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Beyond the bedside: A review of translational medicine in global health

Richard S Hoehn, Daniel E Abbott

Richard S Hoehn, Daniel E Abbott, Department of Surgery, University of Cincinnati, Cincinnati, OH 45267, United States

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Correspondence to: Daniel E Abbott, MD, Department of Surgery, University of Cincinnati, 231 Albert Sabin Way, ML 0558, Cincinnati, OH 45267,

United States. abbottde@ucmail.uc.edu

Telephone: +1-513-5587865

Fax: +1-513-5840459

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syndrome, and non-communicable diseases. Laboratory research has excelled in many of these areas and is struggling in a few. Where successful therapies have been discovered there are often problems with appropriate use or dissemination to groups in need. Also, many diseases would be better prevented from a population health approach. This review highlights successes and struggles in the arena of global health, from smallpox eradication to the impending epidemic of cardiovascular disease, in an attempt to illustrate of the various phases of translational research.

Key words: Global health; Human immunodeficiency virus; Translational research; Vaccines; Cancer; Non-communicable diseases

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Core tip: This review summarizes efforts in translational research as applied to the major global health issues of vaccines, human immunodeficiency virus and acquired immunodeficiency syndrome and non-communicable diseases. Historical perspective as well as current efforts are presented in an effort to describe the success and challenges that are concurrent with translational medicine on the international stage.

Abstract

Translational research is a broad field of medicine with several key phases moving from scientific discovery to bench research and the hospital bedside, followed by evidence-based practice and population-level policy and programming. Understanding these phases is crucial when it comes to preventing and treating illness, especially in global health. Communities around the world struggle with a variety of health problems that are at some times similar and at others quite different. Three major world health issues help to outline the phases of translational research: vaccines, human immunodeficiency virus and acquired immunodeficiency

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INTRODUCTION

To address the breadth of translational research, the United States National Institutes of Health has recently endorsed a 5-phase model that describes the process

of moving from scientific discoveries to population health (Figure 1)^[1]. The process starts with scientific discovery of a problem or pathology, termed T0. From there, T1 and T2 encompass the classic “bench to bedside” process of finding a candidate treatment, test, or clinical intervention (T1) and then comparing safety and efficacy of the candidate against a placebo or existing therapy in randomized trials or other study designs (T2). Lastly, T3 research focuses on implementation and dissemination of evidence-based interventions and T4 examines population-level health impact and cost-effectiveness^[2].

The purpose of this review is to describe the impact of the phases of translational research on global health. Communities around the world suffer from health issues that are at times very different but can also be quite similar. Some therapies are readily available in resource-rich countries but scarce in less affluent countries. Other therapies simply do not work in certain parts of the world due to disease specificity or cultural issues. Though there are many blights, three major issues affecting the health of the global population are vaccine development, the human immunodeficiency virus and acquired immunodeficiency syndrome (HIV/AIDS), and non-communicable diseases (NCDs). Here we will review the history and current status of research in these areas, highlighting the role of various phases of translational research with respect to their effects on global health.

VACCINES

Early successes

The history of vaccine development and distribution exemplifies translational research. Edward Jenner discovered the smallpox vaccine in the 1790s by treating patients with “matter” from the sores of cowpox^[3]. This technique of sharing the live cowpox virus between patients lasted through the 19th century until the development of a live attenuated vaccine in the early 20th century^[4]. In the coming decades the World Health Organization (WHO) would sponsor a smallpox vaccination program, and eradication of the virus was formally declared in 1980 (the only other disease to be declared eradicated is Rinderpest, an RNA virus that affected cattle and water buffalo, mostly in Africa; vaccines for this virus were developed in the early 20th century and two major attempts at mass vaccination led to eradication in 2011).

A second vaccination “victory” resulted from the work of one of the fathers of bacteriology, Robert Koch, who demonstrated that the bacterium *Bacillus anthracis* was the cause of “wool-sorters’ disease” in 1876^[5]. Anthrax had plagued livestock for millennia, and humans involved in wool and hide processing were at risk of infection. Following this discovery, Louis Pasteur described a randomized controlled trial in which he treated livestock with an attenuated anthrax vaccine prior to inoculating them with a virulent strain

of the bacteria^[6]. The results were dramatic; 48 h after inoculation, all vaccinated sheep survived and all un-vaccinated sheep were dead. Virtual eradication was made possible by livestock quarantining and vaccination, but recent terror attacks using the anthrax spore have generated interest in newer vaccines^[7-9]. While the vaccine is only available for at-risk patients (veterinarians, researchers, certain military personnel, etc.) due to difficulties with production and storage, research is underway to develop a stable, needleless vaccine for widespread use^[10].

Works in progress

Worldwide, diarrheal illness is the second leading cause of death in children under the age of five years (760000 deaths each year)^[11]. There are many causes of diarrhea and a significant portion of the disease burden can be prevented through public health efforts to create safe drinking water and adequate sanitation. Infectious causes are well described, and three major sources have been the focus of vaccination efforts in recent decades.

Rotavirus is a leading cause of child mortality worldwide, especially in low-income regions; in children under the age of five in 2008, 5% of all mortality and over one-third of diarrhea-related mortality were attributable to rotavirus infection. Early experiments led to development of monovalent live-oral vaccines with variable success^[12]. In 1998 the rhesus rotavirus tetravalent vaccine (RRV-TV) was licensed for administration in children after successful trials. However, after several cases of bowel obstruction and intussusception following vaccine administration, the Centers for Disease Control and Prevention advised against using the vaccine and in 1999 the manufacturer withdrew the vaccine from market^[13]. Since that time, three live-attenuated oral vaccines have been approved for use. The monovalent (RV1) and pentavalent (RV5) rotavirus vaccines have been evaluated in several large trials and subsequently approved for use in most countries including the United States and the European Union^[14]. A third, the Lanzhou lamb rotavirus vaccine, has been approved for use in China only^[15].

Another major cause of diarrheal illness is typhoid fever, caused by *Salmonella enterica typhi* (*S. typhi*). Two vaccines, injectable (Vi PS) and oral (Ty21a), have shown efficacy and safety in clinical trials and field settings in Chile, Indonesia, and India, but are not ready for widespread immunization protocols^[16]. The Vi PS vaccine is non-immunogenic in children under 2 years, and many *S. typhi* strains are negative for the Vi polysaccharide. The Ty21a vaccine is not recommended for children under 5 years, and its acid-labile nature creates challenges with oral administration. Several newer typhoid vaccines are currently in phase 1-3 trials worldwide, but not licensed for use^[17].

Cholera, caused by *Vibrio cholerae*, is another area of focus for vaccine manufacturers. Dukoral®, an oral killed whole-cell vaccine, was licensed after

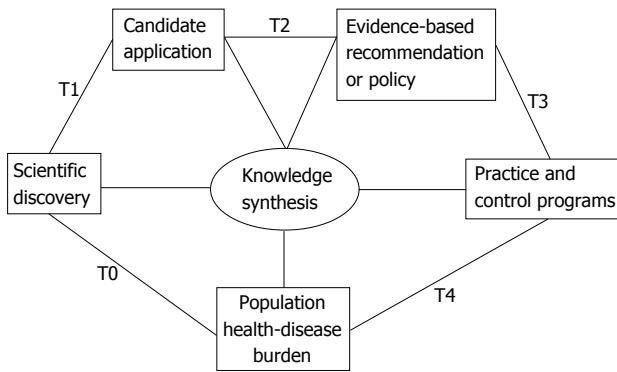


Figure 1 Epidemiology and the phases of translational research. T0: Scientific discovery research; T1: Translational research from discovery to candidate application; T2: Translational research from candidate application to evidence-based recommendation or policy; T3: Translational research from recommendation to practice and control programs; T4: Translational research from practice to population health impact. Source: Khoury *et al.*^[2]

a large randomized controlled trial in Bangladesh in 1990^[18]. The per-dose cost of United States \$5.25 was felt to be prohibitive for use in low-income regions, and subsequent development of Shanchol® at United States \$1.85 per dose was deemed fiscally feasible; Shanchol® use was then adopted after randomized controlled trials in Vietnam and India confirmed safety and immunogenicity^[19,20]. Four single dose, live attenuated oral cholera vaccines are in active clinical programs with hopes to improve efficacy, hasten onset and increase duration of protection^[21].

Ongoing challenges with "the big three"

Despite some vaccination success as a result of collaborative translational research implementation, malaria, tuberculosis (TB), and HIV/AIDS are three of the top ten causes of death worldwide^[22]. As such, much attention and funding has been directed towards finding vaccines for these diseases. Progress has been made, but there are still significant challenges.

There are approximately 250 million reported cases of malaria every year, including almost one million deaths in Sub-Saharan Africa, mostly in children^[23]. Many different vaccines are currently in various trials and they all face a similar challenge; *Plasmodium falciparum*, the causative agent of malaria, has a complex life cycle, with polymorphic antigens expressed in separate phases of the cycle^[24]. The best current vaccine candidate, RTS,S/AS01, is a combination of a portion of the circumsporozoite protein that helps the parasite invade human liver cells and the hepatitis B surface antigen, as well as the liposomal formulation adjuvant AS01. A phase II b trial of the RTS,S malaria vaccine showed safety and efficacy at 20 mo^[25] and phase III trials have demonstrated 31%-56% efficacy for one year, with protection from clinical malaria for at least 3.5 years^[26,27]. However, efficacy of the vaccine wanes with time and also varies based on the age of the vaccinated child^[28]. Despite mediocre results,

RTS,S will likely be the first malaria vaccine to receive regulatory approval^[29].

The bacille Calmette-Guérin (BCG) vaccine for *Mycobacterium TB* is one of the earliest developed vaccines and has been given to over four billion people to date^[30,31]. Despite this fact, TB kills 1.4 million people annually and drug-resistant TB is becoming a major problem^[32]. BCG protects infants from tuberculous meningitis and miliary TB, but is less effective against pulmonary TB in adolescents and adults. There are currently almost twenty candidate vaccines in various phases of clinical trials, all designed to prevent active TB disease^[32,33]. Some of these are live recombinant vaccines that have been genetically engineered for enhanced efficacy and/or safety, meant to replace BCG. Others are proteins or viral vector expressing antigens that are meant to serve as an immune booster following initial treatment with the BCG vaccine^[24]. A common challenge among trials evaluating TB vaccines is that the disease has a long latent period; thus, trialing preventative vaccines is slow and expensive^[31].

HIV kills two million people annually and infects approximately 7000 people per day, making it one of top causes of death worldwide^[34]. Naturally, a significant portion of the world's research dollars are directed toward treating and preventing this disease. Challenges facing researchers looking for a vaccine against HIV include: global variability of HIV, lack of a validated animal model with appropriate immune response, large variety of infected cells that develop as a result of HIV genome integration into the host's DNA, and destruction of immune cells by HIV^[24]. Phase III trials of most vaccines have failed to show efficacy^[35,36] or reduce viral loads^[37,38], and some have actually shown increased HIV infection among vaccine recipients^[39]. The most exciting results are from a randomized controlled trial comparing placebo to a recombinant canarypox vector vaccine (ALVAC-HIV) and two boosters of a recombinant glycoprotein 120 subunit vaccine (AIDSVAX B/E). In a population of greater than 16000 healthy Thai volunteers, this AIDSVAX B/E showed 31% vaccine efficacy vs placebo by reducing the cumulative probability of infection, but did not reduce viral loads^[40]. Nevertheless, finding a safe and effective vaccine against HIV is proving to be one of the most daunting tasks in research today^[41].

Targeted efforts in resource-poor environments

Even when early-phase translational research has found a safe and effective vaccine for a given disease there are still challenges to widespread availability, and the major barrier to vaccine development for low-income regions is cost^[42]. Patients in poor countries cannot afford new, expensive vaccines and pharmaceutical companies are not incentivized to invest capital in developing treatments that will be too expensive for the prospective customers to afford^[43]. Not only that, but the opportunity costs of delaying more profitable projects

are not appealing to industry. To solve this problem, major institutions in global health came together in 1999 to create the Global Alliance for Vaccines and Immunization (GAVI)^[44]. One aim of the alliance is to support new vaccine research, as outlined by their approach to meningitis.

The North African “meningitis belt” is an area that includes countries from Senegal to Ethiopia with a population of around 350 million people^[45]. Annual outbreaks in these countries claim hundreds to thousands of lives and are caused mostly by *Neisseria meningitidis* (*N. meningitidis*) serogroup A^[46]. Controlling these outbreaks requires identifying the culprit strain of *N. meningitidis* and producing a specific polysaccharide vaccine to treat the population at risk. These polysaccharide vaccines are poorly immunogenic in young children, do not prime immunologic memory, and do not lead to “herd immunity”^[47]. To combat this problem, in 2001 the Bill and Melinda Gates Foundation gave United States \$70 million to the WHO to establish the Meningitis Vaccine Project^[48]. The goal was to eliminate epidemic meningitis in Africa through the development of a serogroup A meningococcal conjugate vaccine that would cost less than United States \$0.50 per dose. Vaccine development began in 2003, clinical phase 1-3 trials were completed, and in 2010 MenAfriVac™ was licensed for use in populations aged between one and 29 years; large-scale immunization campaigns began immediately. In 2011, no case of meningococcal A disease occurred in a vaccine recipient in Burkina Faso and the percentage of meningococcal infections in Niger due to serogroup A dropped from 98.6% to less than 2%^[49].

The GAVI alliance has also addressed hepatitis B virus (HBV) in China. HBV is a significant problem in low-income countries, and China accounts for up to half of the HBV-related deaths worldwide^[50]. Over 260000 people die annually in China from HBV-related liver cancer and cirrhosis. A full 60% have a history of infection, and around 10% are chronic carriers^[51]. However, because of high costs, vaccination rates were substantially higher in major cities and wealthy provinces in Eastern China. In 2002, China added HBV vaccination to its National Immunization Programme, and at the same time the China Ministry of Health teamed with GAVI to start the China-GAVI project with a goal of providing free HBV vaccination to people in the poor and western provinces of China. From 1997-2003, overall vaccination coverage increased from 70.7% to 89.8% and timely coverage increased from 29.1% to 75.8%. In the 22 provinces targeted by the China-GAVI Project, timely coverage increased from 64% in 2004 to 81% in 2006, and complete coverage increased from 52% in 2001 to 92% in 2006^[52]. National HBV vaccination programs have had similar effects in other countries^[53] and have been shown to greatly reduce the incidence of hepatocellular carcinoma in these populations^[54].

These examples of meningitis and HBV illustrate

the benefit of targeted funding towards developing specific therapies or distributing existing therapies to a population in need. Fifty years after the Sabin and Salk vaccines were developed, polio has been eradicated in much of the world and vaccine campaigns are now addressing the few remaining countries with recently documented cases^[55,56]. The Bill and Melinda Gates Foundation has given United States \$1.5 billion to the Children's Vaccine Program for research initiatives in malaria, TB, diarrheal diseases, measles, hookworm, and meningitis^[56]. GAVI is targeting the 74 poorest countries in the world with a three-fold approach: improving vaccination infrastructure, purchasing necessary vaccines, and supporting research and development^[56].

For many diseases, notably HIV, TB, and malaria, challenges in vaccine development still need to be overcome. For many others, effective vaccines exist and simply need to be distributed effectively. Through this combination of research and distribution it is possible to use discoveries in the lab to prevent and even eliminate the burden caused by these historically tragic diseases.

HIV/AIDS

In 1981 scientists discovered HIV as the causative agent of AIDS, typified by uncommon opportunistic infections in otherwise healthy young men^[57]. Since then, highly active antiretroviral therapy (HAART) has been proven to significantly reduce morbidity and mortality by suppressing HIV replication and improving CD4⁺ T cell counts. Population studies in developed as well as developing countries have shown a significant effect of HAART treatment on reductions in both viral load as well as HIV transmission and new diagnoses^[58-63]. The WHO has made evidence-based recommendations for HIV treatment and prevention^[64] and the international community has contributed substantially through organizations such as Global Fund to Fight AIDS, TB and Malaria and PEPFAR, the President's Emergency Plan for AIDS Relief^[65]. Despite this progress, over two million people per year become newly infected with HIV worldwide^[66].

A major barrier to defeating HIV is the highly mutagenic and drug-resistant nature of the virus^[64]. The availability of fixed-dose combination pills and simplified treatment schedules can decrease resistance development by increasing adherence to HAART regimens, but resistance is still developing and can be difficult to monitor^[67]. Genotypic testing and viral load monitoring are often not available in resource-limited settings due to cost-constraints and lack of adequate technology. As such, in resource-limited settings the WHO recommends monitoring early warning signs associated with developing drug-resistance: adherence to first-line regimens, changing regimens, inconsistent filling of prescriptions, and missing appointments^[67]. Diagnosis of drug resistance in resource-limited settings is a clinical observation and research is now investigating empiric second- and third-line HAART

options for patients with suspected resistance^[64].

Ultimately, prevention will be the only way to definitively eradicate HIV. Efforts in vaccine development were discussed previously, but another strategy being investigated is prompt treatment of exposed or at-risk individuals with HAART to prevent viral transmission^[66]. Treatment of mothers and children in the peri-natal period has been demonstrated to safely and effectively reduce HIV transmission at birth and during breastfeeding^[68-70]. HIV post-exposure prophylaxis (PEP) taken within 72 h of an occupational exposure has been shown to prevent transmission in the great majority of cases^[71]. Animal trials and observational studies in humans also demonstrate a benefit for non-occupational exposures such as sexual encounters and intravenous drug use^[72]. There is promise of using antiretroviral therapy as pre-exposure prophylaxis for certain high-risk groups, but phase I - III trials have shown variable protection from HIV transmission, likely due to poor drug adherence^[73]. Potential problems with widespread availability of HIV PEP include increased drug resistance, risky behavior, and decreased cost-effectiveness^[66]. To address these issues, dozens of trials in a variety of countries are either planned or ongoing^[74].

NCD EPIDEMIC

In the year 1900, the three leading causes of death in the United States were pneumonia, TB, and diarrhea/enteritis. These diseases caused one third of all deaths, of which 40% were among children under five years of age. Over the next century scientists would discover microorganisms and their role in infectious disease as well as determine ways to treat them. Subsequently the burden of disease has shifted; pneumonia, influenza, and HIV were responsible for 4.5% of deaths in the United States in 1997. Conversely, 54.7% of deaths in that year were a result of heart disease and cancer^[75].

This shift in disease burden is not unique to the United States or even wealthy, industrialized countries. The incidence of many NCDs such as cardiovascular disease (CVD), cancer, and diabetes is growing so fast in developing countries that many have called it an epidemic^[76,77]. From 1909 to 1999, global mortality caused by cancer and CVD increased from 15% to 53%^[78]. In China, for example, the percentage of mortality attributable to CVD tripled from 1957-1990^[79]. The causes are many and include a worldwide surge in life expectancy, lifestyle changes, urbanization, altered diets, increased tobacco use, poor fetal and childhood nutrition, and diminished physical activity^[77].

Epidemiological studies in developing countries have highlighted the substantial presence of risk factors for CVD, many of which are modifiable^[80-83]. These risk factors are less prevalent in developing countries than in developed nations and the incidence of NCDs is lower as well. Nonetheless, incidence is increasing and developing nations are also at risk of a NCD epidemic^[84]. This provides a unique opportunity to halt disease

progression in these regions. Research from developed countries highlights the benefits of preventative medicine in population-based interventions^[85], and national public health programs have successfully improved population health in developed as well as developing countries by disseminating information regarding risk factors^[86,87]. Social education is especially necessary to confront cultural misconceptions in areas where health professionals are distrusted and obesity is seen as a sign of affluence^[88].

When prevention fails, management of affected or high-risk individuals will always be necessary. While there are many known treatments for diabetes, hypertension, hypercholesterolemia, and other chronic diseases, the incidence of these diseases continues to increase both in the United States and worldwide^[89-91].

Another challenge to curbing this epidemic is delivery of appropriate therapy. For example, the results of the β -Blocker Heart Attack Trial were published in the United States in 1981, and 15 years later only 62.5% of patients who had had a myocardial infarction were appropriately being prescribed beta-blockers^[92]. Despite wide availability of an inexpensive, safe, efficacious intervention, less than two-thirds of patients receive appropriate treatment.

Studies in United States have shown health benefits using one-on-one lifestyle teaching^[93] and even text and email reminders^[94] to encourage patients to exercise, modify diet, and take medications as instructed. However, these tactics may not apply to resource-limited countries with insufficient supplies of doctors and medicines^[95]. As is the case with vaccines, governments in these countries can improve health by investing in cost-effective initiatives to develop and provide medications for their citizens at an affordable price^[96-98]. Developing regions have had success improving the management of NCDs by focusing on primary care systems improvements and non-physician-led community initiatives^[95,99-102].

Prevention and management of NCDs is a complicated problem, and the challenges faced by developing and developed countries are both similar and different. There is a unique opportunity in the developing world to prevent an epidemic that is currently evolving^[84]. Risk factors are increasing, but the prevalence of NCDs in developing countries is still quite low, and research has shown that prevention, risk factor modification, and policy change can prevent the NCD epidemic from equaling others the world is currently battling.

Ethical issues

The considerations regarding ethical translational research in global health are diverse. Clearly, the exploitation of economically disadvantaged individuals is egregious, but there are many nuances to consider. When a resource-rich country funds research in a resource-poor country, how do you define the standard of care? Is it wrong to inject live malaria parasites into HIV-positive patients to study the effect CD4⁺ T cell counts, even in an area where malaria is endemic^[103]?

Is it fair to randomly assign some malnourished men to receive vitamin-fortified bread and others standard bread when they normally would not have the fortified option anyway^[104]? When conducting HIV vaccine trials in high-risk populations, is it necessary to provide condoms or safe-sex counseling^[105]? Is it ethical to test a new therapy against subjects who go untreated because they cannot afford medicine which is standard of care^[106]? Early-phase translational research has the potential to harm subjects, and that risk increases when crossing international boundaries^[107].

Another ethical conundrum is the notion of disproportionate profiting from discoveries made in resource-poor countries host^[107]. Not only does it seem wrong to expose patients to a potentially life-altering treatment they could never afford, but such discoveries can further exacerbate international disparities in health, as well as create inequalities in care within the host country^[107]. However, international research partnerships can also improve the quality of care in host nations. For over 30 years Cornell has collaborated with the Haitian Ministry of Health on research that started with AIDS and TB but has since expanded to include maternal-child health, family planning, cancer prevention and treatment, immunization, and education. In that time they have successfully reduced rates of HIV and other sexually transmitted infections, increased the number of patients on HAART, and trained thousands of medical personnel, all the while enjoying uninterrupted NIH support since 1983 and generated more than 150 peer-reviewed publications^[108]. By funding symbiotic partnerships it is possible not only to generate research data but also to improve population health and reduce the "implementation gap" that plagues global health^[109].

CONCLUSION

The history and current struggles of research in vaccine development, HIV, and NCDs emphasize the importance of the five phases of translational research. Many vaccines have been successfully discovered and their effectiveness proven, some are works in progress, and all must have the potential to be efficiently delivered to populations in need. Treatment for HIV has evolved rapidly and become increasingly effective, but HIV is far from eradicated. Therapies for NCDs are well studied in the developed world, but there is much work to be done in prevention and population health in both developing and developed countries. Moving from clinical observation to bench research to bedside intervention is a hallmark of academic medicine in resource-rich countries. However, stopping at the bedside will not improve population health. Region-specific epidemiologic research can highlight needs, opportunities, and challenges that vary due to economic and cultural differences between communities, and goal-directed funding and research can find solutions to these problems. Research in developing countries can inform policy in developed countries, and vice-versa. As

the field of global health grows, research and resources will be shared more efficiently and the successes of smallpox and polio will be translated to HIV and cardiovascular disease.

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Epigenetics and DNA methylation in cancer

Laura Lattanzio, Cristiana Lo Nigro

Laura Lattanzio, Cristiana Lo Nigro, Laboratory Cancer Genetics and Translational Oncology, Medical Oncology, S. Croce University Hospital, 12100 Cuneo, Italy

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Correspondence to: Cristiana Lo Nigro, PhD, Laboratory Cancer Genetics and Translational Oncology, Medical Oncology, S. Croce University Hospital, Via Carle 25, 12100 Cuneo, Italy. lonigro.c@ospedale.cuneo.it

Telephone: +39-0171-616338

Fax: +39-0171-616331

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Abstract

Epigenetic is the study of those alterations regulating gene expression without altering DNA sequence and inherited by transmission through cell division. Mutational and epimutational events that alterate cellular growth and division are combined in carcinogenesis. Advances in genome and epigenome-wide analysis identify DNA hypomethylation, hypermethylation of tumor suppressor genes, aberrant histone modifications and/or specific miRNA expression profiles to contribute to tumor initiation and progression. The major challenge for cancer researchers is to enlighten the complex relationship between the epigenetic and genetic machinery in order to

optimize combined therapies, reducing chemoresistance and minimizing adverse effects in cancer patients. In this review we will cover many distinct aspects of epigenetic phenomenon. Firstly, we will globally explain the most common epigenetic events and their effects on gene expression regulation. Secondly, we will review the evidence of the correlation between epigenetics and cancer progression, focusing in particular on the effect of aberrant hypo- and hyper-methylation. We will also consider the main methods currently used for methylation analysis, covering both locus-specific technologies and genome-wide analysis. Finally, we will discuss the introduction of novel epigenetic drugs in combination with conventional treatments in order to develop more effective cancer therapies. Such information could help in understanding the important role of epigenetics in cancer.

Key words: Epigenetics; DNA methylation; Cancer; Regulation of transcription; Prognostic markers

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Core tip: Carcinogenesis occurs through a combination of mutational and epimutational alterations involving key pathways in cellular growth and division. Tumour cells exhibit two main differences from normal cells in DNA methylation: a global reduction in DNA methylation and the hypermethylation of specific sequences, mainly CpG islands, that cause the transcriptional silencing of tumour suppressor genes, thus directly driving the carcinogenic. In this review, we'll focus on our current understanding of this process, aiming to discuss how the analysis of cancer methylomes and the re-expression of epigenetically silenced genes have potential uses in developing more effective cancer therapies.

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INTRODUCTION

In the early 1940s the word “epigenetics” was introduced in the biological vocabulary to describe those phenomena that traditional genetics could not completely explain. Conrad Waddington (1905-1975) defined epigenetics as “the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being”. Today the most common definition for “epigenetics” is “the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence”^[1].

The epigenetic field covers chromatin-based events that regulate DNA-templated processes. Specific chromatin-modifying enzymes highly regulated modifications to both DNA and to histones, proteins involved in DNA packaging into structural units defined nucleosomes^[2]. Table 1 describes the most well-known DNA and histone modifications and their correlated functions.

Epigenetic modifications play critical role in regulating DNA transcription, repair and duplication. Genomic alterations leading to deregulated expression patterns in chromatin regulators could be responsible for cancer induction and progression^[2].

Earliest studies on gene expression and DNA methylation indicated the possible link of epigenetics to cancer, as detailed in the history of cancer epigenetics by Feinberg *et al.*^[3] and confirmed by recent results from the International Cancer Genome Consortium (ICGC). The analysis of genomes of various cancers allowed us to identify recurrent somatic mutations causing a loss or gain of function in tumour suppressor genes and in oncogenes, respectively. The so called “driver” mutations often present at a high prevalence, are recurrently found in various tumours^[1]. Recent studies identified many of these mutations also in numerous epigenetic regulators. Feinberg hypothesised that epigenetic changes may induce genetic alterations causing cancer initiation and/or progression.

Differently from somatic mutations, epigenetic changes occur without changing DNA sequence; they include chromatin structure variations, due to methylation or histone variants, nucleosome remodelling and non-coding regulatory RNAs changes (Figure 1).

It is now well demonstrated that epigenetic events are heritable changes in gene structures aimed to perpetuate altered activity states^[4]. These alterations in the state of chromosomal regions are called epimutations and could play a significant role in carcinogenesis as they have been commonly found in epigenetic regulators.

The first epigenetic mark studied in correlation with cancer was aberrant DNA methylation causing deregulation in normal gene expression^[5]. DNA methy-

lation is a covalent chemical change that causes the addition of a methyl (CH₃) group at the 5' carbon position of a cytosine ring. The presence of methyl groups determines the turning off of gene transcription and thus the silencing of these genes. The methylation pattern is inherited by the daughter cells during mitosis, allowing maintenance of gene transcription regulation after replication and generating a stable gene silencing mechanism.

