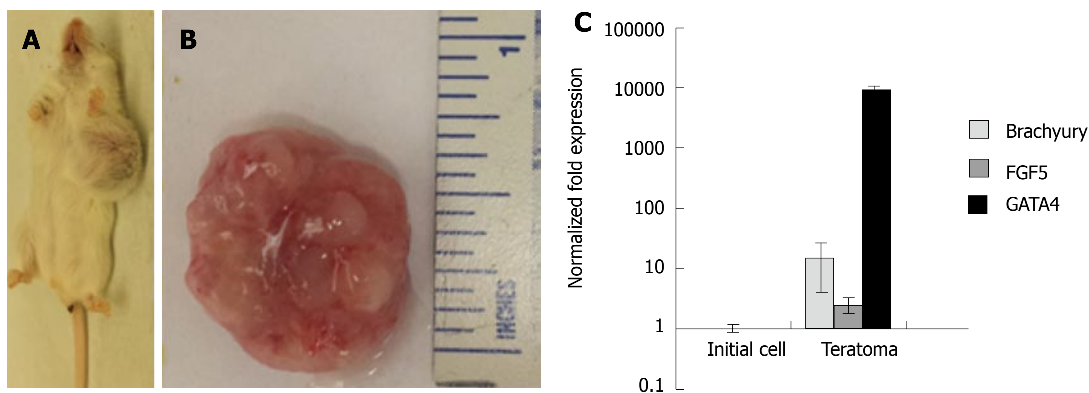


Figure 6 Three-dimensional grown embryonic stem cells formed teratomas in severe combined immunodeficient-beige mice. Explanted teratoma tissues were analyzed for the expression of three germ layer markers. A: Gross images of tumor growth resulting from injection of 3-D grown ESCs. Tumor growth was observed in all mice injected (n = 3); B: Explanted tumor at 4 wk showed encapsulated, lobular and well-circumscribed gross morphology consistent with teratoma growth; C: Expression of germ layer markers, Brachyury, FGF5, and GATA4 representing mesoderm, ectoderm, and endoderm was analyzed by qRT-PCR. Results of tumor explants were expressed as fold expression ± SE (n = 3) normalized to reference genes Gapdh and β-Actin, and compared to initial cells injected *in vivo*. ESCs: Embryonic stem cells; qRT-PCR: Quantitative real time polymerase chain reaction..

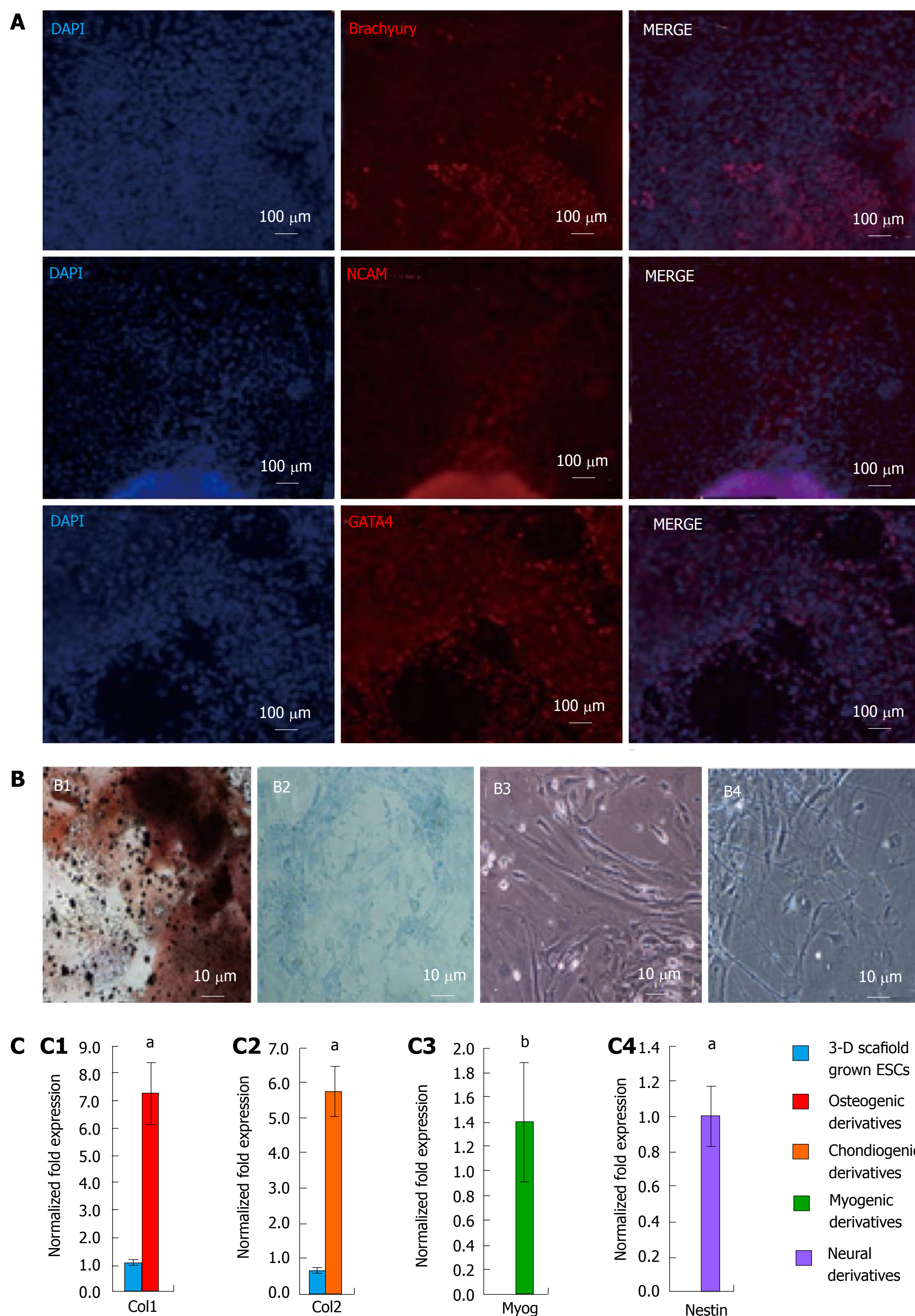


Figure 7 Differentiation potential of three-dimensional grown embryonic stem cells. A: Embryoid bodies derived from ESCs grown in the 3-D scaffolds were allowed to spontaneously differentiate and analyzed by immunofluorescence staining with antibodies against all three germ layers. Confocal images depicted the presence of Brachyury, NCAM, and GATA4 protein expression, showing the potential of ESCs to differentiate into mesoderm, ectoderm, and endoderm, respectively; B: 3-D scaffold grown ESCs were also directed to differentiate *via* EB formation and selective induction media. Shown are light micrographs depicting cell morphology of ESC derivatives, osteogenic, chondrogenic, myogenic, and neural (B1, B2, B3, and B4 respectively). Osteogenic and chondrogenic derivatives were cultured for 4 wk and analyzed by von Kossa, and alcian blue staining, respectively. Osteogenic cell-specific ECM stained dark brown for calcium deposition (B1). Proteoglycan produced by chondrogenic derivatives stained blue; C: Expression of cell-specific markers in differentiated ESC derivatives expressed Col1 (C1), Col2 (C2), Myog (C3), and Nestin (C4) markers for osteogenic, chondrogenic, myogenic and neural cells, respectively. Results represent the fold expression ± SE (n = 3) normalized to reference genes Gapdh and β-Actin where a significant ($^{a}P < 0.05$ and $^{b}P < 0.01$) increase of marker expression was compared to initial 2-D grown ESCs (day 0), using one-way ANOVA with the non-equal variance hypothesis. ESCs: Embryonic stem cells; ANOVA: Analysis of variance..