A family of DNA methyltransferases (DNMTs) regulate DNA methylation in the CpG dinucleotide by catalyzing the addition of CH₃ groups from S-adenosyl-L-methionine to the 5' position of cytosine. Methyl-binding domain (MBD) proteins (MeCP2, MBD1, MBD2, and MBD4) are able to bind to methylated CpGs, causing transcriptional silencing^[1]. Aberrant methylation frequently occurs in cancer and the most common types of alteration are hypo-, hypermethylation and loss of imprinting (Table 2).

In somatic cells DNA methylation mainly occurs at cytosines usually concentrated in islands (CGIs) which frequently correspond to the promoters of tumour suppressor genes (TSGs) (Figure 2A), which are unmethylated in normal cells.

In cancer cells methylation levels are frequently reduced in specific repetitive elements or in target chromosomal regions (Figure 2B). *LINE-1* elements hypomethylation has been described in colorectal, urothelial and hepatocellular cancers, disrupting normal patterns of gene expression. Moreover, *Alu* elements are hypomethylated with *LINE-1* elements in prostate adenocarcinomas, pancreatic endocrine tumors, and carcinoid tumors^[6]. Hypomethylation of these elements is strongly linked to tumorigenesis through insertional mutagenesis, genomic rearrangements, deletions or inversions causing genomic instability and gene activation^[7]. Although hypomethylation has been clearly correlated to cancer development, the chemical process resulting in the removal of methyl groups (demethylation) and its role in gene regulation are still unclear. The family of enzymes Ten-eleven translocation [TET (TET1, TET2 and TET3)] has been identified to be active in initiating demethylation. They are 2-oxoglutarate-/Fe(II)-dependent oxygenases that convert the 5-methylcytosine (5mC) into 5-hydroxymethylcytosine with mechanisms still not well described^[8].

DNA hypermethylation is the most well studied abnormality of DNA methylation. This method of gene inactivation is the most common mode used by cancer cells to silence TSGs, thus affecting DNA repair, apoptosis, angiogenesis, cell cycle regulation, and capability of invasion. TSGs that are cancer-specifically silenced by CpG island hypermethylation of their promoters are, for example, retinoblastoma, *CDKN2A* (*p16*), *hMLH1*, and *VHL* genes^[6]. However, hypermethylation could also hit DNA repair genes and transcription factors indirectly affecting downstream targets, thus leading to genetic errors and tumori-

Table 1 Most common chromatin modifications with their reader motifs and function

Chromatin modification	Nomenclature	Chromatin-reader motif	Attributed function
DNA modification			
5-methylcytosine	5mC	MBD domain	Transcription
5-formylcytosine	5fC	Unknown	Unknown
5-hydroxymethylcytosine	5hmC	Unknown	Transcription
5-carboxylcytosine	5caC	Unknown	Unknown
Histone modification			
Acetylation	K-ac	Bromodomain Tandem PHD fingers	Transcription, repair, replication, and condensation
Methylation (lysine)	K-me1, K-me2, K-me3	Chromodomain, tudor domain, MBT domain, PWWP domain, PHD fingers	Transcription and repair
Methylation (arginine)	R-me, R-me2s, R-me2a	Tudor domain	Transcription
Phosphorylation (serine and threonine)	S-ph, T-ph	14-3-3, BRCT	Transcription, repair and condensation
Phosphorylation (tyrosine)	Y-ph	SH2	Transcription and repair
Ubiquitylation	K-ub	UIM, IUIM	Transcription and repair
Sumoylation	K-su	SIM	Transcription and repair
ADP ribosylation	E-ar	Macro domain, PBZ domain	Transcription and repair
Deimination	R→Cit	Unknown	Transcription and decondensation
Propoline isomerisation	P-cis↔P-trans	Unknown	Transcription
Crotonylation	K-cr	Unknown	Transcription
Propionylation	K-pr	Unknown	Unknown
Butyrylation	K-bu	Unknown	Unknown
Formylation	K-fo	Unknown	Unknown
Hydroxylation	Y-oh	Unknown	Unknown
O-GlcNAcylation (serine and threonine)	S-GlcNAc; T-GlcNAc	Unknown	Transcription

Adapted by Dawson *et al*^[2], 2012. 5mC: 5-methylcytosine; 5hmC: 5-hydroxymethylcytosine; 5caC: 5 carboxylcytosine; 5fC: 5 formylcytosine; me1: Monomethylation; me2: Dimethylation; me3: Trimethylation; me2s: Symmetrical dimethylation; me2a: Asymmetrical dimethylation; Cit: Citrulline; MBD: Methyl-CpG-binding domain; PHD: Plant homeodomain; MBT: Malignant brain tumor domain; PWWP: Proline-tryptophan-tryptophan-proline domain; BRCT: BRCA1 C terminus domain; UIM: Ubiquitin interaction motif; IUIM: Inverted ubiquitin interaction motif; SIM: Sumo interaction motif; PBZ: Poly ADP-ribose binding zinc finger; SH2: Src Homology 2.

Table 2 Abnormal DNA methylation patterns in cancer cells and related consequences

DNA hypomethylation	Consequence
Global hypomethylation	Reactivation of endoparasitic and repetitive genomic sequences Chromosomal and genomic instability
Hypomethylation of gene bodies	Activation of incorrect sites of transcription initiation
Loss of promoter methylation	Activation of metastasis and tumour promoting genes
DNA hypermethylation	Consequence
Promoter CpG island (CpGI) methylation	Tumour-suppressor gene silencing Inhibition of transcription factors suppressors
Loss of imprinting	Abnormal transcriptional inactivation Deregulation of imprinted genes

Adapted by Cock-Rada *et al*^[8], 2013.

genesis^[7].

A less studied epigenetic event is the loss of parental allele specific monoallelic expression of genes, the so-called loss of imprinting (LOI); this may be caused by hypomethylation of one of the two parental alleles (Figure 3). Insulin-like growth factor 2 LOI has been associated with an increased risk of colorectal cancer^[5,9] and other neoplasias. Data demonstrated that LOI can also cause tumour suppressor gene silencing; for example, *ARHI*, a candidate breast tumour gene, shows aberrant allele-specific silencing. Moreover, *LIT1*, an untranslated RNA, undergoes LOI in about half of patients with Beckwith-Wiedemann syndrome, determining downregulation of *CDKN1C* (which encodes KIP2, also known as p57)^[3]. Table

3 shows some of the most well known genes epigenetically regulated in cancer.

ABERRANT CGI HYPERMETHYLATION IN CANCER AND INACTIVATION OF TSGS

The fact that aberrant hypermethylation in cancer causes TSG silencing is supported by three important evidences: (1) hypermethylation has been observed alongside inherited germ line mutations and could be the specific “hit” that completely disables TSG activity (*i.e.*, *CDNK2A-p16/ARF*); (2) in sporadic cancers the tissue specificity of TSG hypermethylation causes predisposition in the specific tissues as the

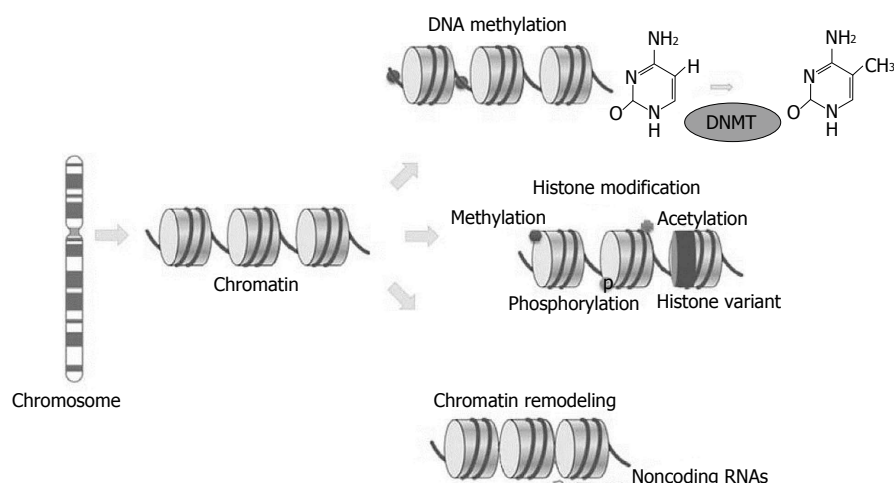


Figure 1 Epigenetic mechanisms. Variations in chromatin structure without DNA sequence modifications by (1) DNA methylation; (2) histone modifications methylation, phosphorylation and acetylation; (3) histone variant composition (dark); and (4) chromatin remodeling (sparse or dense nucleosome occupancy), and noncoding RNAs. DNMT: DNA methyltransferases. Adapted by Choi *et al*^[1], 2013.

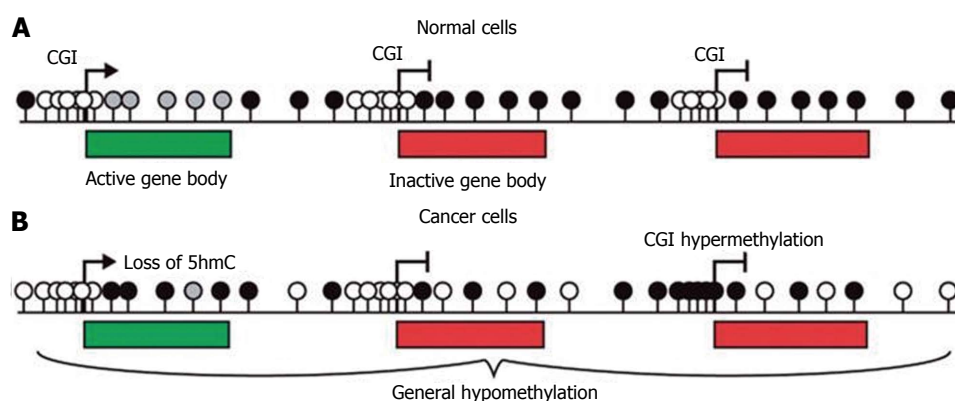


Figure 2 The methylation scenario of normal and cancer cells. A: In the mammalian genome the amount of CpGs is low and most these sites are methylated (black lollipops). CGIs are usually located in gene promoters and are generally unmethylated (white lollipops), irrespective of gene expression status. The bodies of active genes are enriched in hydroxymethylated CpGs (grey lollipops); B: In cancer cells both DNA methylation and hydroxymethylation are reduced even if some CpG island have been found to be aberrantly hypermethylated. Adapted by Sproul *et al*^[4], 2013. 5hmC: 5 hydroxymethylcytosine.

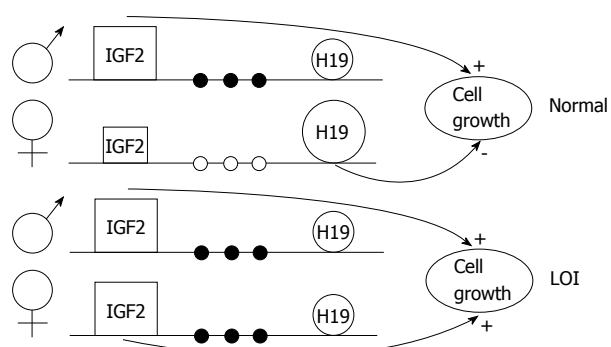


Figure 3 Model of loss of imprinting of insuline-like growth factor 2, H19 and methylation of the H19 promoter in Wilms' tumor. In normal cells, the paternal IGF2 and maternal H19 genes are expressed (shown large). Several sites upstream of H19 are methylated on the paternal allele (filled circles) and unmethylated on the maternal allele (open circles). In tumors with LOI, the maternal chromosome reverses to a paternal epigenotype, with a paternal pattern of methylation of the H19 promoter, IGF2 turned on, and H19 turned off, causing increased cell growth. LOI of H19 on the maternal chromosome, when it occurs, could occur independently or could be influenced by events in the paternal chromosome. Adapted by Steenman *et al*^[9], 1994. LOI: Loss of imprinting; IGF2: Insuline-like growth factor 2.

inherited mutations in these same genes. For example, *MLH1* mutations and hypermethylation predispose to colorectal cancer, the latter been limited to colorectal tumoural tissues. Similarly, *BRCA1* mutations predispose to breast and ovarian tumours and hypermethylation is limited to those tissues; and (3) the strongest evidence that aberrant DNA hypermethylation contributes to silencing of TSG in cancer is that demethylation of promoters is able to reactivate those genes. Many studies demonstrate the ability of 5-aza-2'-deoxycytidine to cause DNMT1 degradation and methyltransferase maintenance, leading to reactivation of hypermethylated gene promoters^[4].

The mechanism(s) responsible for aberrant promoter hypermethylation in cancer are still unclear. However, genomewide analyses of normal and tumour cells demonstrate two principal processes: active mechanisms targeting specific factors to CGIs, or passive ones deriving from a loss of protection against *de novo* methylation.

Over-expression or increased activity of DNMTs

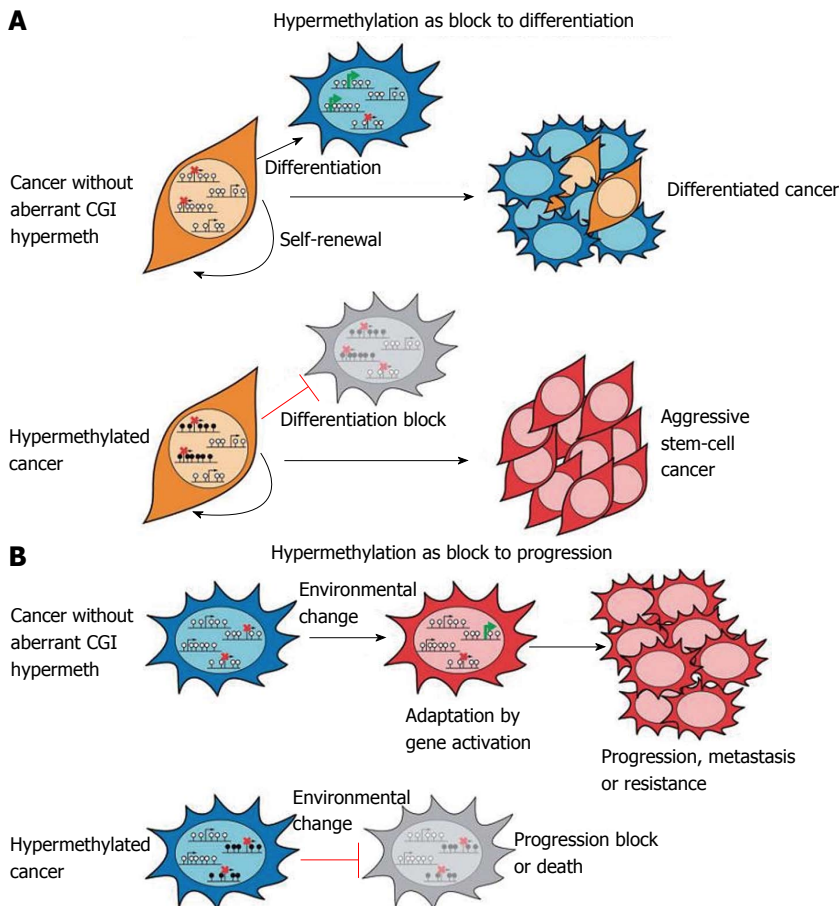


Figure 4 Hypermethylation consequences in cancer. A: Key genes required for normal cellular differentiation become hypermethylated in cancer, resulting in a block to their activation and to normal differentiation processes. Thus cancer cells express a more aggressive, stem-cell like phenotype; B: Hypermethylation of repressed CpG island promoters might prevent the activation of genes facilitating survival in changing conditions such during metastatisation. Thus, widespread hypermethylation might restrict the potential for epigenetic adaptation and result in block to progression. Adapted by Sproul *et al*^[4], 2013.

might cause aberrant CGI hypermethylation. Many studies initially reported DNMTs to be increased, but more recently it has been attributed to cell cycle regulation or to an increased number of cycling cells.

Moreover, methylome analyses demonstrate that hypermethylation is not random, but hits specific set of genes. The promoters of such genes seem to be relatively poor of retrotransposons (transposable elements that duplicate *via* RNA intermediates and are reverse-transcribed and inserted at new genomic locations) compared with hypermethylation-resistant promoters^[4].

EPIGENETIC PLASTICITY OF CANCER CELLS

Many results have demonstrated epigenetic plasticity of cancer cells, suggesting that hypermethylation could act as a block to differentiation and to progression. This derives from studies reporting gene expression profiles of aggressive tumours to be similar to those of embryonic stem (ES) cells. Thus, it has been proposed that in cancer the hypermethylation in ES polycomb repressive complexes targets might impact differentiation and maintain stem-cell-like state (Figure

4A). Moreover, hypermethylation could act in cancer progression (Figure 4B)^[4]. In fact, dissemination of tumour from the primary site requires the re-modelling of gene expression profiles. Moreover, drug resistance might result from secondary activating mutations and/or epigenetic alterations. Hypermethylation causing gene repression might provide a protection to these events and favour cancer progression^[4].

EPIGENETIC REGULATION OF EPITHELIAL-MESENCHYMAL TRANSITION

Epithelial-mesenchymal transition (EMT) and the otherway process, mesenchymal-epithelial transition, (MET) are important during cellular growth and in physiological tissue repair (wound healing) but they also play a crucial role in carcinogenesis. In normal tissues, many intercellular junctions (desmosomes, adherens, tight junctions) ensure tissue homeostasis and stability, linking epithelial cells together and to the extracellular matrix. In particular conditions, such as physiological circadian changes or tissue loss or damage, epithelial cells can acquire a mesenchymal phenotype, including

Table 3 Genes that are epigenetically regulated in cancer

Cancer-associated pathway	Gene
Cell cycle	Rb, p16 ^{INK4a} , p16 ^{INK4b} , 14-3-3, cyclin E, p14 ^{ARF}
Signal transduction	<i>ErbB2</i> , <i>RASSF1</i> , <i>LKB1/STK11</i> , <i>APC</i>
Apoptosis	<i>DAPK</i> gene, <i>Caspase-8</i> gene
DNA repair	<i>MGMT</i> , <i>MHL1</i> , <i>BRCA1</i> , <i>FNACF</i>
Carcinogen metabolism	<i>GSTP1</i> gene
Hormonal response	Oestrogen receptor gene, progesterone receptor gene, <i>RAR-β</i> gene
Senescence	<i>TERT</i> , <i>TERG</i>
Invasion/metastasis	<i>TIMP-3</i> gene, <i>E cadherin</i> gene, <i>VHL</i> gene
Transcription	Runx3, Twist, Er α , Er β , PR, RAR, vitamin D receptor
Drug responsiveness	Glutathione S-transferase, thymidylate synthase

Adapted by Choi *et al.*^[1], 2013.

an intermediate stem cell phenotype, such as in embryogenesis. Recently, many studies have aimed at understanding the role of EMT and MET in cancer progression and, in particular, in the initial processes of tissue invasion and extravasation. EMT, in fact, is minutely regulated by networks of activating/deactivating signalling pathways and also by epigenetic alterations (DNA methylation, histone modifications and by miRNAs). For this reason, anomalies in regulating those mechanisms might cause cancer initiation and progression, depending on the capability of cells to react to external and internal stimuli.

One of the main mechanisms used by epithelial tumor cells to convert into de-differentiated, mesenchymal cells is by silencing epithelial genes, such as E-cadherin, and losing cell-cell contacts. Loss of E-cadherin happens in early tumor progression, so that the EMT process is strictly related to metastatic invasion. The replacement of E-cadherin by N-cadherin (cadherin switching)^[10] depends on multiple cellular signaling mechanisms [Hedgehog, Wnt, Notch, transforming growth factor β , fibroblast growth factor (FGF), epidermal growth factor and platelet-derived growth factor]. Moreover, many epigenetic events are involved in the EMT program and are responsible of the silencing of specific epithelial markers, leading the epithelial cells to be aggressive and invasive (Figure 5)^[11].

DNA METHYLATION IN ANGIOGENESIS AND METASTASIS

During tumorigenesis, cells acquire metastatic potential following angiogenesis, induction of cell surface metalloproteases, decrease in the expression of cell-cell adhesion molecules, and increased expression of cell surface receptors that aid in motility. E-cadherin and α -4 integrins, two of the most common cell adhesion receptors, are silenced by methylation in several cancers^[12]. Similarly, intracellular basement membrane proteins (*i.e.*, NID1 and NID2) are also

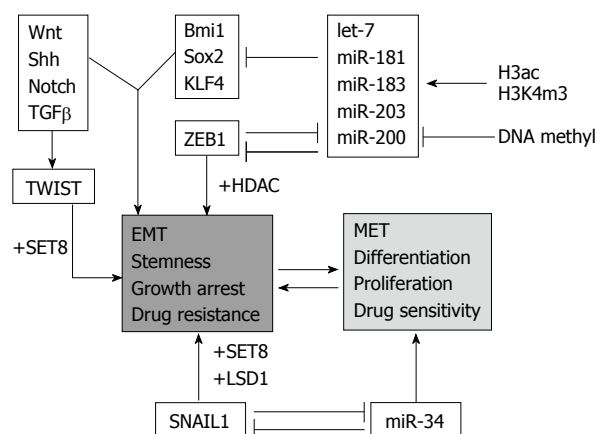


Figure 5 Molecular regulators regulating epithelial vs mesenchymal cell phenotypes and reverse process. In gray/italicised factors/processes involved in epigenetic control are highlighted. ac: Acetylation; EMT: Epithelial-mesenchymal transition; MET: Mesenchymal-epithelial transition; miR: miRNA; m3: Trimethylation; Shh: Sonic hedgehog; TGF β : Transforming growth factor beta; KLF4: Kruppel-like transcription factor4; HDAC: Histone deacetylase inhibitor; LSD1: Lysine-specific demethylase 1. Adapted by Kiesslich *et al.*^[11], 2013.

silenced by methylation in cancer. Therefore, it is evident that epigenetics could also play a critical role in the metastatic process^[13]. The phenomenon of metastasis is a complex process involving several distinct steps: tumor cells, supported by angiogenesis, infiltrate the basement membrane.

Aberrant methylation of metastasis initiation genes could be responsible of tumor invasiveness (for a detailed review, refer to Cock-Rada and Weitzman, 2013^[8]).

Several genes have been identified which regulate the metastatic process, can predict prognosis and metastasis and are used in daily clinical practice^[8]. These genes are usually involved in regulation of extracellular matrix (ECM) and angiogenesis, regulation of cell adhesion and invasion, and repressive and activating histone modifications.

In particular, in the beginning of tumour progression, cellular matrix metalloproteinases (MMPs) are able to degrade the ECM for angiogenesis. The loss of MMP regulation and release of angiogenic stimuli (FGF-2 and vascular endothelial growth factor) contribute to this process^[14]. Tissue inhibitor of metalloproteinase 2 (TIMP-2) is a MMP inhibitor suppressed in some solid and lymphoid tumours by CpGI hypermethylation^[15,16]. TIMP-3 was also found to be silenced by DNA methylation in gastric and oesophageal cancers and to correlate with poor survival^[17].

Cells migrate through the ECM, invade adjacent structures and traverse into lymphatic or blood vessels, so that are able to disseminate to distant sites, form micrometastases and eventually colonise the new organ with macrometastases (Figure 6).

EPIGENETICS AND RADIATION BIOLOGY

Exposure to ionizing radiation (IR) could cause alteration in gene expression, deregulation of cell

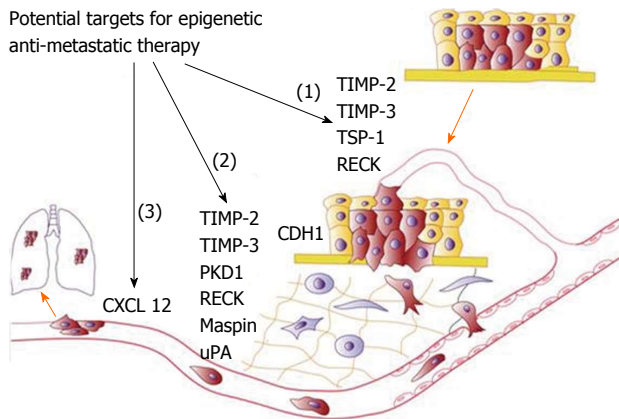


Figure 6 Schematic representation of progressive steps from initial tumour formation to establishment of metastasis include (1) tumour growth, angiogenesis and localised invasion; (2) intravasation and survival; and (3) extravasation and formation of distant tumours. For each step some genes promoting these processes and regulated by DNA methylation are indicated. Adapted by Cock-Rada *et al.*^[6], 2013. TIMP-2: Tissue inhibitor of metalloproteinase 2.

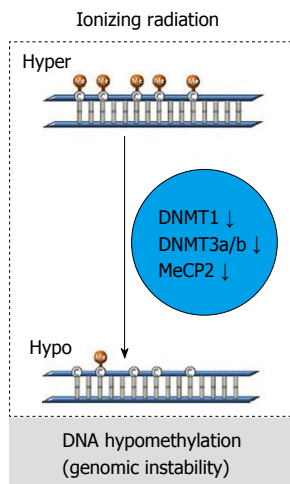


Figure 7 The figure represents the general change of global DNA methylation after radiation exposure in cancer cells. Radiation might induce a decrease in DNA methyltransferases, including DNA methyltransferase 1 (DNMT1), DNMT3a, DNMT3b, and methyl CpG binding protein 2 leading to global DNA hypomethylation and genomic instability. Adapted by Kim *et al.*^[6], 2013.

cycle, and apoptosis. Nonetheless, although several studies demonstrate that IR could also alter DNA methylation^[6], the epigenetic events following IR still need to be defined at the molecular level.

In vivo studies show that IR causes a dose-dependent and sex/tissue-specific global hypomethylation, together with a decrease in methyltransferases (DNMTs; DNMT1, DNMT3a, and DNMT3b) and methyl CpG-binding protein (MeCP2) level (Figure 7). Thus, radiation exposure could be strictly correlated to DNA hypomethylation patterns resulting in genomic instability^[6].

Interestingly, recent studies in colon cancer report a correlation between the DNA methyltransferase inhibitor 5-aza and radio-sensitivity^[18]. In breast cancer cells, fractionated IR caused DNA methylation alterations at specific loci (*TRAPP9*, *FOXC1*, and *LINE1*)^[19]. More

recently, it has been shown that radiosensitive and radioresistant cancer cells present differential DNA methylation alterations^[20]. Nevertheless, the epigenetic mechanisms at the basis of those alterations and site-specificity in DNA methylation, is still not clear and requires further studies, with the final aim of identifying useful methylation target for developing cancer targeting therapies^[6].

EPIGENETIC REGULATION OF miRNAs IN CANCER

Currently, many studies are focusing on the role of microRNAs (miRNAs) in cancer development and metastasis. Here we refer the reader to several excellent recent reviews^[21-25].

miRNAs are small, non-coding RNAs involved in post-transcriptional gene expression regulation through binding to complementary sequences in the 3'-untranslated region of messenger RNAs (mRNA). This interaction leads to mRNA cleavage or inhibited protein synthesis, thus reducing protein expression of the targeted gene. When affecting expression of oncogenes and tumor suppressor genes, common breakpoints and fragile sites (preferential sites of chromatid exchange, deletion, translocation, amplification, or integration of plasmid DNA and tumor-associated viruses), the up- or down-regulation of miRNAs could be critical for tumorigenesis and cancer progression. Indeed, a large set of aberrantly regulated miRNAs have been already identified in several tumor entities, although the biological mechanisms at the basis of miRNA regulation are still poorly studied^[21].

Recent studies demonstrated that many miRNAs could act as TSGs, but others are frequently over-expressed in human tumors possibly exerting a tumorigenic function. For example, miR-17-92 cluster shows an oncogene function, is transactivated by the *c-MYC* oncogene, and accelerates lymphomagenesis in murine models^[26]. Moreover, miR-155 has been shown to induce leukemia in transgenic murine models and plays a critical role in inflammation and immune response^[27]. miR-21 has been found in several tumor types as a regulator of important TSGs such as *PTEN1* and *PDCD4*^[25].