SH and PEG-4-Acr scaffolds to promote differentiation of ESCs[23]. However, we determined that 5% w/v polymer concentrations were ideal to provide a scaffold microenvironment with a greater degree of flexibility and suitability for ESC growth. Mixing of cells with PEG-4-Acr prior to scaffold molding yielded homogeneous distribution of cells in both fixed and floating scaffolds. The 3-D scaffold grown ESCs displayed undifferentiated compact round colony morphology, even upon prolonged culturing. Previously, Gerecht *et al*[26] reported the potential of hyaluronic acid-based hydrogels in maintaining human ESCs with an undifferentiated morphology for 20 d. However, polymerization of hyaluronic acid-based hydrogels was achieved with ultraviolet light, a potent carcinogen. Whereas, self-assembly of the Dex-SH and PEG-4-Acr scaffold does not require a mutagenic catalyst, and ESCs can be maintained for over 6 wk. They also reported that at least 5×10^6 cells/mL to 10×10^6 cells/mL were required for cell growth and colony formation in the hyaluronic acid-based scaffolds. In contrast, we found that use of Dex-SH and PEG-4-Acr scaffolds supported ESC growth at concentrations as low as 1×10^4 cells/mL or 1×10^3/cm^2 in 3-D scaffolds. At concentrations below 1×10^4 cells/mL successful encapsulation in the scaffold was difficult to assess due to low cell density. Furthermore, low concentration may also affect the viability of the cells. In traditional 2-D culture, optimal maintenance of ESCs requires a much higher cell density (3×10^4 to 5×10^4 cells/cm^2)[33].

ESC self-renewal was not affected by the duration of growth in 3-D scaffolds as long as the integrity of the scaffold was maintained. Scaffold integrity also influenced the cell proliferation rate and colony size. ESCs grew at a slower rate with smaller colony size in fixed scaffolds. However, cell growth rate and colony size was less restricted in floating scaffolds. In agreement with previous reports[35,36], we found that mouse ESCs doubled nearly every 12 h in 2-D culture, whereas the generation time for ESCs in the 3-D culture system was approximately 36 h. It has been reported that the growth rate of stem cells *in vivo* vary from a few hours to months depending upon the niche[37]. In 2-D culture, ESCs reached near confluency in 3 d and started to differentiate, whereas the growth rate in 3-D was slower; the cells did not differentiate and continued to grow for up to 6 wk, limited only by scaffold degradation. Using the exponential cell growth formula $N = N_0 \times 2^g$, where N is the final cell number, N_0 is the initial cell number (2×10^6), and g is the number generations (25), the expected total number of cells after 6 wk of growth would be 134×10^{12} cells. The fact that ESCs grew for an extended period without the loss of cell viability and their self-renewal potential suggested that the 3-D culture system described here was superior to 2-D culture, which requires routine passaging.

In addition to the concentration of the scaffold components and degree of thiol substitution, cell concentration significantly affected the rate of scaffold degradation. Consistent with previous studies[23], scaffolds without cells degraded earlier compared to seeded scaffolds (13 d *vs* 27 d, respectively) suggesting that the addition of cells improved the stability of the scaffolds. Moreover, scaffolds encapsulating a higher concentration of ESCs degraded faster and swelled less than scaffolds with lower concentration of ESCs. Consequently, ESC colony size was smaller in scaffolds with a higher concentration of cells as compared to scaffolds prepared with a lower cell concentration. In addition, encapsulated ESCs in floating scaffolds grew rapidly with larger colony sizes as compared to fixed scaffolds of similar composition. These results suggested that the swelling plasticity of the self-assembling scaffolds favorably promoted the growth and maintenance of ESC colonies. Previous studies also indicated that cell encapsulation increased integrity of the scaffold as compared to scaffolds without cells[23]. However, in this report the effect of cell concentrations on scaffold integrity was not reported. Our observations indicated that higher cell concentrations inversely effected scaffold integrity suggesting that the cells may be producing factors that de-stabilize the scaffolds. The nature of these factors remains unknown.

ESCs grown in the 3-D scaffolds for 6 wk maintained their pluripotency and differentiation potential, as determined by their ability to spontaneously differentiate into cell types comprising all three germ layers. The EBs differentiated into osteogenic, chondrogenic, myogenic, and neural cell types, expressing cell-specific markers. These results indicated that the self-assembling scaffolds support the self-renewal and pluripotency of ESCs, even after prolonged periods of culturing. The pluripotency of 3-D grown ESCs was further confirmed by teratoma formation *in vivo*. Teratomas explanted from immune-compromised mice following injection of 3-D grown ESCs showed specific gene expression representing all three germ layers.

Interestingly, the expression of three pluripotency markers, Oct4, Nanog and Klf4, was higher in 3-D cultured ESCs as compared to 2-D grown ESCs both at a transcriptional and translational level. The expression of these genes remained at higher levels throughout the extended growth period (up to 6 wk) until the onset of scaffold degradation. In contrast, Sox2 expression levels in 3-D grown ESCs was found to be similar to the control (2-D grown ESCs). Although high expression of Nanog and Klf4 has been reported to enhance self-renewal and pluripotency[38], overexpression of Oct4 has been implicated in spontaneous ESC differentiation[39]. Contrary to this, we did not observe differentiation of ESCs grown in 3-D scaffolds. When 3-D grown ESCs were subcultured under 2-D culture conditions, expression of pluripotent markers returned to levels similar to 2-D grown cells. Previous reports[40] also described differential regulation of stemness genes, with an upregulation of Oct4 but downregulation of Sox2 in ESCs cultured in three different scaffolds prepared

using PGLA, collagen and chitosan; whereas Nanog was only highly upregulated in chitosan scaffolds. Taken together with our findings, it can be argued that the scaffold microenvironments play an important role in influencing cell-matrix communication, thus effecting gene expression. It is also possible that cellular proteins may have interacted with thiol groups during the encapsulation step of self-assembly of the scaffolds resulting in alteration of their activities. Nevertheless, the underlying molecular mechanism responsible for the upregulation of pluripotent genes in ESCs grown in 3-D Dex-SH and PEG-4-Acr scaffolds remain unknown and warrants further investigation.

The findings reported here demonstrate a novel, efficient, reproducible, and simple approach to ESC cultivation. However, these results were obtained using mouse ESCs as a model, further studies are required to expand these results to human pluripotent stem cells. Previous attempts at 3-D cultivation of ESCs have required routine passaging[19,25,26]. To our knowledge this is the first report that described a robust system for 3-D culturing of ESCs for extended periods without passaging or manipulation. These improvements in the maintenance of ESCs will help their use in translational research, disease modeling, stem cell therapies, and regenerative medicine.

ACKNOWLEDGMENTS

Oakland University Provost Graduate Student Research Award provided support for C. McKee. We would like to thank Dr. Bashir Kaskar for help with the synthesis and analysis of Dex-SH, Naimisha Reddy Beeravolu for her assistance with the western blot analysis and Dr. Harvey Qu for his evaluation of the statistical analysis.

COMMENTS

Background

Embryonic stem cells (ESCs) are capable of differentiating into any of the over 200 cell types found in the body, which makes them an ideal source for cell-based therapies and regenerative medicine. These applications require efficient and reproducible expansion of ESCs *ex vivo* for high quality cells, which then can be uniformly differentiated for clinical use. However, propagation of ESCs is technically challenging, laborious and expensive. Traditional ESC culture techniques are heavily dependent on cell adherence to two-dimensional (2-D) plastic culture plates, regular passaging (every two to three days) and xenogeneic feeder layers. This often results in differentiation and loss of cell lines.

Research frontiers

In order to overcome the problems associated with 2-D cell culture, few studies have reported the use of three-dimensional (3-D) scaffolds to propagate ESCs. However, these methods are not efficient and require the use of carcinogenic catalysts for polymerization of the scaffolds. Moreover, these techniques required routine passaging in order to maintain the pluripotency of ESCs.

Innovations and breakthroughs

A novel 3-D culture system was developed for propagation of ESCs for an extended period of time. In this system, ESCs were encapsulated upon self-assembly of 3-D scaffolds prepared with thiol-functionalized dextran and polyethylene glycol tetra-acrylate. The 3-D scaffold microenvironment supported the growth of ESCs while maintaining their pluripotency and self–renewal potential for extended periods without passaging and extensive manipulation, which is required for 2-D culture of ESCs.

Applications

This new culture system should help develop methods of expansion for high quality and homogenous populations of ESCs, which are critically important for regenerative medicine and therapeutic applications.

Terminology

A scaffold is an engineered matrix formed from biomaterials mimicking the microenvironment and capable of supporting cell growth. In this study, self-assembling scaffolds were formed upon combination of two polymers, polyethylene glycol tetra-acrylate and thiol-functionalized dextran.

Peer-review

The authors designed a simplified and efficient system, which mimics the microenvironment *in vivo* for long-term proliferation and maintenance of ESCs. Furthermore, the culture system is efficient and reproducible and is expected to lead to further innovations.