On the basis of their correlation with cancer, miRNAs are divided as: oncogenic, tumor-suppressive, and "context-dependent" miRNAs^[1]. In cancer cells, the loss of miRNA regulation could activate oncogenes or repress target tumor suppressor genes. Moreover, mutations could occur also in miRNA sequences, leading to lack of recognition of its binding target and thus to oncogene activation and/or tumor suppressor repression. miR-155, miR-21, and miR-17 to -92 are, for example, oncogenic miRNAs and their expression has been found to be amplified in several tumor types; furthermore, tumor-suppressive miRNAs (miR-146, -15 and -16) appear to be down-regulated in cancers.

miRNA mutations are also known to target epigenetically modifying enzymes, such as EZH2 and DNMT3. Alterations of miRNA expression, including miR-101 and miR-29, may cause extensive alterations in histone acetylation or DNA methylation of other miRNAs that target oncogenes and TSG. Since a correlation between miRNA expression and tumorigenesis has been demonstrated, miRNA might be useful therapeutics, replacing tumor-suppressive miRNA or targeting the oncogenic ones^[1].

The study of the influence of DNA methylation on miRNA transcription on a genome-wide level has been hampered by poor miRNA promoter annotation. Recently, large collaborations (ICGC and The Cancer Genome Atlas), have created extensive data sets of genetic, epigenetic, and transcriptome profiles of different tumor entities and cell lines. Furthermore, the Encyclopedia of DNA Elements (ENCODE) consortium profiled a variety of cell lines for 12 histone modifications and variants including H3K4me3 and acetylation of histone 3 at lysine 9 (H3K9ac) to disclose regulatory regions in the human genome^[28].

The resulting data allow us to extend the knowledge on tissue-specific and ubiquitous miRNA promoters. Analysis of 329 miRNA promoters revealed that 300 overlapped with or were close to a DNase I-hypersensitive site. All these analyses might permit us to estimate the number of tissue-specific miRNA promoters as suggested for miR-21^[21].

For example, miR-9-1 has been associated with a CpG island 200 bp upstream and has been found to be hypermethylated in breast cancer, melanoma, and head and neck cancer. Also, miR-200 family members have been found to be near to a CpG island^[29]. CpG island methylation correlated with down-regulated miRNA expression in breast and prostate cancer cell lines^[30]. Moreover, a correlation between loss of miRNA expression and acquisition of mesenchymal features have been observed in tumour progression^[21].

The ENCODE consortium is about to publish the genomewide DNA methylation data, completing analysis of epigenetic regulation of all gene classes including miRNAs in cell lines. In addition, cancer methylomes are analyzed and will be made publicly available by the ICGC which, for example, provided the methylomes of patients with chronic lymphatic leukemia. In conclusion, integrating data sets from different sources will enable scientists to estimate the global influence of DNA methylation on the regulation of miRNA and their aberrant behaviour in cancer^[21].

Furthermore, there has been demonstrated a possible role of miRNAs in IR-induced response *in vitro* and *in vivo*. Indeed, in murine models IR cause sex- and tissue-specific alterations in miRNA expression^[6].

miRNAs are likely epigenetically regulated but it is already well known that they can also affect expression of epigenetically regulated genes by targeting key enzymes responsible for epigenetic reactions. This group of miRNAs is called epi-miRNAs (Figure 8)^[25].

ANIMAL MODELS OF CARCINOGENESIS

Recently, many *in vivo* models of carcinogenesis have been developed in order to investigate epigenetic mechanisms and cancer progression. These models are usually derived from transgenic manipulation or toxicant exposure, inducing a tissue-specific cancer. As a consequence, these models could be useful in characterizing molecular pathways of carcinogenesis and elucidating the contribution of epigenetic and genetic alterations transforming carcinoma *in situ* to metastatic disease^[7].

Recently, these mouse models have been used to study the efficacy of epigenetic-modifying drugs (*i.e.*, 5-azacytidine, decitabine and zebularine), as well as to determine their toxicity, by treating xenograft mice and evaluating tumour size or metastasis formation. However, these results cannot be directly translated into the clinic, since because tumour biology and response to drugs in mice may be substantially different from patients^[8]. In oncology, the greatest challenge is the integration of human and animal results from translational research. This integration may shed light on how or when epigenetic dysregulation could occur in tumor and how environmental and dietary hits may influence the tumour phenotype. Additionally, these studies will provide information on susceptibility to therapy that target epigenetics (DNA demethylating agents, histone deacetylases inhibitors, or a other promising epigenetic therapies currently in trials). Thus, investigation of genetic and epigenetic profiles in cancer patients is a crucial step in the improvement of any personalized cancer therapy^[7].

EPIGENETICS AS A SOURCE OF BIOMARKERS

A biomarker is an indicator of normal biological processes, pathogenic processes, or pharmacologic response to therapeutic intervention. Biomarkers have many valuable applications in disease detection and monitoring, even if the validation and qualification of biomarkers for use with patients is time-consuming. Currently, many validated biomarkers should be used for personalized therapy. In cancer, several biomarkers have been used to reflect the extent of tumor growth and metastasis or as tools for screening and monitoring of disease. For example, our group identified *TFPI2* gene as a novel biomarker of metastatic melanoma, demonstrating that its methylation correlates with metastatic state of the disease. Moreover, we observed that circulating, methylated *TFPI2* DNA was undetectable in sera from healthy individuals but detectable in sera from patients with primary and metastatic melanomas. In particular, the presence of methylated *TFPI2* DNA in serum was strongly associated with metastatic disease, thus defining *TFPI2* a sensitive and specific biomarker of metastatic melanoma^[25].

New biomarkers, useful in clinical oncology and

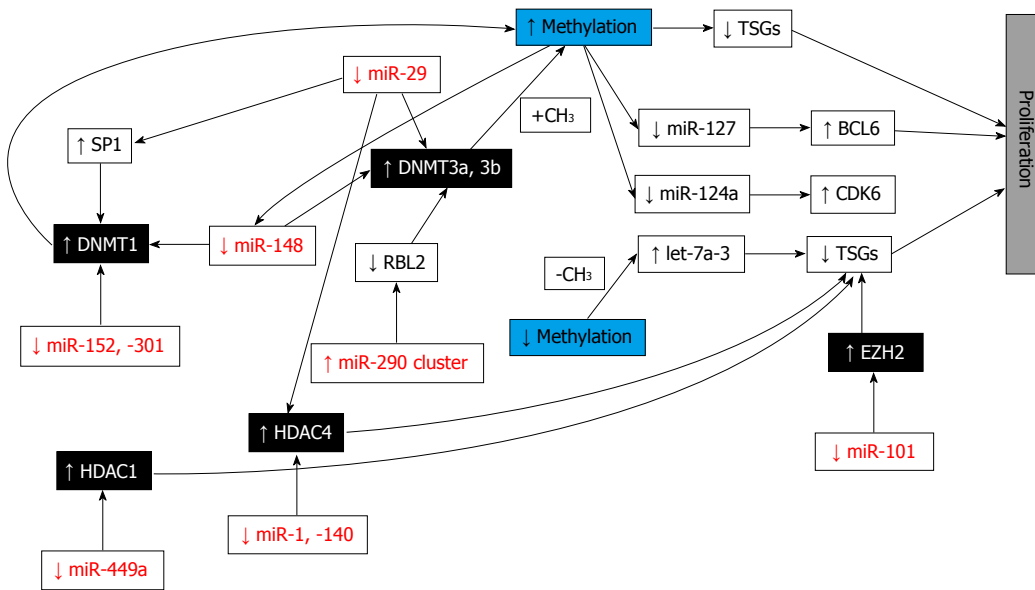


Figure 8 Epi-miRNA functions in cancer cells. Epi-miRNAs (in red) directly target epigenetic effectors (black boxes) and indirectly affect the expression of epigenetically regulated miRNAs and protein coding genes (white boxes), contributing to carcinogenesis. TSGs: Tumor suppressor genes; DNMT: DNA methyltransferase; HDAC: Histone deacetylase; EZH2: Enhancer of zeste homolog 2; BCL6: B-cell CLL/lymphoma 6; CDK6: Cyclin-dependent kinase 6; SP1: Sp1 transcription factor; RBL2: Retinoblastoma-like 2 (p130); CH₃: Methyl group. Adapted by Fabbri *et al*^[25], 2013.

based on DNA methylation, are coming from epigenomic analyses. As a consequence of the large set of alterations in methylation discovered in different tumours, a myriad of DNA methylation-based biomarkers of several human neoplasia have been reported, principally involving hypermethylation of tumor suppressor CGIs^[5].

DNA methylation is an epigenetic event that usually occurs in specific genes or in viral genome regions that are quite promising as independent diagnostic and prognostic markers. Several of these markers could be in common with two or more cancers, while others appear to be tumor-specific, providing an opportunity to determine the origin of metastases of uncertain origin. Moreover, information derived by new biomarkers could help in distinguishing similarities or differences between diseases. However, there is a growing need for evaluation and selection of the most appropriate biomarker sets, standardisation of the methods for assessment of each type of alteration, and clinical validation^[31]. This could hamper and delay implementation of useful epigenetic biomarkers.

We suggest that the readers refer to a detailed review concerning the discovery and validation of clinically relevant DNA methylation biomarkers in cervix and prostate cancers^[32].

VALIDATED METHODS FOR METHYLATION ANALYSIS AND CLINICAL SIGNIFICANCE

One of the open questions in the epigenetic field is which method of analysis of DNA methylation should be the standard in order to show evidence of clinical utility.

Healthy cells show a specific DNA methylation

pattern; alterations in this pattern, such as hypomethylation or hypermethylation, can lead to diseases, including cancer. Methylation status is currently used to classify and characterize cancers and could be of clinical significance at three levels: detection, prognosis, and prediction of treatment responses. In recent years, different methods have been developed to identify aberrant methylation signatures and may be used to identify specific biomarkers useful for tumor subtypes classification. All these technologies have been commonly classified as: (1) global approaches for detection of gross DNA methylation; (2) locus-specific methods for analysis of specific methylated CpG regions; and (3) genome-wide approaches developed to identify methylation hot-spots in the whole genome sequence (Figure 9)^[33].

Two of the most used analyses in DNA methylation are methylation-specific polymerase chain reaction (PCR) (MSP) and bisulphite sequencing PCR. These methods needed an initial bisulphite reaction converting unmethylated cytosines to "uracil" bases read as thymidines (T) after amplification by PCR. This allows to not modify methylated cytosines ("C") in 5mCpG dinucleotides that remain "C". Thus, a hypothetical bisulphite-converted sequence of 5'-AATCmCGTACTmCGCCTG-3' would be read as 5'-AATTCGTATTCGTTTG-3', where the Ts *in italics* derive from unmethylated Cs, whereas methylated CpG remains CpG (here underlined). After bisulphite transformation, DNA could be analysed to specifically distinguish between methylated and unmethylated cytosines.

In MSP, two distinct set of primers containing at least two CpG dinucleotides within the primer sequences are used: U primers detect unmethylated CpGs while M primers detect methylated CpGs. In a

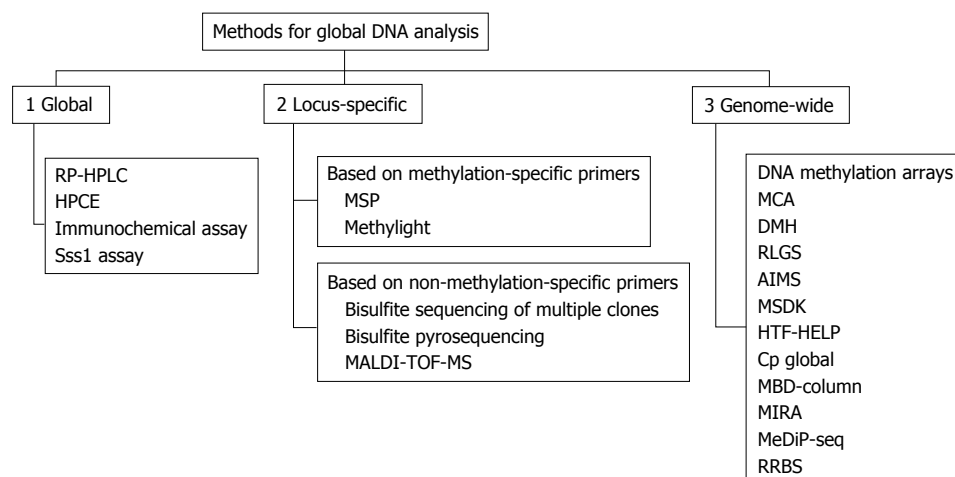


Figure 9 Methods for genomic DNA methylation analysis, classified as global, locus-specific and genome-wide. In the case of locus-specific approaches, techniques were divided depending on the use of methylation-specific primers or not. RP-HPLC: Reverse-Phase high-performance liquid chromatography; HPCE: High performance capillary electrophoresis; Sss1 assay: Methyl group acceptance assay; MSP: Methylation-specific polymerase chain reaction (PCR); MALDI-TOF-MS: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MCA: Methylation CpG island amplification; DMH: Differential methylation hybridization; RLGS: Restriction-landmark genomic scanning; AIMS: Amplification of inter-methylated sites; MSDK: Methylation-specific digital karyotyping; HTF-HELP: HpaII tiny fragment enrichment by ligation-mediated PCR; MBD-column: Methylated DNA binding column; MIRA: Methylated CpG island recovery assay; MeDiP-seq: Methyl-DNA immunoprecipitation and sequencing; RRBS: Reduced Representation Bisulfite Sequencing. Adapted by Toraño *et al.*^[33], 2011.

methylated sample, only M primers produce a PCR band but not U primers; vice versa in samples not methylated. Requiring a simple PCR machine, MSP is adequate in the analysis of large numbers of clinical samples and has been successfully used in tumour methylation studies. The main limit of MSP is that the result obtained is purely qualitative. For quantification of methylation levels, bisulphite sequencing PCR has been developed. In this method, after PCR, amplified DNA need to be appropriately cloned into a vector and then 5-10 clones independently sequenced in order to read all CpG sites included in the amplified sequence, giving a global representation of the cellular methylation status^[34].

More recently, many methods focusing on specific single-CpG have been developed, such as combined bisulfite restriction analysis (COBRA)^[35], MethyLight^[36], and bisulfite pyrosequencing^[37]. In COBRA bisulfite conversion and PCR amplification are maintained; then, PCR product are digested by restriction enzyme in a methylation-dependent manner. Digestion proceeds in the recognition sequence only if the CpG site is protected from bisulfite conversion by methylation. Thus, the presence of restriction products indicates methylation in the PCR amplicon. MethyLight is a bisulfite-dependent, fluorescence-based, quantitative real-time PCR method for DNA methylation. This technique includes specific priming combined with methylation-specific fluorescent probing, allowing one to sensitively detect very low frequencies of hyper-methylated alleles.

Another method based on chemical modification of genomic DNA with sodium bisulfite is pyrosequencing. This technique allows quantification of methylation at individual CpG dinucleotides into an amplified DNA fragment. Different from MSP, primers for amplification are designed from regions which contain no CpG

dinucleotides and differences between methylated and unmethylated sequences are seen only after pyrosequencing. The main advantage of this method is that it allows high-resolution analysis of methylation and detection of small changes in methylation at each CpG, and also in samples containing large amounts of normal DNA.

All the above-mentioned methods are sensitive, specific, and relatively inexpensive, but none allows one to analyse the whole genome, which includes about 28 million CpGs. For a global analysis, recent microarray-based methods have been designed, including direct hybridization^[38], methylated DNA immunoprecipitation (MeDIP)^[39] and HELP assay (HpaII tiny fragment enrichment by ligation-mediated PCR)^[40]. Direct hybridization to CpG island arrays is able to detect DNA methylation in several CpG sites. It is based on the use of methylation-specific oligonucleotides arrayed on glass slides, detecting all possible methylation in target genes. MeDIP is a genome-wide method based on an antibody that recognises 5-methylcytosine in methylated DNA sequences. This technique is used for either array-based hybridization (MeDIP-chip) or high-throughput sequencing (MeDIP-seq). However MeDIP presents a significant limitation: restricted resolution typical of array-based technology. The HELP assay is comparative isoschizomer profiling of DNA methylation. DNA is digested by HpaII in parallel with MspI (resistant to DNA methylation), and then the HpaII and MspI products are either amplified by ligation-mediated PCR and hybridized using separate fluorochromes to a customized array, or directly sequenced^[41].

All these methods based on next generation sequencing technology which produces a huge amount of information on methylomes. Generally, genome-wide technologies are very useful for genome-wide DNA methylation analysis but they are relatively expensive

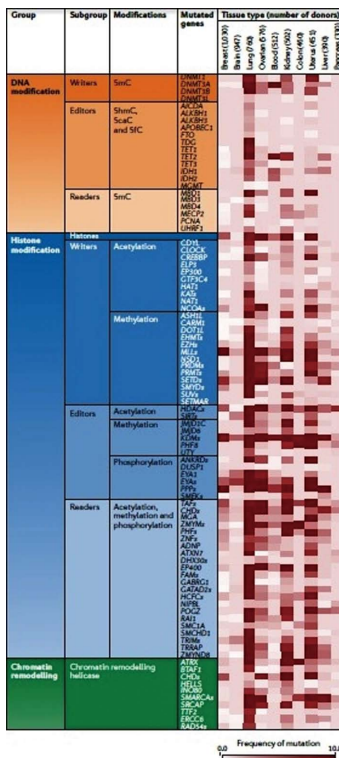


Figure 10 Some of the most known mutated genes classified in groups based on DNA modification, histone modification and chromatin remodelling enzymes. The number of analysed tumour tissues is given. Several epigenetic enzymes present high frequencies of mutations in distinct tumor types. These data are not adjusted for chromosomal instability or mutator phenotypes, hence the frequencies reflect a combination of probable driver mutations in epigenetic regulators and the background mutation rate for the tumour type. 5caC: 5 carboxylcytosine; 5fC: 5 formylcytosine; 5hmC: 5 hydroxymethylcytosine; 5mC: 5 methylcytosine. Adapted by Plass *et al.*^[42], 2012.

and cannot be currently introduced in routinely clinical studies (Table 4). However, methylation profiling could be a useful tool to better understand the biological mechanism at the basis of tumorigenesis and provide insight into prevention strategies to reduce the burden of cancer.

MUTATIONS IN REGULATORS OF THE EPIGENOME

Thanks to advances in sequencing technologies thousands of cancer genomes and methylomes have been re-sequenced and new coding-gene mutations, genetic rearrangements, DNA copy-number alterations and alterations in either regulatory sequences or epigenetic patterns have been discovered. In addition, abnormalities in epigenetic enzymes and pathways, including DNA methylation or demethylation, histone modification, and chromatin remodelling processes, have been highlighted (Figure 10). For example, novel gene mutations have been uncovered in different tumours (*IDH1* or *DNMT3A* in acute myeloid leukemia, mitochondrial succinate dehydrogenase in paragangliomas or gastrointestinal stromal tumours, AT-rich interactive domain 1A-ARID1A in NSCLC; CREB-binding protein-CREBBP, E1A-binding protein p300

Table 4 Comparison of methylation arrays *vs* ultra-deep sequencing for DNA methylation analysis

	Methylation arrays	Ultra-deep sequencing
CpG coverage	+	+++
Sensitivity	+++	++/+ (antibody-based)
Time consuming	++	++
Data analysis	+++	+
High-throughput	+++	+
Price	++	+ / ++ (price decreasing)

Adapted by Toraño *et al.*^[33], 2011.

and MLL in small-cell lung cancer; H3F3A in paediatric glioblastoma; and MLL2 and SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin (SMARCA4) in medulloblastoma^[42].

A major challenge for researchers will be to investigate the role of these mutations in tumorigenesis.

ROLE OF SNPS ON EPIGENETIC REGULATION AND CANCER

Beside the discovery of new mutations, genome-wide association studies have identified various single nucleotide polymorphisms (SNPs) correlated with increased risk of cancer. Interestingly, those SNPs are preferentially located in functional enhancers in ES cells and might confer cancer susceptibility by altering the cellular chromatin setting. In fact, a correlation between genetic variations and in gene expression changes have been demonstrated to involve chromatin accessibility of transcription factor (TF) binding sites, such as SNPs (CpG SNPs) that create or delete CpGs and influence the binding of specific TFs. Further and deeper studies on this association could reveal the functional link among epigenetic, genetic variation and phenotype^[43].

METHYLTRANSFERASE INHIBITORS IN CANCER THERAPY

The major clinical impact of the rising knowledge of epigenetic mechanisms is the possibility of defining epigenetic cancer therapy which inhibit methylation events in order to increase therapeutic efficacy. Recently, many epigenetic-modifying drugs have been introduced in combination with standard chemotherapy treatments in cancer patients. Nonetheless, those drugs may lack specificity, since they modulate global expression more than being gene-specific. However, different from other drugs, they could be able to restore TSG expression or loss-of-function phenotypes; thus, combined therapy could be a good therapeutic strategy.

Unfortunately, it is evident that epigenetic biology is complex; indeed, there are quite a number of scientific and pragmatic challenges, many of which are summarized in Table 5^[44].

Currently, the most common epigenetic drugs are the DNMT inhibitors azacytidine and decitabine,

Table 5 Epigenetic drug discovery challenges

Category	Issues
Target selection	Few activating mutations, translocations or syntethic lethal relationships known limited high-quality antibodies to epigenetic proteins and histone marks (<i>e.g.</i> , confirm target expression linkage of target to mark) Biology driving cancer phenotype unknown or poorly understood Post-translation modification of histone <i>vs</i> non-histone substrates by "epigenetic" targets unclear
Chemistry	Existing chemical librairies may not have adequate diversity to provide goog strating points Few crystal structures solved; are structrues relevant if not reflecting complete complex?
Assay development	Few reference compunds to establish assy signal window, sensitivity, reproducibility Are binding or enzyme configured to properly reflect physiological context? Production of active enzymes is difficult, may require multimeric complex and specific sunstrate (nucleosome, histone, non-histone)
<i>In vivo</i> biology	Limited high-quality antibodies to epigenetic proteins and histone marks (quantify mark or target gene product) Histone marks and target genes slow to change, require longer-duration studies to assess engagement (PD biomarker) May necessitate higer compund requirement to conduct studies, earlier optimatation of PK properties than traditional paradigm May require novel models for tumors with mutation or traslocations
Toxicology	Acute and/or chronic liabilities of specific isofrom targed epigenetic therapies currently unknown Knockout animal data limited; inducible knockouts, dominant negatives preferred but more scarce and technically challenging
Clinical	Identify and implement appropriate patient selection markers, more challenging if not activating mutation (overexpression, gene profile?) Identify and implement suitable PD marker (posttranslational modification or mark, target gene, surrogate tissue or tumor?) Epigenetic changes at metastatic sites can differ from primary tumor, which should be targed clinically?

Adapted by Campbell *et al*^[44], 2014.

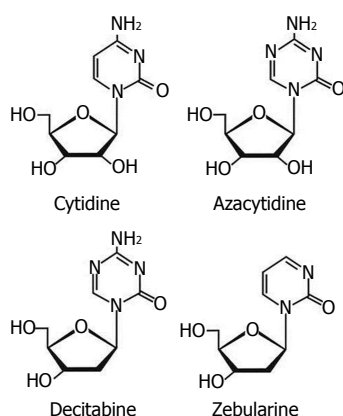
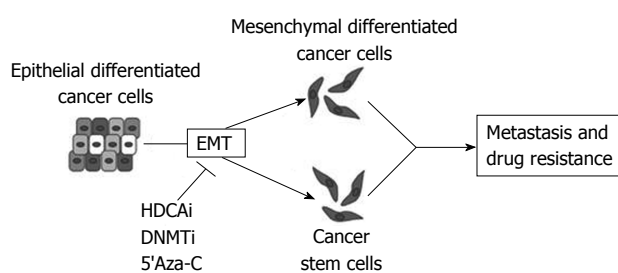
**Figure 11 DNA methyltransferase inhibitors, analog to nucleoside.**

Figure 12 Different classes of epigenetic drugs could inhibit epithelial-mesenchymal transition which plays a crucial role in tumor progression generating both mesenchymal differentiated and stem cancer cells. HDACi: Histone deacetylase inhibitor; DNMTi: DNA methyltransferase inhibitor; 5'Aza-C: 5-azacytidine are given as examples of demethylating agent. Adapted by Kiesslich *et al*^[41], 2013.

although their clinical efficacy is limited by toxicity and chemical instability. Zebularine [1-(β -D-ribofuranosyl)-1,2-dihydropyrimidin-2-one] is a DNMT inhibitor characterized by more stability and less toxicity with a

inhibitory effect on cytidine deaminase (Figure 11).

All these drugs are based on the rationale that, unlike genetic mutations, epigenetic alterations is potentially reversible, thus being an attractive target for cancer therapy.

Since hypermethylation of tumor suppressor genes and overexpression of DNMTs are crucial events for tumor progression, the possibility of de-methylating DNA sequences seems a good strategy for cancer therapy. DNMT inhibitors, in fact, can allow re-expression of aberrantly silenced genes and restore their normal function. Azacytidine (Vidaza; Celgene) and decitabine (5 aza 2' deoxycytidine) (Dacogen; SuperGen) have been approved by the Food and Drug Administration (FDA) for current management of acute myeloid leukemia and myelodysplastic syndrome. Azacytidine has also been approved by the FDA and the European Medicines Agency for use against chronic myelomonocytic leukemia. More recently these two drugs have also been introduced in clinical trials in patients with solid tumors.

Zebularine is a novel member of the nucleoside DNMT inhibitor family, not yet used routinely in clinical practice. Although much *in vitro* data show good results, especially in terms of less toxicity compared to azacytidine or decitabine, zebularine use for future clinical trials is needed^[45].

Moreover, recent studies have also demonstrated a possible use of epigenetic-modifying drugs in targeting invasion, metastasis and drug resistance, all involving EMT (Figure 12)^[11].

CONCLUSION

It is already well known that cancer is a heterogeneous disease and an integrated genome, epigenome, and

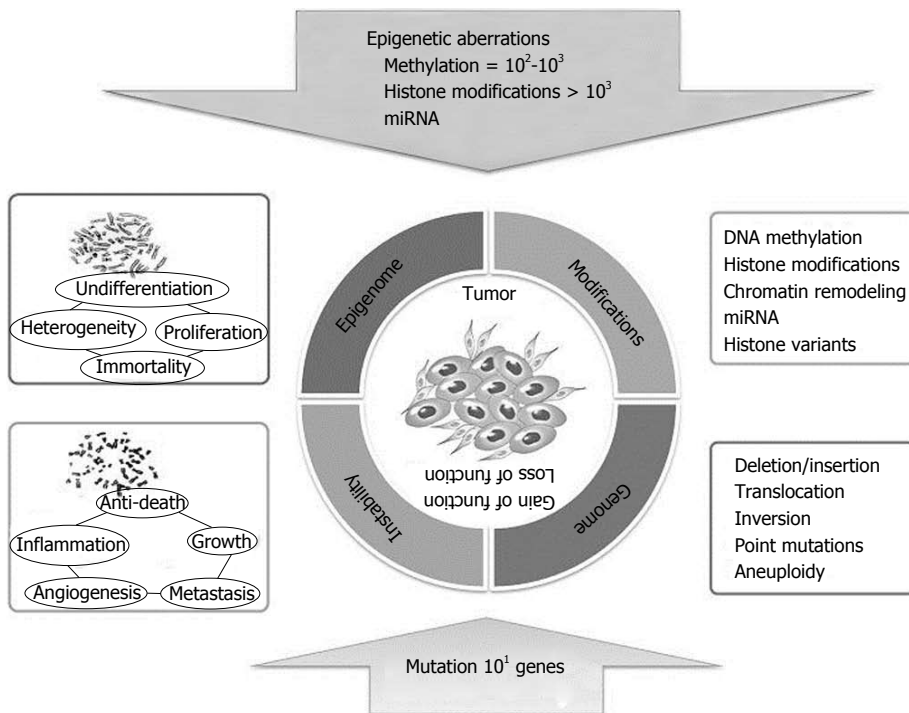


Figure 13 Epigenetics and genetics cooperates in cancerogenesis. Adapted by Choi *et al*^[1], 2013.

transcriptome analysis seems necessary to help clinicians to find good therapeutic strategies to treat this complex disease.

Only in recent years have researchers begun to integrate data deriving from both genetic and epigenetic alteration analyses, including mutations, CNVs, structural changes, epigenetic profiles, and expression changes in both coding and non-coding RNAs. Thanks to these studies, it is now well accepted that epigenetic abnormalities can play a crucial role in tumor initiation and development. In addition, it is now recognized that tumor cells present epigenetic silencing at higher frequency than mutations. Indeed, in tumour cells, hyper- or hypomethylation, histone modifications, and miRNA expression dysregulation are present in thousands of genes, while mutations affect only tens of genes, although all of them determine gene inactivation (Figure 13).

Epigenetic events can be useful biomarkers for detecting disease and predicting therapeutic efficacy. The epigenome undergoes all the above described during tumor initiation driving tumor cell heterogeneity, and consequently progression. Notably, those patterns are stable but reversible depending on the cellular environment, while mutations remain irreversibly locked into the cancer genome. For this reason, epigenetic events could be “drugable” targets for reversing epimutational effects and associated phenotypes.

Basing on the hypothesis that epigenetic agents may enhance sensitivity to conventional drugs (e.g., platinum or taxane chemotherapy), many efforts have been made for using epigenetic agents to re-sensitize tumours recurrent or refractory to first-line treatment.

Advances in genome-wide methylation analyses

and the combination of new epigenetic drugs with conventional therapies could offer new hope for cancer patients, providing in the near future more effective patient-tailored treatments.

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Therapeutic targets in gastrointestinal stromal tumors

Jia-Qing Zhu, Wen-Bin Ou

Jia-Qing Zhu, Wen-Bin Ou, College of Life Sciences, Zhejiang Sci-Tech University, Hangzhou 310018, Zhejiang Province, China

Wen-Bin Ou, Zhejiang Provincial Key Laboratory of Applied Enzymology, Yangtze Delta Region Institute of Tsinghua University, Jiaxing 314006, Zhejiang Province, China

Author contributions: Zhu JQ preformed research, analyzed data, consulted literatures and wrote the paper; Ou WB consulted literatures, provided ideas, designed research, contributed new reagents, analyzed data, and wrote the paper.

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Correspondence to: Dr. Wen-Bin Ou, College of Life Sciences, Zhejiang Sci-Tech University, 866 Yuhangtang Road, Hangzhou 310018, Zhejiang Province, China. ouwenbin@tsinghua.org.cn
Telephone: +86-573-82582765

Fax: +86-573-82582765

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Abstract

Gastrointestinal stromal tumors (GISTs) are the most common type of mesenchymal tumor of the gastrointestinal tract. The tumorigenesis of GISTs is driven by gain-of-function mutations in *KIT* or platelet-derived growth factor receptor α (*PDGFRA*), resulting

in constitutive activation of the tyrosine kinase and its downstream signaling pathways. Oncogenic *KIT* or *PDGFRA* mutations are compelling therapeutic targets for the treatment of GISTs, and the *KIT*/*PDGFRA* inhibitor imatinib is the standard of care for patients with metastatic GISTs. However, most GIST patients develop clinical resistance to imatinib and other tyrosine kinase inhibitors. Five mechanisms of resistance have been characterized: (1) acquisition of a secondary point mutation in *KIT* or *PDGFRA*; (2) genomic amplification of *KIT*; (3) activation of an alternative receptor tyrosine kinase; (4) loss of *KIT* oncoprotein expression; and (5) wild-type GIST. Currently, sunitinib is used as a second-line treatment for patients after imatinib failure, and regorafenib has been approved for patients whose disease is progressing on both imatinib and sunitinib. Phase II/III trials are currently in progress to evaluate novel inhibitors and immunotherapies targeting *KIT*, its downstream effectors such as phosphatidylinositol 3-kinase, protein kinase B and mammalian target of rapamycin, heat shock protein 90, and histone deacetylase inhibitor. Other candidate targets have been identified, including *ETV1*, *AXL*, insulin-like growth factor 1 receptor, *KRAS*, *FAS* receptor, protein kinase c theta, *ANO1* (*DOG1*), *CDC37*, and aurora kinase A. These candidates warrant clinical evaluation as novel therapeutic targets in GIST.

Key words: Gastrointestinal stromal tumors; Tyrosine kinase inhibitors; *KIT*; Platelet-derived growth factor receptor α ; Targets

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Core tip: Oncogenic *KIT* and platelet-derived growth factor receptor α (*PDGFRA*) mutations are compelling therapeutic targets in gastrointestinal stromal tumors (GISTs), and the *KIT*/*PDGFRA* kinase inhibitors imatinib, sunitinib, and regorafenib are the standards of care for patients with unresectable or metastatic GIST. However, most patients eventually develop resistance to these kinase inhibitors, resulting in an urgent need to identify biologically rational targets for novel therapies.

Herein, we review advances in the research on GIST and the therapies that are used to treat it. Additionally, we discuss novel agents, targets, and strategies for the future treatment of GIST.

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INTRODUCTION

Gastrointestinal stromal tumors (GISTs) were originally described as smooth muscle or neural tumors of the gastrointestinal (GI) tract; however, in 1983, Mazur *et al*^[1] referred to GISTs as "stromal tumors"^[2,3]. Subsequent studies identified the interstitial cells of Cajal as the origin of GISTs. In 1998, activating mutations of the *KIT* receptor tyrosine kinase (RTK) were found in GISTs^[4]. In 2003, platelet-derived growth factor receptor α (*PDGFRA*) mutations, an alternative target, were identified in GISTs that lacked *KIT* mutations^[5].

GISTs are the most common mesenchymal tumors of the GI tract and are frequently seen in the stomach (60%), small intestine (25%), colorectum (5%-10%) and occasionally in the esophagus and appendix^[5]. Histologically, GISTs may be composed of spindle cells (70%), epithelioid cells (20%), or a mixture of these types (10%)^[6]. Morphologically, GISTs may be mistaken for smooth muscle neoplasms, such as leiomyoma and leiomyosarcoma (Figure 1)^[6]. Consensus guidelines for GIST prognosis, accentuate risk stratification based on the tumor volume and mitotic index of the primary tumors (Table 1)^[2].

The majority of GISTs contain oncogenic mutations of *KIT* (approximately 85%) or *PDGFRA* (approximately 5%-10%)^[2,4-6]. The resulting mutant oncoproteins are crucial for GIST oncogenesis, proliferation, and survival, as demonstrated by the clinical successes of small molecule therapeutics targeting *KIT* and *PDGFRA*^[7-9]. Imatinib, sunitinib, and regorafenib are the standard first-, second- and third-line therapies, respectively, in patients with inoperable GISTs^[10-12], and adjuvant imatinib is used in patients with localized GISTs with a high risk of recurrence^[13].

Except from imatinib, sunitinib, and regorafenib, which target the activated oncoproteins *KIT* and *PDGFRA* in inoperable or metastatic GIST, the increasing novel drugs are currently in clinical trials, and additional potential therapeutic targets have been identified. Herein, we summarize these agents, targets, and strategies for the future treatment of GIST.

KIT AND PDGFRA ARE MAJOR THERAPEUTIC TARGETS IN GISTS

Oncogenic mutant *KIT* and *PDGFRA* play a critical function in the initiation of the transformation event that leads to

GIST. Mutations in *KIT* are usually found in the regulatory and dimerization domains, which are located in the extracellular region encoded by exon 9 (approximately 13% of GISTs), the juxtamembrane region encoded by exon 11 (approximately 66% of GISTs), or the tyrosine kinase (TK)[I] [adenosine triphosphate (ATP) binding pocket]; and TK[II] (activation loop) domains encoded by exon 13 (approximately 1% of GISTs) and exon 17 (approximately 0.6% of GISTs), respectively^[2,14,15]. Five percent to ten percent of GISTs contain mutations in *PDGFRA* exon 12 (juxtamembrane region) (1.5%) or exon 18 (activation loop) (5.6%). The remainder (10%-12%) are wild-type for both *KIT* and *PDGFRA*^[2,6]. The percentage of population of *KIT* and *PDGFRA* mutations is shown in Figure 2^[2].

GISTs harboring insertions, deletions, and missense mutations in *KIT* exon 11 can be found throughout the GI tract^[16]. A enhanced metastasis and proliferation has been associated with loss of heterozygosity at the *KIT* locus^[17,18]. The vast majority of GIST cases with alterations of *KIT* in exon 9 involve an insertion of six base pairs, resulting in the duplication of Ala and Tyr residues. These mutations often occur in high-risk primary GISTs of the small intestine^[17,19,20], advanced or relapsed GISTs^[18,21]. A recent study demonstrated that GISTs harboring *KIT* exon 17 and exon 13 mutations show slightly overrun population among a subset of GISTs. Most of single base pair substitution *KIT* mutations in exon 13 and 17 in small intestinal GISTs, have no marked effects on the clinicopathologic characteristics when compared to the "average" small intestinal GIST^[22].

The majority of *PDGFRA* exon 14 and 18 alterations are missense mutations. GISTs harboring *PDGFRA* mutations are confined to the stomach and omentum. These tumors are shortage of *KIT* expression, they typically present an epithelioid morphology, and they are commonly associated with a benign prognosis^[23,24]. GISTs harboring a D842V *PDGFRA* exon 18 mutation are resistant to imatinib and other RTK inhibitors^[25-28].

Inhibition of *KIT* or *PDGFRA* kinase activity by imatinib results in an objective response in approximately 80% of metastatic GIST patients (approximately 50% partial response, approximately 30% stable) with a 3-year survival rate of 69%-74%^[8]. However, the median survival of metastatic GIST patients was 19 mo in the pre-imatinib period^[10,15]. Constitutive activation of *KIT* or *PDGFRA* results in the activation of downstream signaling intermediates necessary for proliferation, survival, adhesion, and blockage of differentiation, including the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) and RAF/mitogen-activated protein kinase (MAPK) pathways. Targeting *KIT*/*PDGFRA* and its downstream intermediates has proven to be an effective strategy in the treatment of GISTs^[29-32].

MECHANISMS OF IMATINIB RESISTANCE

Imatinib, an ATP-competitive inhibitor of *KIT* and *PDGFRA*, is the first-line therapy for patients with

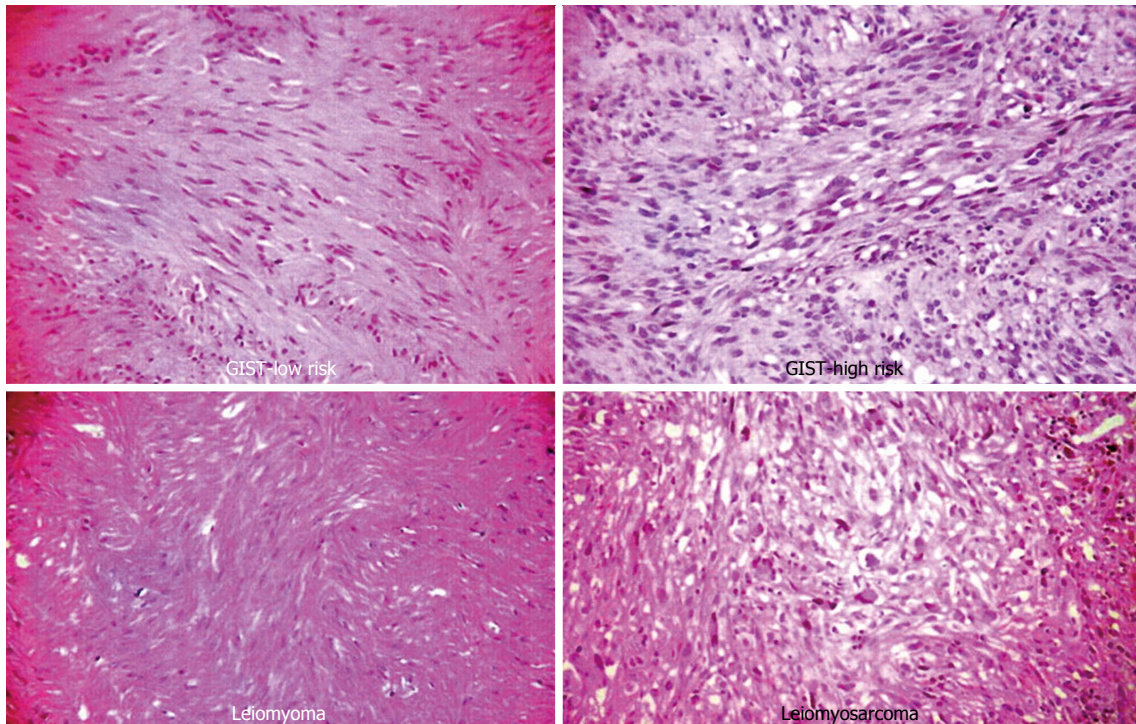


Figure 1 Morphologic similarities of low-risk gastrointestinal stromal tumor and leiomyoma and of a high-risk gastrointestinal stromal tumor and leiomyosarcoma. GIST cells can be divided into 3 types: spindle cell (70% of cases), epithelioid cell (20% of cases), and mixed cell (containing a mixture of spindle and epithelioid cells). GIST: Gastrointestinal stromal tumor.

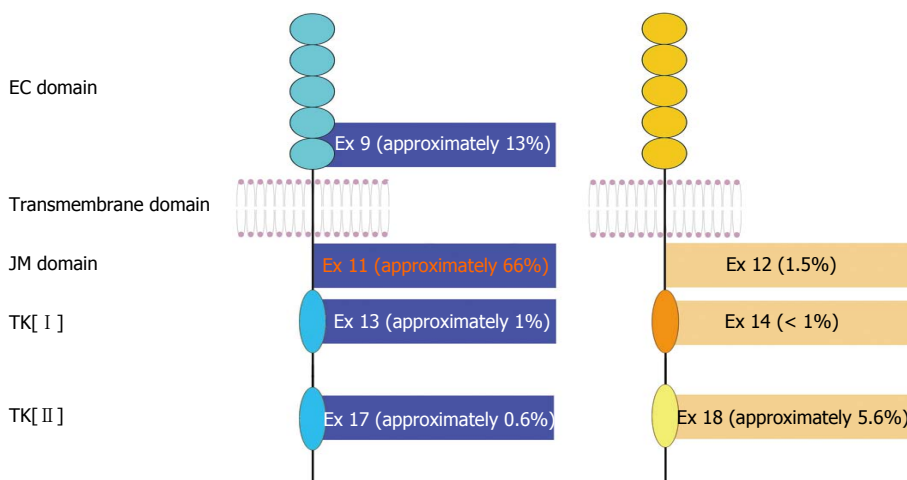


Figure 2 Schematic structure of *KIT* and platelet-derived growth factor receptor α receptor tyrosine kinases and distribution of *KIT* mutations in gastrointestinal stromal tumor. EC: Extracellular; JM: Juxtamembrane; TK[I]: Tyrosine kinase domain I; EX: Exon.

advanced GIST or primary GIST with a significant risk of recurrence after surgery^[28,33-35]. Among patients with advanced GIST, 75% to 90% will show a response to imatinib^[15]. Analysis of the crystal structure of the *KIT*-imatinib complex reveals that the drug fills a hydrophobic region of the ATP binding pocket, effectively blocking ATP binding and inactivating *KIT* and its downstream signaling^[36,37].

Despite the dramatic clinical success of imatinib, most inoperable GIST patients eventually develop resistant to imatinib. Imatinib resistance in GIST is classified as either primary or secondary imatinib

resistance. Approximately 10% of GISTs demonstrate primary imatinib resistance of clinical progression within 3 to 6 mo of the start of treatment^[28,38]. Primary imatinib resistance is usually observed in tumors that lack *KIT* or *PDGFRA* mutations (wild-type GISTs), but it is also common in tumors harboring *KIT* exon 9 mutations^[28,38]. Approximately 40% to 50% of GIST patients experience secondary imatinib resistance of clinical progression after 12-36 mo of response or disease stabilization. Molecular studies showed that activated *KIT* expression in imatinib-resistant tumors was similar to or greater than those typically found in

Table 1 Risk stratification of primary gastrointestinal stromal tumor by mitotic index, size and anatomic location^[2]

Prognosis of primary GIST		
Risk	Size (cm)	Mitotic count (per 50 HPF)
Very low risk	< 2	< 5
Low risk	2-5	< 5
Intermediate risk	< 5	6-10
	5-10	< 5
High risk	> 5	> 5
	> 10	> Any mitotic rate
	Any tumor	> 10

HPF: High power fields; GIST: Gastrointestinal stromal tumor.

untreated GISTs^[15]. Secondary *KIT* mutations were rare in GISTs with primary resistance but often found in GISTs with secondary resistance (10% vs 67%; $P = 0.002$). Polyclonal secondary kinase mutation was detected in 18.8% patients. The secondary kinase mutations were nonrandomly distributed and were associated with attenuated imatinib sensitivity compared with *KIT* exon 9 and exon 11^[15]. Mechanisms of acquired resistance include secondary mutations in *KIT* or *PDGFRA*, genomic amplification of *KIT*, or activation of an alternative RTK^[6,14,39-46]. An even more challenging resistance mechanism, seen in approximately 5%-10% of clinically progressing *KIT*-mutant GISTs involves a transition from dependence on oncogenic *KIT* to a new imatinib-insensitive oncogenic driver, accompanied by the loss of former *KIT* expression^[39,40].

NOVEL INHIBITORS IN PRE-CLINICAL MODELS AND CLINICAL TRIALS

Tumorigenesis is a complex, multi-step process, and oncogenic RTK proteins frequently play key roles^[47] (Table 2). Oncogenic RTK mutations can lead to constitutive kinase activation and thereby enhance growth and survival in cancer cells^[48,49]. Tyrosine kinases can be divided into two categories: receptor tyrosine kinases and non-receptor tyrosine kinases. At present, approximately 90 types of TK members have been identified, including 58 RTKs, such as PDGFR, epidermal growth factor receptor (EGFR), fibroblast growth factor receptor, and 32 non-RTKs^[50]. Oncogenic RTK mutants are useful therapeutic targets, as shown by the clinical benefit of small molecular inhibitor therapies in chronic myeloid leukemia (BCR-ABL)^[51], metastatic breast cancer [human epidermal growth factor receptor 2 (HER2)]^[52], GIST (*KIT*/*PDGFRA*)^[8], and non-small-cell lung cancer [EGFR, hepatocyte growth factor receptor (MET), anaplastic lymphoma kinase, HER2]^[47,53-62].

Sunitinib is an oral multi-target tyrosine kinase inhibitor (TKI) with activity against *KIT*, *PDGFRA*, FMS-Like Tyrosine Kinase 3, Vascular endothelial growth factor receptor (VEGFR), and orphan receptor tyrosine

kinase^[63]. Sunitinib is approved for use as a second-line therapy for patients with imatinib-resistant GIST^[9,64,65]. A clinical benefit of sunitinib was seen in common primary GIST with *KIT* exon 9 (58%), *KIT* exon 11 (34%), and wild-type *KIT*/*PDGFRA* (56%)^[9]. Progression-free survival (PFS) was greater improvement for patients with a wild-type genotype ($P = 0.0356$) or with primary *KIT* exon 9 mutations ($P = 0.0005$) than for those with *KIT* exon 11 mutations. Overall survival (OS) showed the similar pattern. The PFS and OS were greater improvement for patients with secondary *KIT* exon 13 or 14 mutations than for those with exon 17 or 18 mutations^[9]. The safety and efficacy of regorafenib in metastatic or unresectable GIST patients after failure of imatinib and sunitinib were evaluated in phase III, and the results showed that regorafenib can markedly improve PFS compared with control in metastatic GIST patients with progression after standard treatments^[12,66]. Currently, regorafenib has been approved for patients whose tumors are progressing on both imatinib and sunitinib. A large number of therapies are in various stages of pre-clinical and clinical trial development and are summarized in Table 2^[10,13,14,21,30,64,67-84]. These therapies can be divided into four groups: TKIs, PI3K/mTOR inhibitors, heat shock protein 90 (HSP90) inhibitors, and others.

Multiple TKIs, including nilotinib, sorafenib, dasatinib, vatalanib, and motesanib, are being investigated as potential therapies for GIST. Nilotinib, an inhibitor of *KIT*, *PDGFRA* and BCR-ABL, has been shown to be active in a small series of imatinib-resistant and sunitinib-resistant GIST patients in a phase I study^[67,71,74,85]. Sorafenib, an inhibitor of RAF kinase, VEGFR, PDGFR, and *KIT*, inhibited *KIT* activity in some *KIT* primary and secondary mutations in a phase II trial in imatinib- and sunitinib-resistant GIST^[69,80,86,87]. Dasatinib, a dual SRC/ABL kinase inhibitor, binds and inactivates wild-type and mutant *KIT* regardless of the conformation of the *KIT* activation loop^[42,43]. Linsitinib (OSI-906) is a selective inhibitor of insulin-like growth factor receptor (IGFR)/insulin receptor. The combination of imatinib and linsitinib has been shown to be effective in wild-type GIST with insulin-like growth factor 1 receptor (IGF1R) overexpression or amplification^[88]. Vatalanib (PTK787) and motesanib (AMG706), multi-kinase inhibitors, have been evaluated in phase II trials for patients who are resistant to both imatinib and sunitinib^[89,90]. Vatalanib has shown activity in patients with imatinib-resistant or both imatinib- and sunitinib-resistant GIST^[89,90]. Motesanib treatment was shown to have acceptable toxicity, and it resulted in disease stabilization in GIST patients^[82].

The PI3K/AKT/mTOR pathway is crucial for proliferation and survival in GIST^[29,30,68,91-93]. Preclinical experiments have confirmed that targeting the PI3K/AKT/mTOR pathway is a rational therapeutic strategy. Early studies with mTOR inhibitors have shown limited success, possibly due to feedback activation of AKT

Table 2 Novel agents are being developed for gastrointestinal stromal tumor therapy^[10,13,14,21,30,64,67-84]

Agent	Molecular target	Phase
Kinase inhibitors		
Nilotinib	KIT, PDGFRs, BCR-ABL	I
Sorafenib	Raf, KIT, PDGFRB, VEGFR, FLT3, RET	71%
Dasatinib	Src, ABL, KIT, PDGFRs	Phase II ongoing in advanced sarcomas and accepting patients
Cediranib (AZD2171)	VEGFR, KIT, PDGFRs	Phase II ongoing
OSI-930	VEGFR, KIT	Phase II ongoing, not recruiting
Linsitinib (OSI-906)	IGF1R	Phase III
Vatalanib (PTK787)	VEGFR, KIT, PDGFRs	67%
Motesanib (AMG706)	VEGFR, KIT, PDGFRs, RET	24%-27%
XL820	KIT, PDGFRB, VEGFR	Phase II ongoing, not recruiting
mTOR and AKT inhibitors		
Perifosine	AKT	Phase II ongoing in combination with imatinib
Everolimus	mTOR	26%
Temsirolimus	mTOR	Phase II ongoing, closed recruitment
Hsp90 inhibitors		
17-AAG	Hsp90	Phase II / III
Ganetespib (STA-9090)	Hsp90	Phase II
AUY922	Hsp90	Phase II
AT13387	Hsp90	Phase II ongoing in combination with imatinib
IPI-504	Hsp90	78%, phase III ended due to safety concerns
Others		
Flavopiridol	Transcription inhibitor	Phase I ongoing in combination with doxorubicin
Clinical benefit is defined as complete or partial response or stable disease		

PDGFRs: Platelet-derived growth factor receptors; PDGFRA: Platelet-derived growth factor receptor α ; PDGFRB: Platelet-derived growth factor receptor β ; VEGFR: Vascular endothelial growth factor receptor; FLT3: FMS-Like Tyrosine Kinase 3; IGF1R: Insulin-like growth factor 1 receptor; AKT: Protein kinase B; mTOR: Mammalian target of rapamycin; Hsp90: Heat shock protein 90; RET: Orphan receptor tyrosine kinase.

after mTORC1 inhibition. Simultaneous targeting of multiple nodes in the PI3K/AKT/mTOR pathway prevents feedback activation and may translate into more complete pathway inhibition. A few therapies targeting this pathway are currently being evaluated in phase I and II clinical trials^[94]. A number of drugs currently in development include inhibitors of pan-Class I PI3K (BKM120 and GDC0941), PI3K/mTOR (BEZ235, SF1126 and GDC0980), AKT (Perifosine), and mTOR (Everolimus/RAD001 and Temsirolimus). Additionally, combined inhibition of KIT and PI3K/AKT/mTOR results in a greater response compared to either intervention alone^[73,94-97].

Heat shock proteins control the proper folding, function, and stabilization of various client proteins. HSP90 optimizes and maintains the folding and localization of many activated tyrosine kinases and also prevents proteasomal degradation^[98]. HSP90 is abundant in eukaryotic cells, comprising up to 1%-2% of total cellular protein, and it plays key roles in regulating cell proliferation, differentiation, and apoptosis^[99,100]. The HSP90 inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AAG), a geldanamycin derivative^[101], binds a ATP-interaction pocket in the HSP90 NH₂-terminal domain^[102] and shows anti-proliferative effects in various human cancers, where it can degrade HSP90-client oncoproteins with high selectivity^[103,104]. Whereas the clinical application of 17-AAG has been hampered by its low water solubility, IPI-504, a 17-AAG derivative, exhibits improved aqueous solubility while maintaining the biological

HSP90-inhibitory properties of 17-AAG^[105]. Furthermore, clinical trials with new-generation synthetic HSP90 inhibitors are ongoing in various cancer types. HSP90 is an attractive target in GIST as it is a key chaperone for KIT and PDGFRA^[79,106]. Targeting HSP90 results in pro-apoptotic and anti-proliferative effects in GIST and is associated with the inhibition of KIT and PDGFRA signaling^[72,79,107,108]. Other HSP90 inhibitors are in development (NVP-AUY922, AT-13387, KW-2478, and SNX-5422) and show promise for GIST treatment, particularly in combination with TKI^[109].

Other drugs are in various stages of development for the treatment of GIST. Flavopiridol, a transcription inhibitor, has been evaluated in an ongoing phase I trial in combination with doxorubicin^[110]. Histone deacetylase inhibitors (HDACIs) alone or in combination with imatinib have shown pro-apoptotic and anti-proliferative effects in GIST and are associated with inhibition of KIT and a reduction in the expression and activities of downstream pathways^[111].

NOVEL CANDIDATE THERAPEUTIC TARGETS

Other therapeutic targets have been identified for the treatment of GIST, including Ets Variant 1 (ETV1), AXL, FAS, IGF1R, protein kinase c theta (PKC θ), RAS, CDC37, cyclin D1, Dog1, and aurora kinase A. Inhibitors targeting these candidates are being developed, and some are being evaluated in clinical trials.

The E26 transformation-specific family member ETV1 is overexpressed in the GIST and is required in the development of both imatinib-sensitive and imatinib-resistant GIST^[112-114]. ETV1 enhancer binding is a master regulator of an ICC-GIST-specific transcription network. Activated KIT cooperates with ETV1 to induce development of GIST, regulating the ETV1 transcriptional program by prolonging ETV1 protein stability through MAPK signaling^[112,114]. Inhibition of ETV1 reduces the expression of KIT, reduces mutagenesis, and stabilizes the GIST genome, thereby inhibiting GIST growth and progression and inducing apoptosis.

AXL (UFO/ARK/Tyro), an RTK stimulated by its ligand growth arrest-specific 6, shows potent oncogenic and transforming activity in normal and cancer cells^[115-117]. AXL also plays a role in tumor cell invasion, metastasis, and survival^[141,118,119]. AXL is active in GIST metastases that lose KIT expression at the time of clinical progression on imatinib^[41,120]. In KIT-independent GISTs, AXL knockdown results in upregulation of p21, p27 and p53 protein expression and shows anti-proliferative effects^[120]. MP470, a KIT/AXL inhibitor, shows a synergistic cytotoxic effect in GIST cells when combined with docetaxel (taxotere)^[41].

Fas and its ligand FasL belong to the tumor necrosis factor family of death receptors. Activation of Fas by FasL induces cell apoptosis through caspase 8 signaling. Down-regulation of Fas is associated with tumorigenesis^[121,122]. Fas and FasL expression were positively correlated in primary GISTs, but there was no association KIT mutation status^[123]. MegaFasL, a hexameric form of soluble FasL, is an active apoptosis-inducing agent and potentiated the apoptotic effects of imatinib in GIST cell lines^[123].

The IGF/IGF1R signaling system has been implicated as a relevant therapeutic target in a variety of cancers. When IGF1 binds with IGF1R, it activates downstream signaling cascades, such as the PI3K/AKT/mTOR and RAF/MEK/MAPK pathways, to trigger protein synthesis, and it also activates anti-apoptotic and proliferative pathways^[124-126]. Recent reports have shown that *IGF1R* is amplified in a subset of GISTs^[127] and over-expressed in wild-type and pediatric GIST^[88,128,129]. Recent studies have shown that the IGF/IGF1R pathway may be a promising therapeutic target for GIST^[127,130-135].