REFERENCES

1 **Stojkovic M**, Lako M, Strachan T, Murdoch A. Derivation, growth and applications of human embryonic stem cells. *Reproduction* 2004; **128**: 259-267 [PMID: 15333777 DOI: 10.1530/rep.1.00243]

2 **Wobus AM**, Boheler KR. Embryonic stem cells: prospects for developmental biology and cell therapy. *Physiol Rev* 2005; **85**: 635-678 [PMID: 15788707 DOI: 10.1152/physrev.00054.2003]

3 **Zhang S**, Zhang Y, Chen L, Liu T, Li Y, Wang Y, Geng Y. Efficient large-scale generation of functional hepatocytes from mouse embryonic stem cells grown in a rotating bioreactor with exogenous growth factors and hormones. *Stem Cell Res Ther* 2013; **4**: 145 [PMID: 24294908 DOI: 10.1186/scrt356]

4 **Keller G**. Embryonic stem cell differentiation: emergence of a new era in biology and medicine. *Genes Dev* 2005; **19**: 1129-1155 [PMID: 15905405 DOI: 10.1101/gad.1303605]

5 **Trounson A**. New perspectives in human stem cell therapeutic research. *BMC Med* 2009; **7**: 29 [PMID: 19519878 DOI: 10.1186/1741-7015-7-29]

6 **Balami JS**, Fricker RA, Chen R. Stem cell therapy for ischaemic stroke: translation from preclinical studies to clinical treatment. *CNS Neurol Disord Drug Targets* 2013; **12**: 209-219 [PMID: 23394533 DOI: 10.2174/1871527311312020007]

7 **Carr AJ**, Smart MJ, Ramsden CM, Powner MB, da Cruz L, Coffey PJ. Development of human embryonic stem cell therapies for age-related macular degeneration. *Trends Neurosci* 2013; **36**: 385-395 [PMID: 23601133 DOI: 10.1016/j.tins.2013.03.006]

8 **Chambers I**, Tomlinson SR. The transcriptional foundation of pluripotency. *Development* 2009; **136**: 2311-2322 [PMID: 19542351 DOI: 10.1242/dev.024398]

9 **Burdick JA**, Vunjak-Novakovic G. Engineered microenvironments for controlled stem cell differentiation. *Tissue Eng Part A* 2009; **15**: 205-219 [PMID: 18694293 DOI: 10.1089/ten.tea.2008.0131]

10 **Lund AW**, Yener B, Stegemann JP, Plopper GE. The natural and engineered 3D microenvironment as a regulatory cue during stem cell fate determination. *Tissue Eng Part B Rev* 2009; **15**: 371-380 [PMID: 19505193 DOI: 10.1089/ten.TEB.2009.0270]

11 **Lutolf MP**, Gilbert PM, Blau HM. Designing materials to direct stem-cell fate. *Nature* 2009; **462**: 433-441 [PMID: 19940913 DOI: 10.1038/nature08602]

12 **Saha K**, Pollock JF, Schaffer DV, Healy KE. Designing synthetic materials to control stem cell phenotype. *Curr Opin Chem Biol* 2007; **11**: 381-387 [PMID: 17669680 DOI: 10.1016/j.cbpa.2007.05.030]

13 **Geckil H**, Xu F, Zhang X, Moon S, Demirci U. Engineering hydrogels as extracellular matrix mimics. *Nanomedicine* (Lond) 2010; **5**: 469-484 [PMID: 20394538 DOI: 10.2217/nnm.10.12]

14 **Jozefczuk J**, Drews K, Adjaye J. Preparation of mouse embryonic fibroblast cells suitable for culturing human embryonic and induced pluripotent stem cells. *J Vis Exp* 2012; **(64)**: pii: 3854 [PMID:

22760161 DOI: 10.3791/3854]
15 **Fecek C**, Yao D, Kaçorri A, Vasquez A, Iqbal S, Sheikh H, Svinarich DM, Perez-Cruet M, Chaudhry GR. Chondrogenic derivatives of embryonic stem cells seeded into 3D polycaprolactone scaffolds generated cartilage tissue in vivo. *Tissue Eng Part A* 2008; **14**: 1403-1413 [PMID: 18462064 DOI: 10.1089/tea.2007.0293]
16 **Lee J**, Cuddihy MJ, Kotov NA. Three-dimensional cell culture matrices: state of the art. *Tissue Eng Part B Rev* 2008; **14**: 61-86 [PMID: 18454635 DOI: 10.1089/teb.2007.0150]
17 **Guilak F**, Cohen DM, Estes BT, Gimble JM, Liedtke W, Chen CS. Control of stem cell fate by physical interactions with the extracellular matrix. *Cell Stem Cell* 2009; **5**: 17-26 [PMID: 19570510 DOI: 10.1016/j.stem.2009.06.016]
18 **Liu Y**, Charles LF, Zarembinski TI, Johnson KI, Atzet SK, Wesselschmidt RL, Wight ME, Kuhn LT. Modified hyaluronan hydrogels support the maintenance of mouse embryonic stem cells and human induced pluripotent stem cells. *Macromol Biosci* 2012; **12**: 1034-1042 [PMID: 22730306 DOI: 10.1002/mabi.201200043]
19 **Li YJ**, Chung EH, Rodriguez RT, Firpo MT, Healy KE. Hydrogels as artificial matrices for human embryonic stem cell self-renewal. *J Biomed Mater Res A* 2006; **79**: 1-5 [PMID: 16741988 DOI: 10.1002/jbm.a.30732]
20 **Fisher OZ**, Khademhosseini A, Langer R, Peppas NA. Bioinspired materials for controlling stem cell fate. *Acc Chem Res* 2010; **43**: 419-428 [PMID: 20043634 DOI: 10.1021/ar900226q]
21 **Benoit DS**, Durney AR, Anseth KS. Manipulations in hydrogel degradation behavior enhance osteoblast function and mineralized tissue formation. *Tissue Eng* 2006; **12**: 1663-1673 [PMID: 16846361 DOI: 10.1089/ten.2006.12.1663]
22 **Hiemstra C**, Zhong Z, van Steenbergen MJ, Hennink WE, Feijen J. Release of model proteins and basic fibroblast growth factor from in situ forming degradable dextran hydrogels. *J Control Release* 2007; **122**: 71-78 [PMID: 17658651 DOI: 10.1016/j.jconrel.2007.06.011]
23 **Jukes JM**, van der Aa LJ, Hiemstra C, van Veen T, Dijkstra PJ, Zhong Z, Feijen J, van Blitterswijk CA, de Boer J. A newly developed chemically crosslinked dextran-poly(ethylene glycol) hydrogel for cartilage tissue engineering. *Tissue Eng Part A* 2010; **16**: 565-573 [PMID: 19737051 DOI: 10.1089/ten.TEA.2009.0173]
24 **Van Tomme SR**, Storm G, Hennink WE. In situ gelling hydrogels for pharmaceutical and biomedical applications. *Int J Pharm* 2008; **355**: 1-18 [PMID: 18343058 DOI: 10.1016/j.ijpharm.2008.01.057]
25 **Lei Y**, Schaffer DV. A fully defined and scalable 3D culture system for human pluripotent stem cell expansion and differentiation. *Proc Natl Acad Sci USA* 2013; **110**: E5039-E5048 [PMID: 24248365 DOI: 10.1073/pnas.1309408110]
26 **Gerecht S**, Burdick JA, Ferreira LS, Townsend SA, Langer R, Vunjak-Novakovic G. Hyaluronic acid hydrogel for controlled self-renewal and differentiation of human embryonic stem cells. *Proc Natl Acad Sci USA* 2007; **104**: 11298-11303 [PMID: 17581871 DOI: 10.1073/pnas.0703723104]
27 **Hiemstra C**, Aa LJ, Zhong Z, Dijkstra PJ, Feijen J. Rapidly in situ-forming degradable hydrogels from dextran thiols through Michael addition. *Biomacromolecules* 2007; **8**: 1548-1556 [PMID: 17425366 DOI: 10.1021/bm061191m]