PKC θ , a member of the protein kinase C family commonly expressed in T cells and myogenic cells^[136,137], is expressed at high levels and activated in GIST irrespective of the *KIT* or *PDGFRA* status. Therefore, PKC θ serves as a diagnostic marker of GIST^[138-141]. PKC θ knockdown is accompanied by inactivation of KIT in KIT+/PKC θ + GIST cell lines. PKC θ knockdown resulted in inhibition of PI3K/AKT signaling, upregulation of pro-apoptotic proteins p21 and p27, cell cycle arrest, and apoptosis, recapitulating the effect of direct KIT targeting^[142]. PKC θ is a compelling therapeutic target in GISTs, including those with mutations that confer resistance to KIT/PDGFRA inhibitors.

Wild-type GISTs often demonstrate primary imatinib

resistance. In some cases, these tumors are succinate dehydrogenase (SDH)-deficient GISTs with mutations in *SDHA*, *SDHB*, or *SDHC*^[143,144], while others have no known genetic mutations. A recent report suggested that *KRAS* mutations might confer imatinib resistance in GIST, and although rare, *KRAS* gain-of-function mutations contribute to clinical imatinib resistance^[145,146]. Serrano *et al.*^[145] used a Sequenom panel to screen for *RAS*, *BRAF*, and *PI3KCA* mutations in 27 wild-type GIST patients. Only one of these 27 GISTs contained a mutation in this pathway, harboring concomitant *HRAS* G12V and *PIK3CA* H1047R mutations^[145]. *KRAS* and *HRAS* can contribute to GIST oncogenesis and indicate the importance of the PI3K/AKT and RAS/RAF pathways in GIST tumorigenesis.

As discussed previously, HSP90 inhibitors strongly inactive KIT kinase activity, but clinical applications in GIST patients have been prevented due to the toxicity resulting from inactivation of HSP90 client proteins beyond KIT and PDGFRA. Genome-scale short-hairpin RNA (shRNA) screening identified CDC37, an HSP90 cofactor, as an essential GIST-specific gene^[147]. Validation studies in treatment-naïve and imatinib-resistant GIST cell lines demonstrated that CDC37 is a viable therapeutic target in GIST, recapitulating the effect of HSP90 inhibition while remaining selective for KIT/PDGFRA and a limited number of other HSP90 clients^[147]. CDC37 inhibition represents a potential HSP90 targeting strategy that limits toxicity for GIST patients.

The strongly expressed DOG1 (ANO1/TMEM16A) has been used as a diagnostic marker to differentiate GIST from other sarcomas^[148-151]. Loss of DOG1 expression occurs together with loss of KIT expression in a subset of GISTs that are resistant to imatinib. Although DOG1 inhibition do not inhibit cell growth *in vitro*, DOG1 knockdown delays the growth of xenograft models of GIST and is associated with the up-regulation of insulin-like growth factor binding protein 5, a potent antiangiogenic factor implicated in tumor suppression^[152]. These findings suggest that DOG1 is a potential target in GIST through its role in IGFR signaling.

A recent analysis of the prognostic significance of aurora kinase A (AURKA) in imatinib-treated patients with advanced GIST suggested that the expression of AURKA may predict recurrence in patients with primary, surgically resected GISTs^[153,154]. AURKA overexpression is a prognostic factor of poor PFS and OS. Inhibition of AURKA suppresses the growth of both imatinib-sensitive and imatinib-resistant GIST cells in a concentration-dependent manner, and it results in a synergistic cytotoxicity with imatinib^[154].

CONCLUSION

Oncogenic KIT or PDGFRA receptor tyrosine kinase mutations are compelling therapeutic targets in GISTs, and the KIT/PDGFRA kinase inhibitors imatinib,

sunitinib, and regorafenib are standards of care for patients with unresectable or metastatic GIST. However, most patients eventually develop resistance to KIT/PDGFR kinase inhibitors, indicating that there is an urgent need to identify novel therapeutic strategies. A number of novel drugs are undergoing clinical trials, and several novel therapeutic targets have been identified, showing promise for the future treatment of GIST.

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Prospective Study

Modern advances in reducing anxiety and pain associated with cystoscopy: Systematic review

Hossein S Mirheydar, Omer A Raheem, Fuad F Elkhoury, Ramzi Jabaji, Kerrin L Palazzi, Nishant Patel, Rogelio Du, Scott Maroney, Kyoko Sakamoto

Hossein S Mirheydar, Omer A Raheem, Kerrin L Palazzi, Nishant Patel, Kyoko Sakamoto, Department of Urology, UC San Diego Health System, San Diego, CA 92103-8897, United States

Fuad F Elkhoury, Ramzi Jabaji, San Diego School of Medicine, University of California, La Jolla, CA 92103-8897, United States
Rogelio Du, Scott Maroney, Kyoko Sakamoto, VA San Diego Healthcare System, San Diego, CA 92103-8897, United States

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Ethics approval: The study was reviewed and approved by the University of California, San Diego Human Research Protections Program Institutional Review Board (IRB).

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Data sharing: Technical appendix, statistical code, and dataset available from the corresponding author at email address: kyoko.sakamoto@va.gov. All participants gave informed consent for data sharing.

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Correspondence to: Kyoko Sakamoto, MD, Department of Urology, UC San Diego Health System, 200 West Arbor Dr. #8897, San Diego, CA 92103-8897, United States. kyoko.sakamoto@va.gov
Telephone: +1-858-5528585

Fax: +1-619-543-6573

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Abstract

AIM: To investigate if music reduces anxiety and pain in the Veterans Affairs population undergoing flexible cystoscopy.

METHODS: This study was reviewed and approved by the University of California, San Diego Human Research Protections Program Institutional Review Board. Patients were prospectively randomized to undergo flexible cystoscopy with or without music. Thirty-eight patients were randomized into either the No Music group ($n = 24$) or the Music group ($n = 14$). We used the state-trait anxiety inventory and the visual analog pain scale, respectively. Statistics were generated and compared using an independent t -test and chi-squared tests. P values < 0.05 were considered statistically significant. Outpatient cystoscopy is a safe and useful procedure employed frequently in Urology for diagnosis and evaluation of genitourinary pathologies. However, cystoscopy-related distress cannot be ignored. Three components of outpatient cystoscopy have been evaluated to improve the cystoscopic experience: local anesthetic control, cystoscopic equipment redesign and environmental modification. We reviewed the literature pertaining to these modifications.

RESULTS: The mean age was 65.3 and 67.1 years for men in the No Music and Music groups, respectively.

Although, the majority of patients in each group self-identified as Caucasians (66%), African American, Hispanic and other ethnicities represented 13%, 8% and 13% respectively. The majority of patients (68%) reported experiencing hematuria. Thirty-four percent had a history of bladder cancer, and eighteen percent had a history of prostate cancer. Ten patients (26%) admitted to taking antidepressants. Physiologic parameters that correlated to pain and anxiety (systolic blood pressure, diastolic blood pressure, and heart rate) were statistically similar in both groups prior to and after flexible cystoscopy. The median delta anxiety between the No Music and Music groups were not significantly different (0.78 *vs* -1.46), and the pain scores between the No Music and Music groups (1.5 *vs* 1.6) were not statistically different ($P = 0.28$ and $P = 0.92$, respectively).

CONCLUSION: Preliminary results demonstrate that music does not reduce anxiety or pain associated with flexible cystoscopy.

Key words: Flexible cystoscopy; Veterans; Anxiety; Music

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Core tip: Flexible cystoscopy is a very common in-office procedure performed in urology. There have been several technological advances made in the instrumentation of flexible cystoscopies, however, there have also been advances made in reducing patient pain and anxiety associated with this procedure such as viscous lidocaine jelly and music. We reviewed the literature on effects of modifiable factors on patient pain and anxiety associated with flexible cystoscopy, and also includes preliminary data on a Veterans Affairs randomized prospective trial evaluating the effect of classical music on pain and anxiety associated with flexible cystoscopy.

Mirheydar HS, Raheem OA, Elkhoury FF, Jabaji R, Palazzi KL, Patel N, Du R, Maroney S, Sakamoto K. Modern advances in reducing anxiety and pain associated with cystoscopy: Systematic review. *World J Transl Med* 2015; 4(1): 38-43 Available from: URL: <http://www.wjgnet.com/2220-6132/full/v4/i1/38.htm> DOI: <http://dx.doi.org/10.5528/wjtm.v4.i1.38>

INTRODUCTION

Cystoscopy is a common urologic examination indicated for a wide variety of genitourinary conditions^[1-4]. Cystoscopy was first conceptualized over two centuries ago^[5] and underwent multiple advancements in technology to allow patients to undergo the procedure with relative comfort. Regardless of the reason for the inspection, however, cystoscopy is invasive and can be

a distressing experience for patients.

Since patient distress negatively impacts adherence to cystoscopy guidelines, many studies have investigated techniques to reduce cystoscopy-associated anxiety and pain^[1]. Three general methods to alleviate pain and anxiety are local anesthetic control, cystoscopic equipment redesign, and environmental modification. Lidocaine lubricants, inhaled nitrous oxide, and anxiolytic medication have been proposed to ameliorate flexible cystoscopy-associated distress, though none sufficiently relieves patient fear, pain, and anxiety^[6-14]. The cystoscopic instrument has evolved from the rigid cystoscope to the flexible cystoscope, changing from analog visualization to digital and more recently digital high definition visualization.

For environmental modification, music is gaining increasing recognition as an effective tool to alleviate perceived pain and has been shown to be beneficial in a variety of clinical settings^[15-23]. Herein, we report the result of a prospective randomized trial of the effect of music on pain and anxiety in the Veterans Affairs patient population during flexible cystoscopy. Furthermore, we reviewed the above-mentioned three factors (local pain control, equipment redesign and environmental modification) incorporated to lessen the anxiety and pain levels during cystoscopy.

MATERIALS AND METHODS

This study was reviewed and approved by the University of California, San Diego Human Research Protections Program Institutional Review Board (IRB). Patient privacy and confidentiality are protected according to HIPAA guidelines. Following IRB approval, male patients at the Veterans Affairs San Diego Medical Center were prospectively randomized to undergo flexible cystoscopy with or without music. Thirty-eight patients were randomly assigned into one of two groups using an adaptive biased-coin randomization method: (1) the No Music group (patients did not hear music during preparation and draping of patient and anesthetization of urethra); or (2) the Music group (patients listened to the same excerpt of classical music). Inclusion criteria were age > 18 years old. There were no women in this study, although that was not an exclusion criterion. Exclusion criteria were current urinary tract infection, anatomic urethral abnormalities, and inability to complete the surveys. Patients' past medical history, including history of bladder cancer, prostate cancer, hematuria, and current or past use of anti-depressants and anti-anxiety medication, was recorded. All patients with a prior history of bladder cancer were confirmed to have had prior cystoscopy and were currently undergoing cystoscopy for surveillance, while those without bladder cancer were undergoing cystoscopy for diagnostic purposes (e.g., microhematuria). Each patient was consented for the study on the day of the procedure.

	Least anxious ←			→ Most anxious
I feel pleasant	[1]	[2]	[3]	[4]
I feel nervous and restless	[1]	[2]	[3]	[4]
I feel satisfied with myself	[1]	[2]	[3]	[4]
I feel I could be as happy as others seem to be	[1]	[2]	[3]	[4]
I feel like a failure	[1]	[2]	[3]	[4]
I feel rested	[1]	[2]	[3]	[4]
I am calm cool and collected	[1]	[2]	[3]	[4]
I feel that difficulties are piling up so that I cannot overcome them	[1]	[2]	[3]	[4]
I worry too much over something that really doesn't matter	[1]	[2]	[3]	[4]
I am happy	[1]	[2]	[3]	[4]
I have disturbing thoughts	[1]	[2]	[3]	[4]
I lack self-confidence	[1]	[2]	[3]	[4]
I feel secure	[1]	[2]	[3]	[4]
I make decisions easily	[1]	[2]	[3]	[4]
I feel inadequate	[1]	[2]	[3]	[4]
I am content	[1]	[2]	[3]	[4]
Some unimportant thought runs through my mind and bothers me	[1]	[2]	[3]	[4]
I take disappointments so keenly that I cannot put them out of my mind	[1]	[2]	[3]	[4]
I am a steady person	[1]	[2]	[3]	[4]
I get in a state of tension or turmoil over my recent concerns and interests	[1]	[2]	[3]	[4]

Figure 1 State-trait anxiety inventory.

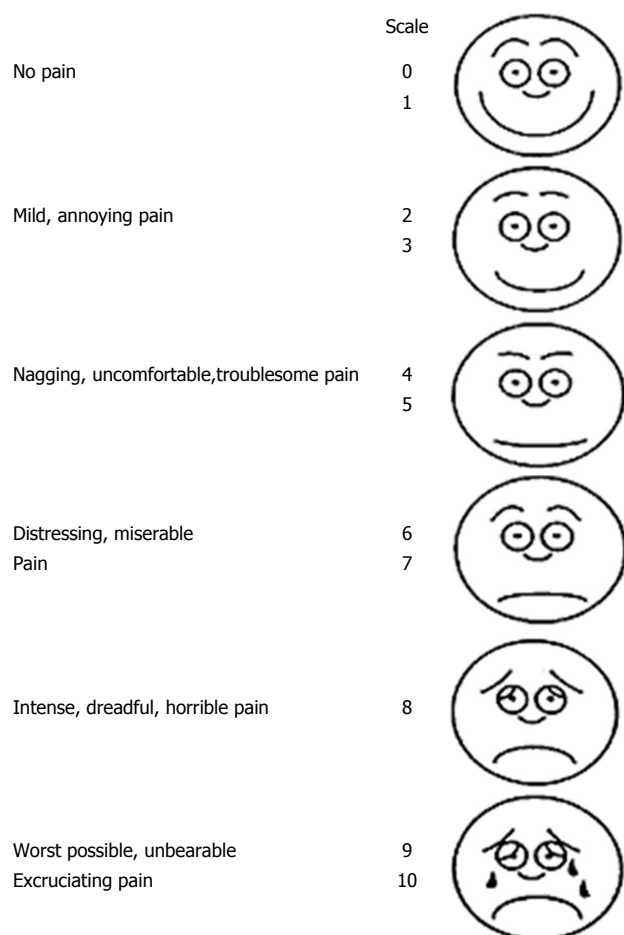


Figure 2 Visual analog pain scale.

Pre-operative and post-operative systolic and diastolic blood pressure, heart rate, and respiratory rate were measured for each patient. The pre-procedure vital signs, including blood pressure, heart rate,

and respiratory rate, were all obtained prior to the cystoscopy and prior to playing music. Post-procedure vital signs were obtained after the cystoscopy was completed. During the 10-min dwell time of the 2% intraurethral lidocaine jelly, the patient either listened to classical music for 10 min or did not hear any music at all. A 15 French Olympus® digital flexible cystoscope was connected to a digital video monitor that was used for all procedures. The music was played prior to the cystoscopy.

To measure anxiety level and pain level, we used the State-Trait Anxiety Inventory (STAI) and the Visual Analog Pain Scale, respectively (Figures 1 and 2)^[24,25]. Both are validated surveys. The State-Trait Anxiety Inventory (range 20-80) measures the transitional emotional status evoked by a stressful situation, such as a medical procedure or surgery. The Visual Analog Pain Scale (range 0-10) quantifies pain level using verbal and visual descriptors. Higher scores indicate higher anxiety and pain for both tests. The patients were asked to complete two anxiety surveys, one pre-procedure and one post-procedure. Patients were also asked to complete a visual analog pain scale survey post-procedure.

Statistics were generated and compared using an independent *t*-test and chi-squared tests. *P* values < 0.05 were considered statistically significant. Delta anxiety score is defined as pre-cystoscopy anxiety score - post-cystoscopy anxiety score.

RESULTS

Thirty-eight patients were randomized into either the No Music group (*n* = 24) or the Music group (*n* = 14). The subjects were all male as noted above. Table 1 outlines the demographics and clinical characteristics of these subjects. The mean age was 65.3 and 67.1

Table 1 Summary of patients' demographics and clinical characteristics of the music and number of music groups *n* (%)

Variables	Groups		<i>P</i> value
	Music group (<i>n</i> = 14)	No music group (<i>n</i> = 24)	
Age, mean + SD	67.1 + 9.9	65.3 + 10.4	0.9541
Race			0.698
Caucasian	10 (71)	15 (63)	
African American	1 (7)	4 (17)	
Hispanics	1 (7)	2 (8)	
Others	2 (15)	3 (12)	
History of bladder carcinoma	10 (71)	3 (1)	0.1488
History of prostate carcinoma	2 (14)	5 (21)	0.3039
History of hematuria	12 (87)	14 (58)	0.2049
Antidepressant or antianxiety medications	4 (29)	6 (25)	0.7092

Table 2 Summary of patients' peri-procedural vital signs between the music and number of music groups

Variables	Groups		<i>P</i> value
	Music group (<i>n</i> = 14)	No music group (<i>n</i> = 24)	
Pre Cystoscopy			
Systolic blood pressure (mmHg), mean + SD	135.5 + 17.7	135.5 + 17.9	0.9834
Diastolic blood pressure (mmHg), mean + SD	79.4 + 12.9	80.0 + 11.5	0.7908
Heart rate (beat/min), mean + SD	73.5 + 13.3	75.3 + 14.3	0.4405
Post Cystoscopy			
Systolic blood pressure (mmHg), mean + SD	139.1 + 22.8	137.2 + 17.3	0.5837
Diastolic blood pressure (mmHg), mean + SD	81.8 + 13.0	83.0 + 11.6	0.5717
Heart rate (beat/min), mean + SD	71.3 + 15.6	74.9 + 14.2	0.16

years for men in the No Music and Music groups, respectively. The majority of patients in each group self-identified as Caucasians. A majority of patients (68%) reported experiencing hematuria. Thirty-four percent had a history of bladder cancer, and 26% admitted to taking antidepressants. Physiologic parameters that correlated to pain and anxiety (systolic blood pressure, diastolic blood pressure, and heart rate) were statistically similar in both groups pre- and post-flexible cystoscopy. Patients' peri-procedural vital signs between the Music and No Music groups are summarized in Table 2. The median delta anxiety between the No Music and Music groups were not significantly different (0.78 vs -1.46), and the pain scores between the No Music and Music groups (1.5 vs 1.6) were not statistically different ($P = 0.28$ and $P = 0.92$, respectively).

DISCUSSION

Outpatient cystoscopy is a safe and useful procedure

employed frequently in Urology for diagnosis and evaluation of genitourinary pathologies. However, cystoscopy-related distress cannot be ignored^[1]. Three components of outpatient cystoscopy have been evaluated to improve the cystoscopic experience: local anesthetic control, cystoscopic equipment redesign and environmental modification. We reviewed the literature pertaining to these modifications. In addition, since flexible cystoscopy is a more tolerable procedure more commonly utilized in the United States, we performed this study to investigate if music reduces anxiety and pain in American veterans undergoing flexible cystoscopy.

A recent study suggests that listening to classical music during rigid cystoscopy enhances patient comfort and decreases post-procedure pain and anxiety^[23]. In addition to this report, prior studies have demonstrated music's ameliorating effect on pain and anxiety in patients undergoing a variety of procedures, including rigid cystoscopy^[16-18,20].

Contrary to these studies, our study did not show reduction of cystoscopy-related anxiety by listening to classical music during flexible cystoscopy, regardless of its indication. The lack of the effect of music in our group may be due to the small number of subjects. In addition, due to the patient demographics of the Veterans Affairs hospitals, the majority of our patients have been Caucasian males. Subsets of patients treated at the Veterans Affairs Hospitals respond to painful stimuli differently than their civilian counterparts, possibly due to the increased prevalence of PTSD and generalized anxiety disorder^[24,25]. A subset of patients with history of bladder carcinoma that required repeated surveillance using office-based flexible cystoscopies were also included in this cohort. We observed that listening to music at time of respective cystoscopy did not influence their peri-procedural STAI anxiety scores when adjusting for other variables. We included blood pressure, heart rate, and respiratory rate as objective representations of the emotional state of the patient in addition to validated questionnaires. These parameters are directly influenced by sympathetic nervous system activation due to emotional distress or anxiety through the release of catecholamines, specifically norepinephrine and epinephrine^[26,27]. Higher catecholamine levels from anxiety and fear result in elevated blood pressure, heart rate, and respiratory rate. Thus, the combination of these objective physiologic indicators with the subjective patient-provided information on anxiety and fear provided a more holistic assessment of the impact of music on how a patient experiences flexible cystoscopy.

Several randomized studies have shown the benefit of intraurethral lidocaine gel in reducing the pain associated with flexible cystoscopy and others have shown no improvement^[6,8-13]. However, a recent meta-analysis^[28] evaluating the effect of lidocaine gel on pain during flexible cystoscopy concluded that intraurethral instillation of lidocaine gel vs plain lubricating gel

reduces the likelihood of moderate to severe pain during flexible cystoscopy. Although fiber optic technology is still utilized for many of the flexible cystoscopes in the country, digital technologies are available. In other endoscopic devices, visualization using digital technology has higher resolution, decreased distortion, improved color representation, and larger image size compared with the standard fiber optic visualization with a narrower field of view^[29]. More recently, digital technology with distal sensor high definition images has been compared to standard digital visualization^[30]. These reductions will likely result in improved patient comfort during outpatient flexible cystoscopy.

Modern advances in flexible cystoscopic instrumentation and peri-procedural instillation of intraurethral lidocaine have both decreased pain associated with flexible cystoscopy. Listening to music during rigid cystoscopy has been shown to reduce pain and discomfort. Our prospective, randomized study explored the effect of classical music on pain and anxiety associated with flexible cystoscopy, and preliminarily demonstrates that music does not reduce anxiety or pain associated with flexible cystoscopy in the Veterans Affairs population.

COMMENTS

Background

Flexible cystoscopy is a very common in-office procedure performed in Urology. Several technological advances have been made in flexible cystoscopy instrumentation, however, there have also been advances in reducing patient pain and anxiety associated with this procedure, such as viscous lidocaine jelly and music. The authors reviewed the literature on effects of modifiable factors on patient pain and anxiety associated with flexible cystoscopy, and also include preliminary data on a Veterans Affairs randomized prospective trial evaluating the effect of classical music on pain and anxiety associated with flexible cystoscopy. This study explored the effect of classical music on pain and anxiety associated with flexible cystoscopy, and preliminarily demonstrates that music does not reduce anxiety or pain associated with flexible cystoscopy in the Veterans Affairs population.

Research frontiers

Flexible cystoscopy is a very common in-office procedure performed in Urology. Several technological advances have been made in flexible cystoscopy instrumentation, however, there have also been advances in reducing patient pain and anxiety associated with this procedure, such as the use of viscous lidocaine jelly and music.

Innovations and breakthroughs

In this systematic review, the authors attempted to evaluate whether listening to music at time of office-based flexible cystoscopy can alleviate pain and anxiety associated with this procedure. Previous studies have highlighted that listening to music during rigid cystoscopy reduces pain and discomfort. In contrast, they study did not show reduction of cystoscopy-related anxiety by listening to classical music during flexible cystoscopy, regardless of its indication. The lack of effect of music in their group may be due to the small number of subjects recruited in this study. Additionally, owing to the patient demographics of Veterans Affairs hospitals, the majority of our patients have been Caucasian males. Subsets of patients treated at Veterans Affairs hospitals respond to painful stimuli differently than their civilian counterparts, possibly due to the increased prevalence of PTSD and generalized anxiety disorder.

Applications

Although listening to music at time of office-based flexible cystoscopy may play a limited role in reducing pain and anxiety in the Veteran population, these findings cannot be generalized to the general patient population, particularly civilian, as responses to pain and/or anxiety associated with flexible cystoscopy

can manifest differently among heterogeneous patient populations.

Terminology

Well described medical and technological terminology commonly known to the general audience and the wider medical community was used. State-trait anxiety inventory; visual analog pain scale.

Peer-review

This study investigated cystoscopy-related distress, reviewing benefits of intraurethral lidocaine use as well as of digital cystoscopes for reducing procedural anxiety and pain. Indeed, the authors performed a study to investigate if listening to classical music reduces anxiety and pain in patients undergoing flexible cystoscopy who were treated at a Veterans Affairs hospital in the United States.

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Editorial Board Member of *World Journal of Translational Medicine*, Mitchell H Grayson, MD, Associate Professor of Pediatrics, Medicine, Microbiology and Molecular Genetics, Division of Allergy and Clinical Immunology, Medical College of Wisconsin, MACC Fund Research Center, Room 5064, 8701 Watertown Plank Road, Milwaukee, WI 53226, United States

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Beijing 100025, China
Telephone: +86-10-85381891
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Component-resolved allergen testing: The new frontier

Matthew T Tallar, Mitchell H Grayson

Matthew T Tallar, Mitchell H Grayson, Section of Allergy and Clinical Immunology, Department of Pediatrics, Medical College of Wisconsin, Milwaukee, WI 53226, United States

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Correspondence to: Mitchell H Grayson, MD, Associate Professor, Section of Allergy and Clinical Immunology, Department of Pediatrics, Medical College of Wisconsin, MFRC Room 5068, 8701 Watertown Plank Road, Milwaukee, WI 53226, United States. wheeze@allergist.com
Telephone: +1-414-2666840
Fax: +1-414-2666437

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Abstract

The discovery that allergen specific IgE (sIgE) identified

individuals who were allergic to specific allergens, revolutionized allergy and immunology. Recently, component-resolved allergen testing (CRD) has shown promise in improving the field yet again. Prior to development of CRD immunoassays, whole allergen extracts were used to detect IgE mediated allergic disease either by oral, cutaneous, or conjunctival provocation. The most widely used immunoassays detect sIgE to either whole allergen sources or individual allergic components. The use of CRD micro-assay technology (not Food and Drug Administration approved in the United States) has been used to evaluate multiple allergens in parallel. This technique allows for determination of primary vs secondary sensitizations from either close sequence homology or cross-reactive carbohydrate determinants. Published studies have shown beneficial uses in hymenoptera venom immunotherapy, anaphylaxis, and food allergy. The use of component testing for aeroallergen immunotherapy has been studied, however clinical use is hampered by lack of allergen components approved for injection. Therefore, although promising in many respects, the frontier of CRD testing requires more data before it can be widely used in clinical practice.

Key words: Component resolved diagnosis; Molecular allergy; IgE; Polysensitization; Immunotherapy; Venom allergy; Food allergy

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Core tip: Component-resolved allergen diagnostic testing is testing for specific allergenic proteins in a given allergen. This testing modality may revolutionize diagnostics and treatment of immediate hypersensitivity reactions. Several promising studies and allergen components have been described for patients with food allergy, venom allergy, and idiopathic anaphylaxis. Some appear to have clinical utility, such as ω -5 gliadin in evaluating wheat dependent, exercise induced anaphylaxis. Components for many of the relevant aeroallergens have been characterized; however, readily available allergen components for injection are lacking, and further research is needed before these practices can be recommended for

widespread clinical use.

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INTRODUCTION

The discovery of IgE in 1966 by the Ishizaka group provided a molecular explanation for the underlying cause of Type 1 hypersensitivity^[1]. Soon after its discovery, *in vitro* methods to detect allergen specific IgE (sIgE) were developed. Prior to this discovery, detection of Type 1 hypersensitivity reactions were based on clinical history and provocation *via* oral, cutaneous, or conjunctival routes, all of which carried a significant risk of allergic reactions including anaphylaxis.

We now know that Type 1 hypersensitivity reactions are secondary to cross-linking of IgE bound to the high affinity FcεRI on mast cells and basophils resulting in the release of inflammatory mediators (*e.g.*, histamine and leukotrienes). Bound IgE in this case is specific to a particular allergen; in fact, it is this IgE that makes an environmentally innocuous substance capable of eliciting an allergic reaction. Through the widespread use of DNA and molecular sequencing we have found that allergen sources such as timothy grass pollen contain many different proteins-some of which drive allergic responses and others that have no pathogenic potential^[2-4]. Current *in vivo* and *in vitro* testing for IgE mediated allergies uses extracts from allergen sources. False negative results can occur because the individual extracts may not contain relevant allergens for a specific patient. These tests are also limited because they do not allow discrimination between true allergy and sensitization from cross-reactive allergens^[5,6].

There are many reviews discussing the more than 130 available allergy components from more than 50 allergy sources^[2,3,7-10]. Components consist of several different protein families including storage proteins, profilins, cross-reactive carbohydrate determinants (CCD), and serum albumin, to name a few. Many of these components have been shown to cross react with plants that are taxonomically unrelated. An example is the Bet v 1 homologue, a pathogenesis-related protein family 10 (PR-10) protein from birch (*Betula verrucosa*), associated with oral allergy syndrome. There is known pollen cross reactivity with *Rosaceae* fruits (apple, cherry, apricot, peach, and pear) and *Apiaceae* vegetables (celery and carrot)^[7]. This cross-reactivity, and the subsequent clinical disease of oral allergy syndrome, highlights the importance that component testing may play-especially in polysensitized patients. Components were already being identified in 1999 when Valenta *et al*^[11] proposed the term com-

ponent resolved diagnostics to refer to the science of determining specific allergenic protein(s) to which a patient is sensitized. Another term applied to this field is "molecular-based allergy diagnostics".

In this editorial review we aim to describe currently available technology and uses for component resolved diagnostic testing. Promise has been shown in the diagnosis and treatment of anaphylaxis, food allergy, and allergen and hymenoptera venom immunotherapy. For the sake of this editorial we will limit our discussion to several representative allergen components. For a more thorough discussion, we recommend one of the comprehensive reviews on this subject^[2,3,5,7,8,10].

SINGLE COMPONENT VS MICROARRAY TESTING

At the present time the FDA has only approved testing for single components (or allergens). Two frequently used enzyme linked-immunoassays are ImmunoCAP™ (Phadia/Thermo Fischer Scientific, Uppsala, Sweden) and Immulite (Siemens Healthcare Diagnostics, Los Angeles, CA, United States)^[12]. Both of these assays are quantitative for serum IgE to a particular allergen source or component, however values cannot be compared between the two due to differences in assay technology. ImmunoCAP uses a 3-dimensional cellulose sponge matrix with either complete allergen sources or allergen components that are covalently bound. In contrast, Immulite uses an allergen source or component coated bead.

In an effort to determine sensitivities to more than one component, the use of microarray technology has been explored. ISAC (Immuno Solid phase Allergen Chip) 112 (Phadia/Thermo Fischer Scientific, Uppsala, Sweden) uses ImmunoCAP technology to detect 112 allergy components^[10]. It has been increasingly used in Europe, although it is not FDA approved. Allergen components are fixed to the microarray slide surface and component sIgE from the serum is then determined, much like single component testing. However, unlike single component testing, microarray technology can evaluate multiple allergen components in parallel. In polysensitized individuals this allows for a more comprehensive view of the sensitization profile, as well as identifying possible cross-reactive pollen or food proteins. Each assay has advantages and disadvantages as shown in Table 1.

Whether using microarray or single component testing, results must be interpreted with care. Positive values denote sensitization, but may be clinically irrelevant. This is especially important with regards to food allergy. To the untrained clinician a positive result despite tolerance in the diet may prompt removal of important protein sources (*e.g.*, milk and soy) needed for proper growth and development. For this reason these tests should be ordered only by those trained to properly interpret the results.

Table 1 Comparison of single component and microarray tests

Single component	Microarray
Serum	Serum or plasma
40 μ L per component	30 μ L total
One allergen at a time	Up to 112 allergens in parallel
Recombinant, natural, or crude protein	Natural or recombinant protein
Quantitative	Semi-Quantitative
Automated	Manual
Can be affected by high total IgE	Interference between IgE and IgG
Highly sensitive	Less sensitive

POTENTIAL USES OF CRD TESTING

There are several proposed uses for CRD testing in allergy including allergen and hymenoptera venom immunotherapy (allergy shots), risk stratification for food allergy, latex allergy diagnosis, and evaluation of anaphylaxis^[10,11,13]. There have been promising results using CRD in many of these areas, as discussed below.

Anaphylaxis

Allergists define anaphylaxis as a life threatening, systemic, immediate hypersensitivity reaction involving more than one organ system. The most commonly implicated triggers are medications, food, and hymenoptera venom^[14]. Fortunately in the vast majority of cases, with the exception of hymenoptera, the correct agent is identified. However, in those patients who have repeated or even delayed episodes of anaphylaxis without any trigger (*i.e.*, idiopathic anaphylaxis) there is a need for a better way to identify causative antigens. In fact, a recent article discussed the use of the ISAC array to identify likely causes of idiopathic anaphylaxis in 20% of patients studied^[15].

In adult patients it has been shown that co-factors are relevant in 39% of food allergy anaphylaxis^[14]. Exercise, the most commonly implicated co-factor, has been associated with reactions to ω -5 gliadin in wheat. In 1999, Palosuo *et al.*^[16], described 18 adult patients with this clinical phenomenon. All 18 patients had sIgE against ω -5 gliadin (Tri a 19) in their sera, and in 15 of them this reactivity was verified by skin prick testing. Wheat avoidance prior to exercise prevented recurrence in 15 of the patients. Interestingly, the three patients who continued to have recurrent symptoms were shown to have unintentionally ingested wheat products prior to the exercise. Using a cutoff of 0.89 kUA/l for ω -5 gliadin achieved a sensitivity of 78% and specificity of 96% for the detection of wheat dependent exercise induced anaphylaxis (WDEIA)^[17]. In 100 patients tested, 40 met this cutoff, and 39 of them were diagnosed with WDEIA. Thus, detection of ω -5 gliadin could be used to diagnose this condition without performing a provocation challenge, and the authors recommend testing for this allergen component in patients with suggestive symptoms^[17].

Prior to the demonstration of galactose- α -1,3-galactose (α -gal, a sugar moiety) as the cause of delayed anaphylaxis from ingestion of "red meat", it was commonly believed that only proteins were the source of IgE mediated anaphylactic reactions. α -gal was first described in 2009 in patients who had immediate hypersensitivity reactions while receiving Cetuximab for treatment of colorectal and squamous cell head and neck cancers^[18]. Cetuximab, a chimeric mouse-human monoclonal antibody against the epidermal growth factor receptor, contains α -gal in the Fab fragment. Of 76 patients who received treatment, 25 patients had immediate hypersensitivity reactions. In 17 of these patients sIgE to α -gal was found to be present in their sera before they began treatment. Further supporting the idea that these antibodies were not raised against Cetuximab, 15 control patients also were found to have sIgE against α -gal in their serum.

α -gal is a carbohydrate moiety present in non-primate mammals such as cows, pigs, lambs, and cats. Based on this knowledge, Commins *et al.*^[19] evaluated patients with delayed anaphylaxis, angioedema, or urticaria following ingestion of "red meat" such as beef, pork, or lamb. Patients in this study had similar clinical presentations with symptoms presenting 3-6 h after ingestion of red meat. It was noted also that a large percentage of patients were from Virginia, Tennessee, North Carolina, Arkansas, and Missouri. Further, over 80% of patients had a history of a tick bite from the Lone Star tick, *Amblyomma americanum*. In a follow-up report it was noted that similar cases have been found in Australia and Europe associated with bites from *Ixodes holocyclus* and *Ixodes ricinus*, respectively^[20]. It is now well accepted that sensitization to galactose- α -1,3-galactose may result from a tick bite, and that having IgE against galactose- α -1,3-galactose causes delayed anaphylaxis to red meat in susceptible individuals. Another similar disease, cat-pork allergy, has also been well described in the literature. In this condition, primary exposure to cat albumin leads to development of cross-reactive IgE antibodies against pork albumin. Upon ingestion of pork, patients may develop symptoms ranging from oral pruritus to anaphylaxis, although, not every ingestion is associated with a reaction. Patients do not have reactions to beef or other meats and, unlike α -gal, there is no association with tick bites^[20].

Food allergy

Milk and egg are the two most common foods causing allergic reactions. In the United States, peanut is third most common, with self-reported peanut allergy rapidly increasing^[21,22]. A complete discussion of all described food component allergens is outside the scope of this editorial, but can be found in many review articles^[2,3,5,7,8,10]. The gold standard diagnosis for food allergy requires a double blind placebo controlled food challenge (DBPCFC). This procedure involves exposing the patient to a known food allergen (or placebo)

while evaluating for reaction. In addition to the risk of a potential life threatening reaction, this procedure is expensive and time consuming^[23]. A patient is said to have passed the food challenge if no reaction is noted for up to 24 h after ingestion. Skin prick and or sIgE testing are routinely used as a means to evaluate when a patient is deemed "low risk" for reacting to a challenge^[21]. Patients with a low risk can undergo an open food challenge rather than a DBPCFC. Open challenges are much less expensive and don't require the use of a placebo. However, because they lack the blinded approach, subjective symptoms can cloud the results of these tests. Further, using the "low risk" stratification the success rate for food challenges is still not 100%^[24]. Dilemmas arise in those patients who never consumed a food to which they were later found to be sensitized to by allergy testing. Why certain patients pass a challenge with a given food and others do not even though their skin test reactivity or sIgE levels are similar has perplexed the field, as well. It has been hypothesized that this difference is due to sensitivity to different components of the various allergens. Thus, the use of CRD testing might provide additional information on risk stratification and could help guide clinical decision making on who will pass (or even be challenged) a food challenge.

Milk is an important food source in the first 6 mo of a child's life, whether it is breast milk or a cow's milk based formula. In cow's milk, casein (Bos d 8) and whey (β -lactoglobulin/Bos d 5 and α -lactalbumin/Bos d 4) are the most relevant allergen components associated with IgE mediated reactions^[2]. Casein is a heat and enzyme stable protein that is associated with a more severe phenotype and persistent food allergy. Alternatively, whey proteins are heat and enzyme labile and are associated with a less severe phenotype. It has been shown that patients who tolerate baked milk have earlier resolution of their allergy than those that do not^[25]. Not surprisingly, sIgE against whey proteins are more prevalent in those that outgrow milk allergies, and those with sIgE against casein are much less likely to pass a baked milk challenge or outgrow their food allergy^[2,26-28]. A recent paper demonstrated that casein sIgE testing, thus, could be useful prior to an open food challenge to predict reactivity to baked milk^[26]. While further studies are needed, these data suggest that CRD testing may provide appropriate stratification of risk, making milk food challenges much safer (and maybe someday obsolete).

Egg is the second most common food allergy and is found in a large number of fresh and pre-packaged food sources. Like milk, egg allergy is typically outgrown prior to adulthood^[29,30]. Historically patients have been tested with whole egg, egg white, and/or egg yolk extracts. Molecular diagnostics have allowed further characterization of allergens in these sources. The two most clinically relevant allergens are ovomucoid (Gal d 1) and ovalbumin (Gal d 2)^[29,31]. Ovomucoid is a heat and enzyme stable protein comprising approximately

10% of egg white and is associated with a more severe phenotype with delayed allergy resolution. Ovalbumin is the most abundant egg white protein, is heat and enzyme labile, and is associated with less severe clinical reactions^[29]. SIgE testing to ovomucoid predicts tolerability to heated/baked egg^[32]. Using microarray technology it was shown that 44 of 47 patients who lacked sIgE to ovomucoid were able to tolerate a boiled egg challenge. Additionally they showed that 20 of 21 patients sensitized to ovomucoid reacted to raw egg^[33]. This supports the notion that the presence of sIgE to ovomucoid is associated with a higher frequency of clinical allergy to egg, whereas its absence predicts patients who could tolerate boiled egg.

Self-reported peanut allergy has been increasing world-wide in modernized societies. In the United States it is the third most common food allergy and increased in prevalence from 0.4% in 1997 to 1.4% in 2008^[23]. A diagnosis of peanut allergy carries significant burden on a patient's quality of life. Many schools require children to sit at special tables at lunch, there is increased scrutiny of birthday treats, and certain restaurants must be avoided. This also imposes an economic and healthcare burden on the patients. Therefore, it is important to distinguish patients with true IgE mediated peanut allergy versus those who are just sensitized but unlikely to clinically react to the food. The peanut storage proteins Ara h 1, 2, and 3 are commonly associated with severe allergic reactions. In contrast Ara h 8 a PR-10 protein (Bet v 1 homologue) that is associated with the oral allergy syndrome and is most likely indicative of birch tree sensitization^[23,34,35].

Ara h 2 has been consistently reported to be the most specific component in diagnosing true peanut allergy^[2,3,7,8,21,23]. In 2013, research teams from the United States and Sweden performed peanut component testing (Ara h 1,2,3,8) on serum from 167 patients with suspected peanut allergy^[36]. All patients underwent oral food challenges with 106 of them having clinical reactions. sIgE to Ara h 2 alone was shown to demonstrate specificity between 85% to 95% for a true peanut allergy, which improved diagnostic accuracy over sIgE to whole peanut. Importantly in this study it was noted that 3 subjects with peanut sIgE ≥ 15 kU_A/L who lacked any sIgE to Ara h 2 tolerated peanut on challenge. Further, 82% of patients with whole peanut sIgE ≤ 15 kU_A/L but a positive IgE to Ara h 2 had clinical reactions on peanut challenge. Clearly the implications of these data are tremendous and have the potential to significantly change the clinical practice of peanut food challenges (*i.e.*, having sIgE to Ara h 2 would need to avoid peanut, otherwise can be challenged regardless of sIgE to whole peanut).

Aeroallergen immunotherapy

The vast majority of patients receiving allergen immunotherapy (AIT) are polysensitized, with multiple positive skin or *in vitro* testing to indoor and outdoor

aeroallergens. With the characterization of various protein families has come the realization that many proteins from taxonomically unrelated species cross-react. In 2013, the World Allergy Organization published a consensus document describing the use of recombinant/purified allergens to help in discriminating between genuine sensitization and reactions due to cross reactive allergens^[10]. In theory, this knowledge may decrease the number of allergens in a patient's AIT prescription, if only the clinician knew the relevant cross-reacting allergens. This is where the ISAC 112 microarray could be of benefit, as there are a large number of components tested at once (without an a priori knowledge of the subject's sensitivities/reactions). Nonetheless, any version of CRD should allow the clinician to get a better picture to what it is that the patient is actually allergic.

Two of the most well studied and relevant outdoor aeroallergens are grass and tree pollens. Of the grasses, timothy has been most studied with regards to allergy component testing. Eight components (Phl p 1, 2, 4-7, 11, and 12) are commercially available. The two most relevant components, Phl p 1 and Phl p 5, are highly cross-reactive with other grass species^[2]. A recent study comparing CRD, sIgE, skin prick testing, conjunctival provocation, and basophil activation testing showed that the use of rPhl p 1, a recombinant Phl p 1, alone was sufficient to diagnose timothy grass pollen allergy in a central European population^[37]. Additionally, a study of patients with grass allergy in southern Spain demonstrated that the use of CRD changed immunotherapy prescriptions for 55% of patients in the study^[38]. To date similar studies have not been published in the United States.

Because of the association between birch tree allergy and the oral allergy syndrome, birch tree components have been studied quite extensively^[2,3,7-9]. There are 4 commercially available birch components: Bet v 1, 2, 4, and 6. The PR-10 protein, Bet v 1, is present in 95% of patients with clinical birch allergy and is a marker of primary sensitization^[2]. PR-10 proteins in other tree pollens such as *Rosaceae* fruits (apple, cherry, apricot, peach, and pear) share sequence homology with Bet v 1 and are known as Bet v 1 homologues. Patients with birch allergy may develop itching of the lips, tongue, and mouth following ingestion of raw fruits; however, symptoms are not present when the fruits are cooked. Both Bet v 2 and Bet v 4 are markers of cross reactivity. Bet v 2, a member of the profilin family has shown cross reactivity with pollens (trees, grasses, weeds) and foods (fruits, peanut, legumes, and vegetables). Bet v 4, a member of the calcium-binding protein family, cross-reacts only with pollens such as trees, grass, and weeds^[2,7].

Hymenoptera venom immunotherapy

CRD testing has shown great progress in the field of hymenoptera venom immunotherapy. Approximately 50% of patients with venom allergy have positive sIgE

testing to both honeybee and yellow jacket^[39]. CCD and homologous protein allergens (e.g., hyaluronidase) are believed to be the causes of this phenomenon^[40]. As a result, a patient's true sensitization profile to venom may be inaccurate, leading to unnecessary allergens being added to their immunotherapy prescription. A recent study evaluated sIgE levels to recombinant honey bee (rApi m 1) and yellow jacket (rVes v 1 and rVes v 5) allergens, all of which lack CCDs^[41]. Of patients with positive sIgE testing to both species, only 47% were found to be sensitized to both honey bee and yellow jacket venom components. Therefore, in over half of these patients, initial skin testing or serum sIgE testing was clouded by cross-reactivity, which could be sorted out using CRD. However, it is important to note that the detection of incidental sIgE to hymenoptera venom may increase with tests such as ISAC. It is recommended in clinical practice that patients without a history of allergic reactions to stings should not have skin prick or sIgE testing performed. The clinical relevance of a positive test without a history of a reaction is similar to the problem with food allergy testing, where positive tests must be correlated with the clinical history. Therefore, a positive anti-venom IgE test in the absence of clinical disease is of unclear significance^[42].

CONCLUSION

CRD testing is a new frontier in evaluating IgE mediated hypersensitivity reactions. Research has shown potential benefits in idiopathic anaphylaxis, food allergy, and venom allergy, while there is some possible utility in aeroallergen allergies. The ISAC 112 has been used to detect pollen cross reactivity in polysensitized patients, which may help focus patients' immunotherapy prescriptions. However, at the present time commercial allergen components are not easily available and many more studies need to be published before CRD becomes a main stay of clinical practice. We believe this is only a matter of time, and expect with increased research in the future, our patients will benefit from the more targeted guidance CRD appears to provide.

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Translating laboratory anti-aging biotechnology into applied clinical practice: Problems and obstacles

Marios Kyriazis

Marios Kyriazis, ELPIs Foundation for Indefinite Lifespans, London SE13 7DQ, United Kingdom

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Correspondence to: Dr. Marios Kyriazis, MD, ELPIs Foundation for Indefinite Lifespans, 75 Embleton Road, London SE13 7DQ, United Kingdom. drmarios@live.it
Telephone: +44-78-50221796

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Abstract

Although the use of biomedical technologies against ageing (rejuvenation biotechnologies) is considered by many as an effective way of controlling all age-related degeneration, in reality this belief cannot be justified. The human body is notoriously resistant to external perturbations and can respond in unpredictable or undesirable ways. Basic concepts of science, evolution and disease must also be considered. In this paper, I discuss some relevant problems associated with the application of any putative rejuvenation biotechnologies

such as stem cell therapies, genetic engineering, tissue manipulation, as well as pharmacological approaches. I conclude that these and other biotechnologies will not be applicable to humans in the community. This is due to a wide spectrum of problems and obstacles, such as unpredictable therapeutic results, unrealistic expectations, lack of infrastructure, cellular network disruption, and many more. Even if some such technologies are developed, the totality of the problems, issues and side effects will prove an insurmountable final hurdle, rendering the development of such therapies, essentially and practically useless.

Key words: Rejuvenation; Biotechnologies; Translational medicine; Ageing; Clinical medicine

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Core tip: Those who rely on biomedical technologies in order to achieve rejuvenation (global reduction of age-related degeneration) are bound to be disappointed. Such a reductionist approach will not have an impact on reducing mortality as a function of age. This is due to problems and obstacles associated with human nature, which are much more complicated than hitherto recognised. The use of biomedical rejuvenation technologies in radically reducing the impact of ageing is conceptually naive, scientifically reductionist, technologically unfeasible, and medically undeliverable.

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INTRODUCTION

Research into ways of treating age-related disease is

progressing in leaps and bounds. Proposed treatments based not only on pharmaceuticals but also on biotechnology give hope to millions of people who have degenerative diseases such as Alzheimer's dementia^[1] osteoarthritis^[2] or cardiovascular disease^[3]. Some researchers and academics^[4] also hope that these disruptive biotechnologies may enable us to repair age-related damage before this becomes clinically relevant, and thus reduce or eliminate the impact of aging on humans, with a consequent dramatic extension of healthy lifespan. However, it is surprising how few people (both the public and academics) actually consider the translational and clinical issues relating to such treatments^[5]. For instance, a PubMed online search of "rejuvenation biotechnologies in aging" reveals 53 papers discussing theoretical or laboratory aspects of rejuvenation biotechnologies, but a search of "clinical applications rejuvenation biotechnologies in aging", reveals just one relevant paper, analysing the clinical application of these technologies.

Laboratory research may appear promising, but when it comes to applying the results of this research onto real patients in the community, then a host of new problems become evident^[6]. The difficulties in developing new pharmaceutical treatments for age-related conditions are well known. Clinicians have already begun to also apply biotechnological therapies in clinical situations, for example treatment of stroke with neural stem cells^[7], Parkinson's disease with stem cells^[8] arthritis with tissue engineering^[9], and diabetes with new drugs^[10] with variable success.

DISCUSSION

It is very plausible that laboratory and clinical research will progress in tandem, mutually providing feedback and adjustments, but only insofar as the treatment is aimed at carefully selected patients suffering from one clinically manifest age-related condition. The issue becomes much more complex if we consider a large number of patients with multiple, clinically-relevant degenerative illnesses, or patients who are disease-free but are subjected to as yet sub-clinical chronic degeneration processes that need to be treated by these technologies^[11]. The rationale of many of these regenerative biotechnologies is based on the assumption that, even if developed, they can easily be applied and used by the public. However, a host of problems, obstacles and ill-defined thinking impedes this application. In a recent paper^[12] we highlighted two principal issues which pose dramatic problems with the practical application of disruptive rejuvenating biotechnologies. These issues are the interference with the complex organic and dynamic properties of the human body, and the actual impracticality of use of these treatments by the general public. We have argued that biomedical technologies applied on humans at large have effects which cannot be predicted and may result in situations where adverse effects and practical

problems become uncontrollable.

For example, in our paper we considered the case of bone marrow transplant of stem cells. I quote: World-wide, there are approximately 60000 bone marrow transplants performed each year. If we assume that an arbitrary minimum 1% of all humans could possibly be treated with marrow transplant-dependent rejuvenation biotechnologies each year, then there will be a need to provide 7000000 such transplants a year. Assuming a reasonable, and perhaps generous, yearly 20% increase in our clinical capability to deliver rejuvenation biotechnologies, it will still take us 10 years to reach a mere 1000000 target patients - and at that point, the procedures would need to be repeated, in order to maintain the status quo. In this scenario we would only be able to treat a total maximum of 0.015% of humans, ever.

Any pre-existing illness involving any organ or tissue may cause the treatment to behave sub optimally and result in unpredictable side effects. Some general problems that can be encountered are outlined in Table 1.

Taken in isolation, each of the proposed biomedical treatments is associated with significant translational problems. However, if we also consider that these therapies must be deployed in association with each other so that to achieve a lasting and curative clinical benefit, we are bound to encounter additional emergent problems at least with respect to practical clinical applications.

At this point, it is worth mentioning that many rejuvenation biotechnologies do not take into account newer concepts such as the heterogeneous process of disease evolution, described by Molecular Pathological Epidemiology (MPE)^[21]. Nor do they consider the role of epigenetic regulation in disease^[22]. Ogino *et al*^[23] quote: "MPE is founded on the unique disease principle, that is, each disease process results from unique profiles of exposomes, epigenomes, transcriptomes, proteomes, metabolomes, microbiomes, and interactomes in relation to the macroenvironment and tissue microenvironment. Although epigenome-wide association study attracts increasing attention, currently, it has a fundamental problem in that each cell within one individual has a unique, time-varying epigenome" (emphasis mine).

In other words, unique individual patterns of disease evolution may lead to unpredictable outcomes, and any future treatments designed against age-related disease must address this, by using tools of personalised medicine developed through MPE concepts. Otherwise, these putative treatments may prove ineffective in some individuals, depending on epigenetic factors, *i.e.*, environmentally-dependent changes of their disease phenotype.

We have been criticised for being too pessimistic about the expected problems and that it could be possible that novel developments may diminish the uncertainties and practical difficulties of such a scenario.

Table 1 Problems and obstacles associated with some biomedical technologies

Biomedical technologies	Applied translational and clinical problems
Tissue engineering	Harvesting of autologous material, transplantation surgery, immunosuppression, infrastructure of delivery ^[13]
Stem cell therapies	Clinical harvesting of cells, delivery (such as problems with bone marrow transplants ^[14]), inadequate integration of transplanted cells ^[15] and earlier-than-planned re-treatments
Immune therapies	Side effects, non-compliance, reluctance to accept as a treatment ^[16]
Genetic therapies	Immunity to vector, inadequate integration and assimilation of genes, unknown variables relating to genetic cross-talk ^[17] and over-expression, practical delivery methodologies
Nanomedicine	Unknown and unpredictable side effects (including immune system disruption), unknown end-results, toxicity, inflammation ^[18]
Pharmacological therapies	Ineffective or complex treatments, tolerance, clinical polypharmacy, side effects, interactions and non-compliance ^[19]
Other disruptive interventions (apoptotic modulation, crosslink breakers, chemotherapy, chromosomal interventions)	Unpredictability of the combined effect, adverse effects, cost, compliance, ethical and psychological problems, inadequate clinical capability to deliver the treatments ^[20]

This is a valid point, however the interventions necessary to have an impact on age degeneration will never be completely free of adverse effects or have an easy applicability. Due to the sheer number of interventions needed, these side effects and translational problems will, even if individually mild in themselves, accumulate and multiply, resulting in a situation where emergent problems affect the predictability and applicability of the treatments. Therefore, even if we consider a less pessimistic scenario where technology may be able to deliver individual therapies with a minimal disruption to the patient and with an effective result, the spectrum of age-associated pathologies is so wide that each one of these minimal problems will be magnified and result in a situation where adverse effects become significant, effects which cannot be reduced back to individual isolated problems. This is a typical example of emergence, a process whereby larger entities, patterns, and regularities arise through interactions among smaller or simpler entities that themselves do not exhibit such properties^[24]. As a result, we have a state where a litany of problems continually appear, making the applicability of rejuvenation biotechnologies a truly impossible approach.

The use of biomedical rejuvenation technologies in radically reducing the impact of ageing is conceptually naive, scientifically reductionist, technologically unfeasible, and medically undeliverable.

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Nutritional determinants of anemia among adults in Eastern China

Zumin Shi, Anne W Taylor

Zumin Shi, Anne W Taylor, Discipline of Medicine, University of Adelaide, SAHMRI, North Terrace, Adelaide SA 5000, Australia

Zumin Shi, Department of Nutrition and Foodborne Disease Prevention, Jiangsu Provincial Center for Disease Control and Prevention, Nanjing 210009, Jiangsu Province, China

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Correspondence to: Dr. Zumin Shi, MD, PhD, Discipline of Medicine, University of Adelaide, Level 7, SAHMRI, North Terrace, Adelaide SA 5000, Australia. zumin.shi@adelaide.edu.au
Telephone: +61-8-83131188
Fax: +61-8-83131228

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Abstract

Historically, Eastern China has the highest prevalence of anemia among adults in China. It is commonly believed

that iron deficiency is the main cause of anemia in China. Iron fortified soysauce is used to prevent anemia. Findings from Jiangsu Nutrition Study suggest that diet is related to anemia in this region. However, iron deficiency is not the main cause. Micronutrients other than iron (*e.g.*, riboflavin) need to be considered in the prevention of anemia in the region.

Key words: Anemia; Diet; Iron; Riboflavin; Magnesium; China

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Core tip: It is commonly believed that iron deficiency is the main cause of anemia in China. Here we summarize the evidence from Eastern China showing that it is not the case.