28 **Zawko SA**, Suri S, Truong Q, Schmidt CE. Photopatterned anisotropic swelling of dual-crosslinked hyaluronic acid hydrogels. *Acta Biomater* 2009; **5**: 14-22 [PMID: 18929518 DOI: 10.1016/j.actbio.2008.09.012]
29 **Hiemstra C**, Zhou W, Zhong Z, Wouters M, Feijen J. Rapidly in situ forming biodegradable robust hydrogels by combining stereocomplexation and photopolymerization. *J Am Chem Soc* 2007; **129**: 9918-9926 [PMID: 17645336 DOI: 10.1021/ja072113p]
30 **Zhang WY,** de Almeida PE, Wu JC. Teratoma formation: A tool for monitoring pluripotency in stem cell research. StemBook [Internet]. Cambridge (MA): Harvard Stem Cell Institute, 2008-2012 Jun 10 [PMID: 23658974 DOI: 10.3824/stembook.1.53.1]
31 **Gertow K**, Przyborski S, Loring JF, Auerbach JM, Epifano O, Otonkoski T, Damjanov I, Ahrlund-Richter L. Isolation of human embryonic stem cell-derived teratomas for the assessment of pluripotency. *Curr Protoc Stem Cell Biol* 2007; **Chapter 1**: Unit1B.4 [PMID: 18785162 DOI: 10.1002/9780470151808.sc01b04s3]
32 **Chaudhry GR**, Yao D, Smith A, Hussain A. Osteogenic Cells Derived From Embryonic Stem Cells Produced Bone Nodules in Three-Dimensional Scaffolds. *J Biomed Biotechnol* 2004; **2004**: 203-210 [PMID: 15467160 DOI: 10.1155/s111072430431003x]
33 **Chaudhry GR**, Fecek C, Lai MM, Wu WC, Chang M, Vasquez A, Pasierb M, Trese MT. Fate of embryonic stem cell derivatives implanted into the vitreous of a slow retinal degenerative mouse model. *Stem Cells Dev* 2009; **18**: 247-258 [PMID: 18442304 DOI: 10.1089/scd.2008.0057]
34 **Wang C**, Hao J, Zhang F, Su K, Wang DA. RNA extraction from polysaccharide-based cell-laden hydrogel scaffolds. *Anal Biochem* 2008; **380**: 333-334 [PMID: 18582431 DOI: 10.1016/j.ab.2008.06.005]
35 **Kidder BL**, Oseth L, Miller S, Hirsch B, Verfaillie C, Coucouvanis E. Embryonic stem cells contribute to mouse chimeras in the absence of detectable cell fusion. *Cloning Stem Cells* 2008; **10**: 231-248 [PMID: 18338954 DOI: 10.1089/clo.2007.0039]
36 **Tamm C**, Pijuan Galitó S, Annerén C. A comparative study of protocols for mouse embryonic stem cell culturing. *PLoS One* 2013; **8**: e81156 [PMID: 24339907 DOI: 10.1371/journal.pone.0081156]
37 **Lane SW**, Williams DA, Watt FM. Modulating the stem cell niche for tissue regeneration. *Nat Biotechnol* 2014; **32**: 795-803 [PMID: 25093887 DOI: 10.1038/nbt.2978]
38 **Nur-E-Kamal A**, Ahmed I, Kamal J, Schindler M, Meiners S. Three-dimensional nanofibrillar surfaces promote self-renewal in mouse embryonic stem cells. *Stem Cells* 2006; **24**: 426-433 [PMID: 16150921 DOI: 10.1634/stemcells.2005-0170]
39 **Radzisheuskaya A**, Silva JC. Do all roads lead to Oct4? the emerging concepts of induced pluripotency. *Trends Cell Biol* 2014; **24**: 275-284 [PMID: 24370212 DOI: 10.1016/j.tcb.2013.11.010]
40 **Wei J**, Han J, Zhao Y, Cui Y, Wang B, Xiao Z, Chen B, Dai J. The importance of three-dimensional scaffold structure on stemness maintenance of mouse embryonic stem cells. *Biomaterials* 2014; **35**: 7724-7733 [PMID: 24930853 DOI: 10.1016/j.biomaterials.2014.05.060]

P- Reviewer: Chen CP, Liu L **S- Editor**: Tian YL
L- Editor: A **E- Editor**: Wu HL

Published by **Baishideng Publishing Group Inc**
8226 Regency Drive, Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com

© 2015 Baishideng Publishing Group Inc. All rights reserved.