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INTRODUCTION

Yangtze delta covers Jiangsu province, Zhejiang province and Shanghai city in Eastern China. Economically, it is one of the most prosperous regions in China. Historically, findings from three Chinese national nutrition surveys (1959, 1982, and 1992) showed that the prevalence of anemia among adults in this region is the highest in China^[1]. Over the past several decades, the prevalence of anemia decreased substantially but remains high. For example, in Jiangsu province the prevalence of anemia was 18.3% in men and 31.5% in women in 2002^[2]. It is commonly believed that iron deficiency (due to low intake and poor bioavailability) is the main

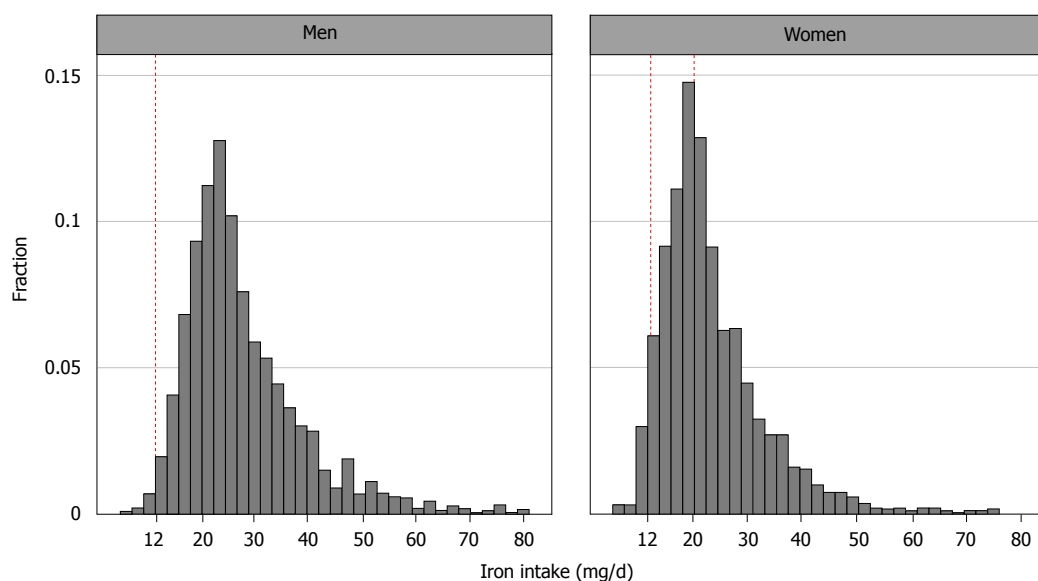


Figure 1 Distribution of iron intake among Chinese adults. Data from Jiangsu Nutrition Study (2002). Dash line represents Chinese recommended nutrient intake for iron.

cause of anemia in the region as the prevalence of Thalassemia and other haematological disorders is low^[3] and hereditary haemoglobinopathy is rare in the Chinese population^[4]. This concept has been challenged by recent findings from the region. Understanding the nutritional determinants of anemia in the region is the key for prevention and treatment.

The current editorial aims to summarize the findings from the Jiangsu Nutrition Study (JIN). The baseline survey of JIN was conducted in six counties and two cities in Jiangsu Province in 2002 as part of the Chinese National Nutrition Survey. In total, 2849 adults had dietary intake and haemoglobin (Hb) information^[5]. Dietary intake was assessed using both Food Frequency Questionnaire and 3-d weighted food record. In 2007, a 5-year follow-up survey was conducted and 1175 participants had fasting blood measured^[6].

IRON INTAKE AND ANEMIA

The Chinese Nutrition Society set the recommended nutrient intake (RNI) for iron as 12 mg/d for adults aged above 18 years and 20 mg/d for women 18-50 years^[7]. The median intake of iron in the region was 23 mg/d, which is well above the RNI especially in men (Figure 1). Among adults, the prevalence of iron intake below RNI was 1.1% in men and 25.8% in women. The distribution of serum ferritin (an indicator for iron storage) also shows a low prevalence of iron deficiency (2.1% in men, 15.1% in women). In contrast, the prevalence of iron overload (ferritin > 150 μ g/L) is 33.0% in men and 11.9% in women (Figure 2). In the sample, only 5.1% of women had both low intake of iron and low level of ferritin while no men had both conditions. The prevalence of iron deficiency anemia was 0.7% in men and 6.3% in women^[8].

The high intake of iron, low prevalence of iron deficiency and high prevalence of anemia puzzled the nutrition community in China. Currently, iron fortified soysauce is used to prevent anemia in China. The efficacy of using NaFeEDTA-fortified soy sauce to prevent anemia was tested in Guizhou, a less developed Chinese province^[9]. The food habits are totally different in our study region as compared with Guizhou. It is unknown whether the findings in Guizhou can be applicable in the Yangtze delta. A recent study shows that the association between dietary intake and anemia is different between migrant students and local students in the region^[10]. Debate on iron supplement is ongoing. Several studies in China have found that a high iron intake, especially heme iron intake or elevated serum ferritin, is associated with increased risk of diabetes^[8,11-14].

FOOD AND NUTRIENTS OTHER THAN IRON AND ANEMIA

Findings from JIN suggest that diet at different levels (dietary patterns, individual foods, and nutrients) is related to anemia.

Dietary patterns and anemia

Cross-sectional studies of dietary pattern and anemia found that a traditional dietary pattern (high intake of rice and vegetable) was positively associated with anemia^[5]. Compared with the first quartile of the traditional dietary pattern, the highest quartile had a higher odds ratio for anemia of 2.60 (95%CI: 1.38-4.88) in men and 3.40 (95%CI: 2.14-5.39) in women. This association was confirmed in the longitudinal study: for 1 unit change in traditional food pattern score, the OR for incident anemia was 1.35 (95%CI: 1.07-1.71) after adjusting for sociodemographic and lifestyle factors.

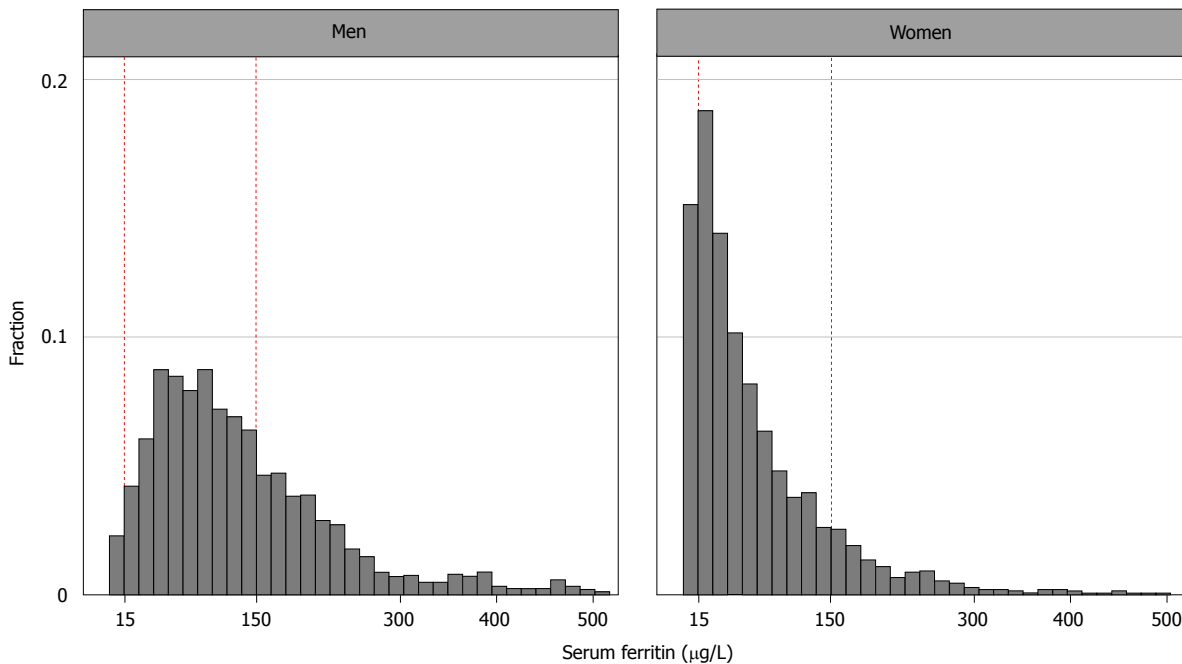


Figure 2 Distribution of serum ferritin among adults in Jiangsu China. Data from Jiangsu Nutrition Study (2002). Dash line represent low and high iron levels.

Individual food intake and anemia

Cross-sectional analysis suggests that meat intake was positively associated with anemia^[15]. However, among the anemic at baseline, compared with the first quartile of pork intake, quartile 3 and 4 were 80% (95%CI: 0.18-1.76) more likely to be non-anemic at follow up. It is likely that a reverse causation between meat intake and anemia exists at baseline. Cross-sectionally, Tofu intake was inversely related to anemia^[16]. The longitudinal association between tofu intake and anemia seems complex. Although Tofu intake was inversely associated with persistent anemia during follow-up, it was positively related to incident anemia but inversely related to anemia resolving. The cause for incident anemia or anemia resolving may be different to persistent anemia. An inverse association between Tofu intake and persistent anemia may be explained by several mechanisms: (1) Tofu intake was positively associated with magnesium intake^[16]; (2) Tofu intake is inversely associated with blood lead levels in the Chinese population^[17]; and (3) soy intake is inversely associated with inflammation biomarkers^[18]. In the sample, there was a positive association between monosodium glutamate (MSG) and increase of Hb among men but not women^[19]. Among anemic participants at baseline, there was a dose response relationship between MSG intake and increase in Hb levels during follow-up. The possible mechanisms linking MSG and anemia may include the increased secretion of gastric acid and leptin^[20,21].

Nutrients and anemia

Riboflavin and anemia: Figure 3 shows the distribution of the intake of riboflavin. The majority of the population had inadequate riboflavin intake (the Chinese RNI for

riboflavin is 1.4 mg/d for men and 1.2 mg/d for women aged above 18 years^[7]). This is in line with findings from the China Nutrition and Health Survey (CHNS), which showed that the mean intake of riboflavin was around 0.7-0.9 mg/d among adults aged 18-45 years in six surveys between 1989 and 2004^[22]. The main reason for the low intake of riboflavin is the low consumption of milk and animal food.

Based on longitudinal analysis, it was found that inadequate riboflavin intake increases the risk of anemia^[23]. There was a significant interaction between riboflavin and iron intake in relation to anemia risk. When riboflavin intake is inadequate, a high iron intake reduces the risk of anemia. However, when riboflavin intake is adequate iron intake is not a determinant of anemia^[23]. The possible explanation for the link is that riboflavin can enhance iron absorption and utilization^[24,25]. If the riboflavin level is low, the ability to mobilise iron from ferritin to synthesis Hb will be limited^[26]. This could be one of the reasons why Hb is low but ferritin is relatively normal in the region. Thus correcting inadequate riboflavin intake may be a priority for anemia prevention in the region. A recent trial in China showed that retinol and riboflavin supplements decreased the prevalence of anemia in pregnant women who were also taking iron and folic acid supplements^[27]. In United Kingdom, it has been found that riboflavin supplementation improves hematologic status among women aged 19-25 years with moderate riboflavin deficiency^[28].

Magnesium and anemia: A high intake of magnesium was associated with a lower prevalence of anemia^[2]. Comparing extreme quartiles of magnesium intake, the odds ratio for anemia was 0.48 (95%CI: 0.31-0.74).

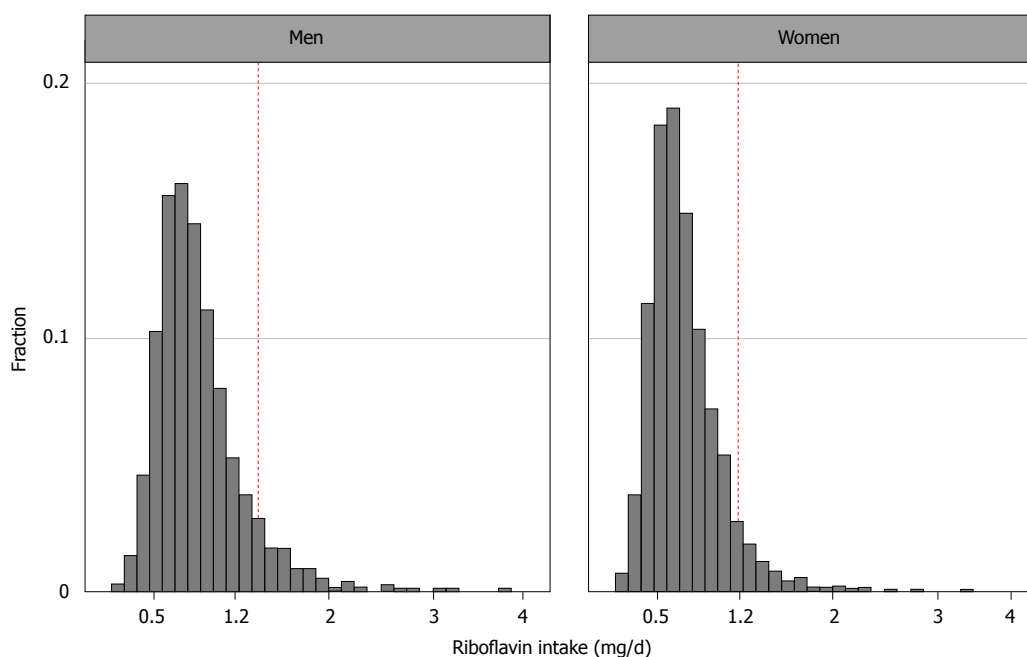


Figure 3 Distribution of riboflavin intake among Chinese adults. Data from Jiangsu Nutrition Study (2002). Dash line represents Chinese recommended nutrient intake for riboflavin.

There was a joint effect between magnesium and iron intake in relation to anemia^[2]. Data from CHNS also shows that serum magnesium is inversely associated with anemia in both men and women^[29].

Vitamin C and anemia: In JIN, there was no association between vitamin C intake and anemia both cross-sectionally and longitudinally.

Other nutritional related factors and anemia: Different from Western countries, both general and central obesity were inversely related to anemia in the region^[30]. Exposure to the Chinese famine in early life was positively associated with anemia in adulthood^[31].

CHALLENGES AND LIMITATIONS

Despite the known health risks of anemia, the adverse effects of anemia in this population are not well studied. There are a limited number of longitudinal studies on nutrition and anemia in the region. Surprisingly, maternal anemia is not associated with neonatal mortality in the region. The stillbirth rate was lower among those with anemia than those without anemia (6.2 vs 9.2 per 1000 births)^[32]. Based on 10-year follow-up of JIN participants, both high and low Hb are related to an increased risk of mortality in both men and women (unpublished data). While emphasizing the importance of low Hb levels, we should not neglect the adverse effects of elevated levels of Hb.

CONCLUSION

Diet is related to anemia among adults in Eastern China.

Iron intake is in general adequate in men as compared with RNI. Micronutrients other than iron (e.g., riboflavin) need to be considered in the prevention of anemia. Prospective cohort studies and randomized clinical trials are needed. Iron supplement should not be the first choice of anemia prevention at the population level.

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World Journal of Translational Medicine
Room 903, Building D, Ocean International Center,
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Telephone: +86-10-85381891
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Contributions of neutrophils to the adaptive immune response in autoimmune disease

Kathryn M Pietrosimone, Peng Liu

Kathryn M Pietrosimone, Thurston Arthritis Research Center, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, United States

Peng Liu, Department of Medicine and Thurston Arthritis Research Center, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, United States

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Correspondence to: Peng Liu, MD, PhD, Department of Medicine and Thurston Arthritis Research Center, University of North Carolina at Chapel Hill, 3300 Thurston Building, CB#7280, Chapel Hill, NC 27599, United States. liupz@med.unc.edu
Telephone: +1-919-9660570
Fax: +1-919-9669269

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Abstract

Neutrophils are granulocytic cytotoxic leukocytes of

the innate immune system that activate during acute inflammation. Neutrophils can also persist beyond the acute phase of inflammation to impact the adaptive immune response during chronic inflammation. In the context of the autoimmune disease, neutrophils modulating T and B cell functions by producing cytokines and chemokines, forming neutrophil extracellular traps, and acting as or priming antigen presentation cells. Thus, neutrophils are actively involved in chronic inflammation and tissue damage in autoimmune disease. Using rheumatoid arthritis as an example, this review focuses on functions of neutrophils in adaptive immunity and the therapeutic potential of these cells in the treatment of autoimmune disease and chronic inflammation.

Key words: Neutrophils; Chronic inflammation; Autoimmune disease; Rheumatoid arthritis; Collagen-induced arthritis

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Core tip: Neutrophils guide the adaptive immune response in persistent inflammation by directly and indirectly interacting with T and B cells. Understanding and manipulating these roles of neutrophils will lead to novel therapeutic approaches to prevent and treat autoimmune diseases, such as rheumatoid arthritis.

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INTRODUCTION

Neutrophils are polymorphonuclear granulocytes comprised of enzyme-containing granules. Neutrophils generate from the bone marrow and account for

50%-70% of circulating leukocytes in humans and 10%-25% in mice^[1,2]. Under acute inflammation, particularly as a result of bacterial infection, neutrophils are the first leukocytes to respond, migrate to the site of inflammation, and kill microorganisms through phagocytosis, degranulation and generation of neutrophil extracellular traps (NETs)^[3-5]. These cells have long been thought of as short-lived cells of the innate immune response.

However, recent research evidence has demonstrated that neutrophils persist beyond acute inflammation to initiate and perpetuate chronic inflammation. The onset of inflammation increases the lifespan of neutrophils in circulation, anywhere from 12 h to several days^[6]. Pro-inflammatory cytokines, such as tumor necrosis factor- α (TNF- α) and interferon- γ , inhibit neutrophil apoptosis^[7]. Neutrophils also change phenotypes as inflammation persists. For instance, Neutrophils isolated from various inflammatory states show altered cell densities in gradient centrifugation^[8,9]. The expression of granulocyte marker CD66b in neutrophils increases in patients with rheumatoid arthritis (RA), and decreases with the treatment by anti-inflammatory glucocorticoids, suggesting neutrophils may alter functions during chronic inflammation^[10]. Glucocorticoids, a class of drugs often prescribed to patients with autoimmune disease, also inhibit apoptosis of neutrophils and increase the neutrophilic production of reactive oxygen species (ROS)^[11].

Tissue-specific autoimmune disease such as RA, multiple sclerosis, and type I diabetes are generated from unknown etiology and impact the quality of life of patients through sustained chronic inflammation driven by innate and adaptive immune responses. Neutrophils bridge the innate and adaptive immune response in autoimmune disease. This review focuses on the functional heterogeneity of neutrophils in autoimmune disease and the contribution of these roles to chronic inflammation, in the context of RA.

RA

RA is an autoimmune disease, in which the body generates antibodies against its own tissues. RA is characterized by tissue-specific autoimmune-mediated chronic inflammation that affects multiple joints and results in destruction of cartilage and bone loss^[12]. Risk factors of RA are multi-dimensional and include genetic defects, infections, and environmental influences^[12].

Population studies of RA estimate that genetics account for about 50% of RA disease susceptibility^[13-15]. The most consistent predictor of susceptibility to RA is the link between specific major histocompatibility (MHC) II-associated alleles of the *DRB1* gene that encode human leukocyte antigen (HLA)-DR4 and the onset of RA^[16]. All of the susceptible alleles encode a conserved amino acid sequences on MHC II^[17]. RA patients with these susceptible HLA-DR4 alleles and subsequent expression of the conserved amino acid sequence on MHC II, develop more autoantibodies associated with

RA-related joint breakdown than patients without these alleles^[18]. Activated antigen-presentation cells (APCs), including dendritic cells and macrophages, upregulate MHC II surface molecule expression, which activates antigen-specific T cells and B cells and initiates the adaptive immune responses in autoimmune disease.

Bacterial infections may trigger the onset of RA. A population-based study in Sweden determined that 45% of inflammatory arthritis patients had an infection prior to the onset of early arthritis^[19]. In Chlamydia-induced reactive arthritis, the microbe primes neutrophils through toll-like receptor signaling, which activates the cell to clear the infection. However, activated neutrophils can also infiltrate the joint and cause chronic inflammation^[20]. Neutrophils may employ the same mechanisms in the onset of RA after an infection. Although many microbial factors have been found in synovial fluid of RA patients, there is no clear agreement that these microbial factors are the causative agents of RA^[21-23].

Environmental factors, such as cigarette smoke, make provoke the development of RA in genetically susceptible populations^[24,25]. Smokers with the HLA-DR shared epitope allele were 3 times more likely to test positive for rheumatoid factor (RF) than non-smokers with HLA-DR shared epitope allele^[25]. Smoking increases the activation and migration of neutrophils^[26,27]. Dysregulated neutrophils as a result of cigarette smoking generate a systemic inflammatory environment that is associated with autoimmune disease, such as RA and systemic lupus erythematosus^[28,29].

Neutrophils account for the majority of inflammatory cells in the synovia of human RA patients, and the joints of collagen-induced arthritis (CIA) mice, a murine model of RA^[30,31]. In both humans and mice, neutrophils accumulate at the pannus-cartilage interface, where much the destruction to both bone and cartilage takes places^[32-34]. Thus, an in-depth understanding of neutrophil function in arthritis is vital to the prevention and treatment of this disease.

Neutrophil identification

The surface marker granulocyte receptor-1 (Gr-1) was previously used to identify neutrophil populations in murine models^[35], as it was thought only mature granulocytes express Gr-1. Later researchers found that Gr-1 antibodies cross-react and bind two Ly-6 family member proteins, Ly6G and Ly6C^[36,37]. While neutrophils express Ly6G, monocytes, memory T cells, and some dendritic cell subsets express Ly6C^[36,38-40]. Studies utilize anti-Gr-1 antibodies to deplete neutrophils and attribute the presence of neutrophils to the ability of cancers cells to acquire metastatic phenotypes^[41,42]. Although neutrophils make up the majority of Gr-1⁺ cells in these studies, the increased metastasis after administration of anti-GR-1 antibodies cannot be attributed solely to neutrophil depletion, as Ly6C⁺ monocyte are also depleted. In CIA, the depletion of neutrophils with anti-GR-1 antibody concluded that neutrophils were necessary for the onset and maintenance of disease,

as this treatment prevented the onset of disease and ameliorated established disease^[31]. Macrophages express Gr-1 and also infiltrate the CIA joint, so this method fails to delineate the roles of neutrophils from macrophages in CIA. Currently, studies utilize antibody clone IA8 to specifically bind Ly6G and deplete neutrophils without an effect on other leukocyte populations^[43].

In general, current murine studies identify neutrophils as CD11b⁺Ly6G⁺Ly6C⁺ cells, and monocytes as CD11b⁺Ly6G⁻Ly6C⁺ cells. Human neutrophils are defined as CD14^{lo/neg}CD15⁺CD16^{hi}CD33⁺CD11b⁺CD15⁺CD66b⁺, and human monocytes are defined as CD14⁺CD16⁺HLA-DR⁺CD66⁻ cells^[44].

Neutrophil effect on T cells

Neutrophils, unlike dendritic cells and macrophages, are not defined as APCs. APCs express antigen to T cells via MHC II molecules and stimulates T cell activation with the aid of co-stimulatory molecules CD80 and CD86. In autoimmunity, autoantigen is presented and antigen-activated T cells quickly expand and migrate to the site of inflammation and induce tissue inflammation through the production of pro-inflammatory cytokines^[45]. In genetically susceptible individuals, the conserved amino acid sequence on MHC II may manipulate the antigen presentation process and cause activation of autoreactive T cells^[46]. In the absence of autoimmune disease, neutrophils isolated from the peripheral blood of healthy controls produce MHC II mRNA, but do not express the cell surface molecule^[47]. Exposure of these healthy neutrophils to synovial fluid from an RA patient elicits surface expression of both MHC II and co-stimulatory molecules^[48]. Contact with T cells induces neutrophilic expression of MHC II and costimulatory molecules^[48]. Since T cells are abundant in the RA joint, neutrophils continually express MHC II and co-stimulatory molecules and act as APCs. This further activates T cells and forms a vicious feedback loop that promotes chronic inflammation and tissue damage in the joints^[48-50]. The continuous activation of T cells in the joint advances joint destruction through the production of pro-inflammatory cytokines and activation of autoreactive B cells^[51,52].

Recently, studies have linked neutrophil functions to Th17 cells. Th17 cells, which produce IL-17, are potent proinflammatory mediators and have been implicated in the pathogenesis of autoimmune disease^[53-56]. IL-17 can induce tissue inflammation by stimulating the recruitment of neutrophils. In the RA joint, IL-17 activates fibroblast-like synoviocytes (FLS), macrophages, and osteoblasts^[53-58]. FLS activation produces the potent neutrophil chemoattractant IL-8^[59,60]. Activation of macrophages produces TNF- α ^[61], and the combination of IL-17 and TNF- α in the joint stimulates synovial endothelial cells to produce more neutrophil chemoattractants^[62,63].

Reciprocally, neutrophils help sustain Th17 cells in the joint through the secretion of Th17 chemokines CCL20 and CCL2^[60]. Human neutrophils purified from the synovial fluid of RA patients express high levels of

these chemokines^[60]. Despite the presence of other chemokines in the joint, Th17 cells preferentially migrate toward CCL20^[64]. Mice that lack the CCL2 receptor (CCR2^{-/-}) develop exacerbated CIA. Furthermore, Th17 and neutrophil populations expand in the lymph nodes and joints of these mice^[65]. Therefore, CCL20 likely plays a more prominent role in Th17 migration than CCL2. The expansion of both neutrophils and Th17 cells in mice with exacerbated arthritis demonstrates the importance of both these cell types to the onset and maintenance of autoimmune arthritis. The reciprocal signaling between Th17 cells and neutrophils causes accumulation and activation of these cells, cultivating an inflammatory microenvironment in the joint^[58,60,66].

Although reducing neutrophils in the joint is beneficial to limit sustained T cell activation, systemic neutropenia can cause infection. Some therapeutics intervene with proinflammatory events that upregulate neutrophils, instead of depleting neutrophil function. A phase II trial that utilizes the anti-IL-17 monoclonal antibody drug, secukinumab, improves symptoms in 46% of RA patients after 16 wk of treatment, and can safely maintain these improvements through week 52^[67,68]. Infection rates of patients on these drugs were 31.9% through week 52. Most infections were mild, but, interestingly, were not associated with neutropenia^[68]. Simultaneously targeting neutrophils may improve IL-17 treatments, as activation of Th17 cells would decrease in addition to just decreasing the IL-17 effector molecule. However, as with many autoimmune therapies, this may leave the patient susceptible to infection.

Neutrophil effect on B cells

One of the major features of autoimmune disease is the presence of autoantibodies in circulation. In RA patients, various autoantibodies against cartilage components, chaperones, enzymes, nuclear proteins, and citrullinated proteins have been identified. However, the clinical significances and pathogenic roles of these antibodies are largely unknown, except for RF, anti-citrullinated protein antibodies (ACPA), and anti-collagen antibodies, which are all associated with joint inflammation^[69-72]. Neutrophils stimulate the activation, proliferation, differentiation, and antibody-production of B cells through the production of the B cell stimulating factor BAFF (also known as BLyS)^[73-75]. Peripheral blood neutrophils from both RA patients and healthy control patients express BAFF as a membrane bound molecule^[76]. However, TNF- α in the RA joint releases surface-bound BAFF from neutrophils and increases the concentration of soluble BAFF^[76]. High levels of soluble BAFF in the serum of RA patients correlates with high concentrations of autoantibodies^[77].

Neutrophils in the joint act on B cells similarly to splenic marginal zone neutrophils, which induce antibody production and immunoglobulin class switching through the production of the B cell stimulants BAFF, APRIL, and IL-21^[78]. These stimulants drive the formation of splenic germinal centers that support the proliferation and

differentiation of B cells. The blockage of BAFF decreases the size and disorganizes splenic germinal centers^[79,80]. Structures similar to splenic germinal centers develop in the synovia of some RA patients^[81]. A therapy that reduces soluble BAFF released from neutrophils may prevent synovial germinal center formation and reduce autoantibody production^[82]. Thus, soluble BAFF from neutrophils plays a critical role in facilitating an environment, both systemically and locally, that activates B cells and perpetuates autoantibody formation.

The release of ROS by neutrophils^[83,84] generates advanced glycation end-products (AGE) through oxidant-induced alteration of the structures of lipids, DNA, and proteins^[83-86]. High levels of AGE in the sera of RA patients correlates with a high disease severity and high levels of inflammation markers^[87]. B cells recognize ROS-modified structures as foreign molecules and produce autoantibodies against these structures^[88,89]. ROS modifies type II collagen, the main structural component in human articular cartilage and induces the production of autoantibodies to ROS-modified collagen^[90-94]. In an *in vitro* study, serum from RA patients could only bind type II collagen after exposure to ROS produced by neutrophils^[95].

Autoantibodies form immune complexes in the joint, which induce neutrophil infiltration and activation through complement-mediated pathways^[96,97]. B cells and neutrophils, therefore, work in concert to maintain inflammation in the RA joint.

Treatment with rituximab, a monoclonal anti-CD20 antibody that depletes B cells, decreases the severity of RA most efficiently in RA patients with high levels of autoantibodies^[98,99]. Some patients experience late-onset neutropenia up to 12 mo following rituximab treatment^[99]. The exact mechanism that causes late-onset neutropenia is not known. One popular hypothesis suggests B cells compete with neutrophils for resources in the developmental niche of the bone marrow as the B cells repopulate after rituximab treatment^[98].

Neutrophils and NET formation

Neutrophils form NETs through a unique model of cell death known as NETosis^[100]. The formation of NETs requires activated neutrophils to lose integrity of intracellular membranes prior to that of the plasma membrane^[101]. In the first steps of NET formation, granules containing cytotoxic antimicrobial proteins decay, and chromatin condenses as the nuclear membrane collapses. The plasma membrane then invaginates, ruptures, and releases NETs comprised of intracellular antimicrobial contents into the extracellular space^[102]. In the context of infection, the antimicrobial proteins trap and kill infiltrating microbes in the extracellular space^[100,103].

NETs release citrullinated histones and proteins into the extracellular space^[104,105]. Citrullination of a protein or histone is a post-translational modification (PTM) that converts arginine residues to citrulline^[104,105]. This process changes the structure and antigenicity

of proteins and histones, as the adaptive immune response can recognize PTM as non-self^[106]. In RA, ACPA are of particular interest as an increase in ACPA correlate with an increased disease severity^[107,108]. An increased propensity for neutrophils to die *via* NETosis correlates with increased levels of ACPA in the serum of RA patients^[105], which suggests NETs are a major source of autoantigen in RA. In fact, proteins extracted NET-induced peripheral blood neutrophils react with sera from RA patients. Sera from RA patients react specifically with citrullinated histone H4^[109].

The discovery of a conserved citrullinated antigen associated with the onset of RA opens up a novel avenue of therapeutic intervention. The enzyme PAD4 controls the citrullination of histone H4^[109]. Interruption of PAD4 function could decrease citrullination of histone H4 and subsequent autoantibody production that is crucial to the development of RA.

NETosis also stimulates FLS to produce the proinflammatory cytokines IL-6, IL-8, and the Th17-associated chemokine CCL20^[105]. Production of IL-6 and IL-8 aid in the polarization of CD4⁺ T cells to Th17 phenotypes, while CCL20 traffics Th17 cells to the site of inflammation. Thus, NET stimulation of FLS shapes a microenvironment favorable to sustained inflammation associated with Th17 cells.

Neutrophils as myeloid-derived suppressor cells

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous group of immature myeloid cells derived from the bone marrow under pathologic conditions that suppress T cell functions^[110]. Murine MDSCs are divided into two subsets based on surface expression of Ly6G and Ly6C; the Ly6G⁺Ly6C⁺CD11b⁺ granulocytic-MDSC (G-MDSC) subset and the Ly6G⁺Ly6C⁺CD11b⁺ monocytic-MDSC (M-MDSC) subset^[111]. The counterparts of M-MDSC subset in humans have been identified as CD11b⁺CD14⁺CD15⁺HLA-DR⁺Lin⁻, while human G-MDSC subset are identified as CD11b⁺CD15⁺CD14⁺HLA-DR⁺Lin⁻^[111] (Table 1). M-MDSCs are immature monocytes that suppress T cell functions through the secretion of inducible nitric oxide synthase (iNOS)^[112-114]. G-MDSCs are neutrophil-like cells that inhibit T cell function through the production of arginase-1^[114].

First described in murine tumor models, MDSCs accumulate in the lymphoid tissues of tumor-bearing mice^[115] and significantly increase in the circulation of cancer patients compared to healthy controls^[116-118]. MDSCs promote tumor growth by inhibiting T-cell mediated immune surveillance and cytotoxic effects on tumor cells^[119]. Recently, evidence indicates MDSCs also contribute to the regulation of autoimmune disease by inhibiting CD4⁺ T cell proliferation and differentiation^[120,121].

Neutrophil-like G-MDSCs are believed to be immunosuppressive in autoimmunity. In the experimental autoimmune encephalomyelitis (EAE) murine model of multiple sclerosis, G-MDSCs express high levels of programmed cell death 1 ligand 1 (PD-L1), a costi-

Table 1 Surface markers used in identification of myeloid-derived suppressor cells

	Human	Mouse
MDSC	CD11b ⁺ CD33 ⁺	CD11b ⁺ Gr-1 ⁺
M-MDSC	CD11b ⁺ CD14 ⁺ CD15 ⁻ HLA-DR ⁻ Lin ⁻	CD11b ⁺ Ly6C ⁺ Ly6G ⁻
G-MDSC	Cd11b ⁺ CD15 ⁺ CD14 ^{lo} HLA-DR ⁻ Lin ⁻	CD11b ⁺ Ly6G ⁺ Ly6C ⁺
Progenitor neutrophil	CD14 ^{lo/neg} CD15 ⁺ CD16 ^{lo/neg}	
Mature neutrophil	CD14 ^{lo/neg} CD15 ⁺ CD16 ^{hi} CD33 ⁺ CD11b ⁺ CD15 ⁺ CD66b ⁺	CD11b ⁺ Ly6G ⁺

MDSC: Myeloid-derived suppressor cell; M-MDSC: Monocytic-MDSC; G-MDSC: Granulocytic-MDSC.

mulatory molecule that negatively regulates T cell proliferation. G-MDSCs inhibit autoantigen-priming of Th1 and Th17 cells in a PD-L1-dependent manner^[122]. In CIA, MDSCs that include mostly neutrophil-like G-MDSCs suppress both T cell proliferation and CD4⁺ T cell differentiation into Th17 cells, mainly through the production of arginase-1^[123]. The depletion of MDSCs increases inflammation and disease severity, while the subsequent adoptive transfer of MDSC ameliorates arthritis^[123]. Furthermore, based on our published^[124] and unpublished data, M-MDSCs as well as G-MDSCs not only suppress T cell functions, but also inhibit B cell proliferation in the context of CIA.

The use of CD11b⁺Gr-1⁺ cells in most functional MDSC studies makes it difficult to confirm G-MDSCs immunosuppressive effects in autoimmune arthritis. Additionally, utilization of Ly6G to recognize G-MDSCs cannot differentiate G-MDSCs from neutrophils. Similar issues exist in human MDSCs, as mature human neutrophils express many of the same surface receptors as G-MDSCs and cannot be distinguished without functional analysis^[125].

Further studies comparing G-MDSC phenotypes vs neutrophil phenotypes are needed to determine the effect of these cells on the adaptive immune response. Current evidence indicates G-MDSCs and neutrophils act in opposing manners on the adaptive immune response^[125]. Uncovering mechanisms that lead to the plasticity of G-MDSCs and neutrophils in autoimmune disease could lead to cell-based therapies that convert pro-inflammatory cells to immunosuppressive cells.

Neutrophils and potential therapeutics

As one of major contributing factors in the establishment of chronic inflammation in RA, neutrophils serve as potential therapeutic targets. Some current therapies for RA interfere with the functions of neutrophils. For example, anti-TNF- α therapies reduce IL-33 receptor expression on neutrophils and subsequently decrease neutrophil migration. Neutrophils from RA patients treated with anti-TNF- α therapies do not respond to IL-33-mediated chemotaxis^[126]. Impaired chemotaxis of neutrophils may lead to a decrease in inflammation and disease severity. The wide array of effector proteins produced by neutrophils, such as BAFF, could also become therapeutic targets for RA.

NETs may serve as a novel therapeutic target for RA and other NET-associated autoimmune diseases. In

addition to targeting enzymes associated with PTM, the use of DNase to breakdown the extracellular DNA and histones associated with NETs has been suggested as a potential therapeutic in NET-associated diseases^[127]. The study of NETs in RA may also reveal currently unknown citrullinated proteins that contribute to RA pathogenesis and could serve as therapeutic targets.

In CIA, the adoptive transfer of MDSCs decrease T and B cell proliferation and decreases the severity of arthritis^[123,124]. The discovery of a mechanism that induces the G-MDSC phenotype from neutrophil-like cells could be crucial for the therapy of autoimmune disease. Manipulation of this mechanism will drive neutrophil-like cells toward an immuno-suppressive MDSC phenotype that impedes the super-active adaptive immune response, and thereby reduces chronic inflammation in autoimmunity. An in-depth understanding of the contributions of the joint microenvironment to the various neutrophil phenotypes, and subsequent neutrophil functions, may aid in the development of neutrophil-based RA therapies.

Similar to many autoimmune therapies, inhibition of neutrophil functions leaves the patients susceptible to infection. For this reason, the inhibition of specific enzymes, such as PAD4, may be a promising therapeutic intervention. If PAD4 can be neutralized, this could curb autoantibody production without completely diminishing the antimicrobial function of neutrophils.

CONCLUSION

Neutrophils are involved in the onset and progression of RA in a complex capacity. Neutrophils engage in several reciprocal signaling events with both B and T cells, which promote a microenvironment conducive to sustained inflammation. The formation of NETs increases the production of ROS and ACPA, which are hallmarks of RA. Neutrophils can act as pro-inflammatory cells influencing chemotaxis and immune cells signaling, but can also have a MDSC phenotype that suppresses the immune response. Since neutrophils affect many aspects of the adaptive immune response and drive chronic inflammation, the disruption of the signals between neutrophils and the adaptive immune response can serve as therapeutic targets for RA.

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Targeting apoptosis is the major battle field for killing cancers

Xiao-Chun Liu, Jiang-Ming Gao, Shan Liu, Li Liu, Jing-Rui Wang, Xian-Jun Qu, Bing Cai, Shu-Lin Wang

Xiao-Chun Liu, Jiang-Ming Gao, Shan Liu, Li Liu, Jing-Rui Wang, Bing Cai, Shu-Lin Wang, the Marine Biomedical Research Institute of Qingdao, Department of Pharmacology, School of Medicine and Pharmacy, Ocean University of China, Qingdao 266071, Shandong Province, China

Xian-Jun Qu, Department of Pharmacology, School of Basic Medical Sciences, Capital Medical University, Beijing 100000, China

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Correspondence to: Shu-Lin Wang, MD, PhD, Professor, the Marine Biomedical Research Institute of Qingdao, Department of Pharmacology, School of Medicine and Pharmacy, Ocean University of China, 23 Hongkong East Rd. Marine Biomedical Research Building, Qingdao 266071, Shandong Province, China. shulinwang@ouc.edu.cn
Telephone: +86-532-80932613

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Abstract

Targeting apoptosis is one of the major strategies for cancer therapy. Essentially, most of the conventional cancer therapeutic drugs that are in the clinical use induce apoptosis and in part necrosis of malignant cells and therefore prevent cancer progression and metastasis. Although these cytotoxic anticancer drugs are important weapons for killing cancers, their toxic side effects limited their application. The molecularly targeted therapeutics that are based on the deeper understanding of the defects in the apoptotic signaling in cancers are emerging and have shown promising anticancer activity in selectively killing cancers but not normal cells. The examples of molecular targets that are under exploration for cancer therapy include the cell surface receptors such as TNFR family death receptors, the intrinsic Bcl-2 family members and some other intracellular molecules like p53, MDM2, IAP, and Smac. The advance in the high-throughput bio-technologies has greatly accelerated the progress of cancer drug discovery.

Key words: Apoptosis; Chemotherapy; Drug targets; Drug resistance; Cancer; Translational medicine

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Core tip: Chemotherapy and radiotherapy are important approaches for cancer therapy and have prolonged the lifespan and reduced the mortality of cancer patients. But chemotherapy and radiotherapy induce apoptosis in both cancer and normal cells, therefore possessing severe toxic side effects. It appears quite important to develop the biological mechanism-based drugs that that can selectively kill tumor cells but not normal

cells. Molecular targets in the apoptotic signaling pathways such as p53, TRAIL, and Bcl-2 have been identified, and molecularly targeting drugs for a variety of tumors based on these pathways are currently under development. Dissecting the genetic alterations in a particular tumor type and designing the rational drug combinations targeting different pathways can help achieve synergy in eradicating cancer cells and reversing drug resistance, and this holds great promise for the personalized treatment of cancer patients.

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INTRODUCTION

The most important concern facing current cancer therapy is the lack of tumor selective agents that kill cancer cells effectively but do not harm normal cells. In the past decade, many of the efforts have been contributed to dissecting the mechanisms underlying the pathogenesis of cancer, particularly the altered signal transduction pathways in different cancers^[1-5]. Recent advances in our understanding of how a normal cell is mutated to become cancerous and the accumulation of mutations in cancerous cells leads to malignancy, progression and metastasis have greatly promoted the identification of new molecular targets for developing tumor selective therapeutics^[6-10].

Apoptosis is a programmed physiological process to eradicate unwanted cells for maintaining homeostasis^[11-14]. The signal transduction pathways mediating apoptosis are frequently deregulated in cancers, enabling cancer cells to evade from apoptosis and become hyper-proliferated. Most of the genetically altered signaling components in the intrinsic and extrinsic apoptotic pathways in cancers are usually the central regulators of apoptosis, which play a key role in arbitrating the fate of a cell^[15,16]. The key components in the apoptotic machinery are the Bcl-2 family members including Bcl-2, Bax and Bcl-xl in the mitochondrial pathway, the extrinsic cell surface receptors such as death receptor KILLER/DR5, and some other intracellular molecules such as p53^[17-25]. These signaling molecules are frequently mutated or deleted in cancers and therefore are the ideal targets for developing novel cancer targeting drugs^[24-28]. Targeting of Bcl-2 with RNAi and BH3 mimetics are currently underway in the clinical trial studies and have shown significant clinical activity in selectively killing cancer cells^[29-32]. High-throughput screening of chemical libraries have identified some small molecule compounds that can target p53 to activate or restore p53 functions, which already showed success in killing some of the cancer cells^[33,34]. Loss of p53 in cancers confers resistance to some

chemotherapeutics and tumors bearing p53 mutations are less sensitive to radiotherapy^[35-37]. Optimal combination of agents targeting different targets in the signal transduction pathways in cancers leads to a synergistic effect on killing tumors and overcoming resistance to single agent treatment^[25,38].

In this review, we summarize the basic understanding of the apoptotic signal transduction cascades in cancers and the crosstalk between different pathways in which the signaling components are mutated in cancers. We also introduce some of the knowledge about the mechanisms of tumor targeted drugs and chemotherapeutic agents in killing cancers. Moreover, we highlight recent advances in the technologies that are applied for identification of cancer drug targets and the strategies for cancer drug screening. The translational designs of drug combination for achieving synergy in eradicating cancer cells and reversing drug resistance will also be discussed.

DYSREGULATED CELL DEATH PATHWAYS IN CANCERS

The complex apoptotic signaling networks play a key regulatory role in maintaining homeostatic cellular progresses in living organisms^[39]. There are two major signaling pathways that mediate apoptosis in mammalian cells: The extrinsic apoptotic signaling pathway and the intrinsic apoptotic signaling pathway (Figure 1). Some intracellular stimuli, such as DNA damaging agents, irradiation or oncogene activation, trigger apoptosis primarily through the intrinsic pathway which requires the activation of apoptotic regulators in the mitochondrion. Extracellular apoptotic pathway is initiated through apoptotic signaling cascades mediated by members of the tumor necrosis factor (TNF) superfamily, usually generated by the cytotoxic cells of the immune system, for example TNF, TRAIL, and FasL. Both pathways require the activation of proapoptotic cysteine proteases, caspases, to execute the cell death process^[40-43]. Most of the genetically mutated intracellular signaling molecules in the intrinsic and extrinsic apoptotic pathways in cancers are the central regulators of apoptosis which arbitrate life-or-death decision of a cell. Mutations in these signaling components allow cancer cells to escape from eradicating by the host immune system and accelerates the proliferation of transformed tumor cells^[44,45].

Some of the mutated molecules in the apoptotic machinery have been identified as important markers for the distinction between normal and cancer cells, and therefore are the targets for developing tumor therapeutic drugs. The alterations in the apoptotic pathways in cancers include the Bcl-2 superfamily members in the mitochondrion. All the Bcl-2 pro-survival family members like Bcl-2 and Bcl-XL are likely to be oncogenic^[15,17,18]. Bcl-2 was found to be overexpressed and implicated in the pathogenesis of myeloid and T-cell leukemia^[21]. Conversely, members of the pro-apoptotic

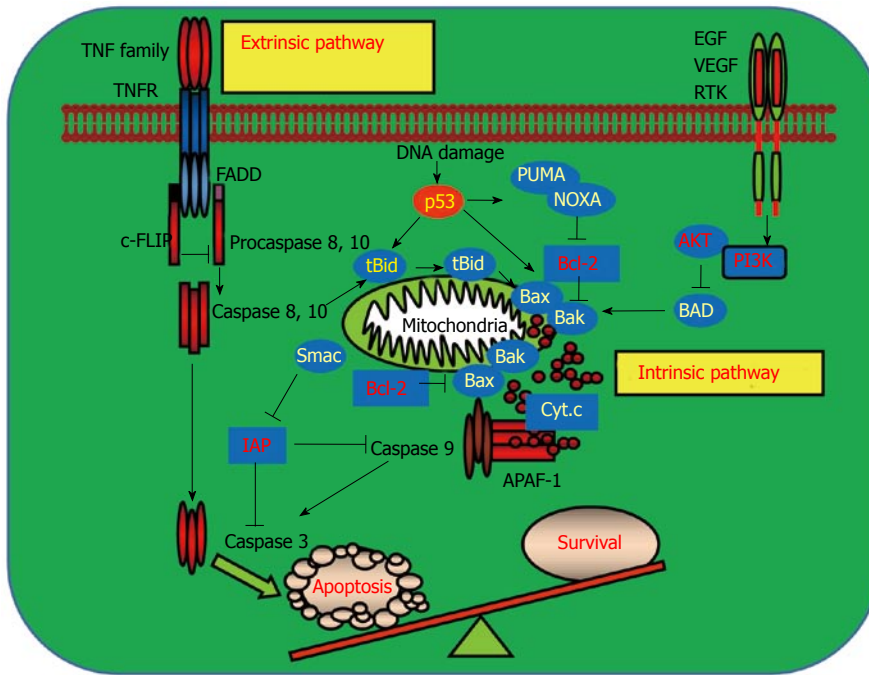


Figure 1 Signaling pathways mediating programmed cell death and cell survival. Apoptosis can be induced by extra- and intra-cellular stimuli. Activation of the cell surface receptors such as tumor necrosis factor receptor (TNFR) upon binding to their ligands leads to activation of the receptor-mediated extrinsic apoptotic pathway. Activation of death receptors triggers the cleavage of pro-caspases 8 and 10 to their active forms by recruiting adaptor protein, Fas associated via death domain (FADD) to form a death inducing signaling complex (DISC). The activated caspases 8 and 10 then activate the downstream effector caspase 9 and result in apoptosis. DISC formation is negatively regulated by cellular Fas-associated death domain-like interleukin-1 β converting enzyme inhibitory protein (c-FLIP) which inhibits apoptosis. Intracellular stresses such as DNA damage, oncogene activation and virus infection activate the intrinsic apoptotic pathway which usually requires p53 function. p53 triggers the intrinsic apoptotic pathway by upregulating its downstream proapoptotic target genes including the p53 up-regulated modulator of apoptosis (PUMA), phorbol-12-myristate-13-acetate-induced protein 1 (NOXA), Bcl-2 associated X protein (Bax) and Bcl-2 antagonist killer1 (Bak), which in turn lead to the release of cytochrome c from the mitochondrion. In the cytosol, the released cytochrome c binds to apoptotic protease activating factor 1 (APAF-1) and caspase 9 to form the apoptosomes. Activated caspase 9 then cleaves and activates the effector caspases 3, 6, and 7 to execute apoptosis. The mitochondrial protein Smac augments apoptosis by interacting and inhibiting inhibitor of apoptosis proteins (IAPs) which block the activation of caspase 9. Akt, an important downstream target in response to the activation of receptor tyrosine kinases, inhibits apoptosis by phosphorylating and inhibiting the proapoptotic Bcl-2 associated antagonist of cell death (BAD) which prevents the activation of Bax and Bak. Bid crosslinks the extrinsic and intrinsic pathways in response to extrinsic stimuli. Activation of caspases 8 and 10 causes the cleavage of BH3 interacting domain death agonist (BID) and the truncated BID is then translocated to the mitochondrion to engage the activation of the intrinsic pathway by interacting with Bax and Bak. EGF: Epidermal growth factor; VEGF: Vascular endothelial growth factor; RTK: Receptor tyrosine kinase.

subfamily such as Bax and Bak are probably tumor suppressors. Bax or Bak is frequently mutated or deleted in some gastric and colorectal cancers. Cell surface death receptor KILLER/DR5 is mutated in some of the head and neck cancers and lung cancers^[46,47]. P53 is mutated or deleted in more than 50% of the human cancers. P53 induces apoptosis through regulating its downstream targets including the proapoptotic Bax, KILLER/DR5 and p53 upregulated modulator of apoptosis (PUMA). Loss of p53 impairs the apoptotic signaling pathways by aberrant control of its downstream apoptotic target genes and confers resistance to chemotherapy or radiotherapy-induced apoptosis^[35-37,48-50]. Oncogenic mutations impair apoptosis indirectly by prompting or repressing the expression of the signaling molecules in cell death pathways and promote tumor progression and metastasis.

TARGETING APOPTOTIC PATHWAYS FOR CANCER THERAPY

Recent advances in our understanding of how apoptosis

is activated and the mechanisms how cancers evade apoptosis have paved new revenues for developing the molecularly targeted cancer drugs^[10,19,26-28]. The first link between apoptosis and cancer emerged when Bcl-2, the gene that is linked to an immunoglobulin locus by chromosome translocation in follicular lymphoma, was found to inhibit cell death^[51]. Later on, many other Bcl-2 family proteins including the proapoptotic Bax, Bak and Bok, and anti-apoptotic Bcl-2, Bcl-XL and Mcl-1 were discovered. The BH3-only proteins such as the proapoptotic Bid, Bad, Bim, PUMA and phorbol-12-myristate-13-acetate induced protein 1 (NOXA) were identified as the other key regulators which control the mitochondria-mediated intrinsic apoptotic pathway^[52]. These Bcl-2 family proteins are important targets for cancer therapy (Figure 2). Several strategies have been developed by targeting the Bcl-2 family members. One approach for modulating Bcl-2 function is to target Bcl-2 with antisense Bcl-2 deoxyoligonucleotides. The other approach is to mimic the binding of a BH3 peptide to its surface groove. For example, ABT-737 and ABT-263 are BH3-mimetics that inhibit Bcl-2, Bcl-XL and Bcl-w

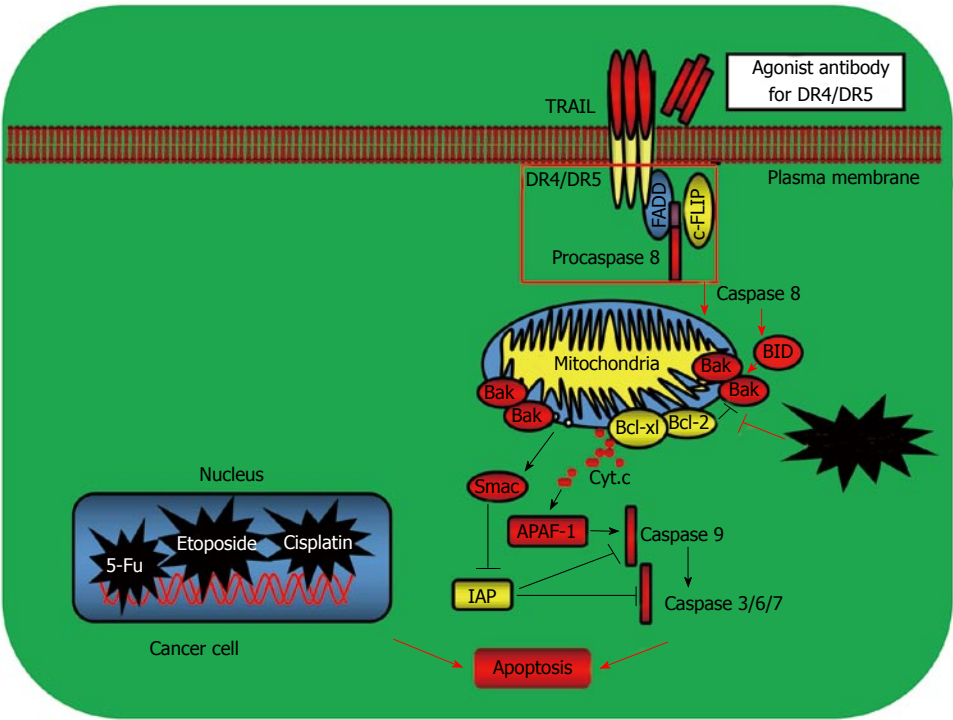


Figure 2 Examples of targeting apoptosis for cancer therapy. The prominent examples for tumor targeted therapy include the approaches by targeting the tumor necrosis factor receptor family death receptor-mediated apoptotic pathway and the mitochondrial B cell lymphoma 2 (Bcl-2) family member-mediated pathway. Activation of death receptors after binding to their ligand tumor necrosis factor related apoptosis inducing ligand (TRAIL) triggers activation of the extrinsic apoptotic pathway leading to cell death. Activation of Bax and Bak causes cytochrome c release from the mitochondrion and induces apoptosis. Bcl-2 and Bcl-XL prevent the activation of Bax and Bak and inhibit apoptosis. The mAbs targeting the TRAIL receptors have been developed and exerted significant clinical activities for some cancers such as non-small cell lung cancer (NSCLC), colon cancer, breast cancer, leukemia and prostate cancer. Small molecule BH3 mimetics activate the mitochondrial apoptotic pathway by interrupting the interaction of Bcl-2 with the proapoptotic Bax and Bak and induce apoptosis. FADD: Fas associated death domain; IAP: Inhibitor of apoptosis protein; APAF-1: Apoptotic protease activating factor-1.

Table 1 Small molecules targeting apoptosis for cancer therapy		
Drug name	Mechanism of action	Drug targets
CP-31398	Restoration of wild type p53 function	Mutant p53
PRIMA-1	Restoration of wild type p53 function	Mutant p53
Nutlins	Prevent p53 degradation	p53/MDM2
RITA	Prevent p53 degradation	p53/MDM2
Oblimersen sodium	Antisense inhibitors	Bcl-2
ABT-737	BH3 mimetics	Bcl-2, Bcl-XL, Bcl-w

to liberate Bax and Bak to induce apoptosis (Table 1). Preclinical studies have shown that ABT-737 was effective as a single agent in killing small cell lung cancer (SCLC) and causing SCLC tumor regression^[29-31,51-55]. ABT-737 has also been shown to synergize with other conventional chemotherapy, radiotherapy or tyrosine kinase inhibitors imatinib and gefitinib to reverse drug resistance and enhance cancer cell apoptosis^[56,57]. One advantage of the BH-3-memetics as cancer therapeutics is to target specific Bcl-2 family members such as Bcl-2 or Bcl-XL which is overexpressed in certain cancer types, therefore increasing the tumor selectivity and reducing the toxicity of the anticancer drugs (Figure 2). p53 is the most commonly mutated gene in human cancers and more than 50% of cancers carry mutations or deletions in p53, making p53 an important target for cancer therapy^[58-60]. Growing evidence has shown that reactivation or restoration of p53 function in cancers will

have significant therapeutic benefit. Several strategies are being explored to target tumors expressing mutant p53. Small molecules that bind to a site in mutant p53 (Y220C) increase the level of p53 with wild type conformation and activity. Other compounds (*e.g.*, PRIMA-1, CP-31398) bind to multiple mutant p53 proteins and interact with DNA binding domain, thereby promoting the proper folding of the mutant protein and restoration of p53 function^[61,62]. Of all the compounds that restore wild type activity, the most progress has been made with PRIMA analogs, with the demonstration of safety in phase I clinical trial study^[63] (Table 1). An alternative approach to target mutant p53 is to remove the protein by enhancing its degradation. HDAC inhibitors such as SAHA show promise in destabilizing mutant p53 by preventing HDAC6 from interacting with Hsp90^[64]. Small molecule activators of SIRT1 have also been shown to lead to the deacetylation of p53 and

reduction of overall mutant p53 levels^[65].

Oncogenic mutations of proteins in the intrinsic or extrinsic apoptotic pathways often lead to resistance to anticancer agents, among these molecules are p53, the Bcl-2 family members, death receptors, inhibitor of apoptosis proteins (IAPs), and the prosurvival factors such as AKT, PI3K and c-FLIP^[66-69]. Mutant p53-carrying tumors showed increased resistance to commonly used chemotherapeutic agents. Overexpression of Bcl-2 confers the resistance of leukemic cells to cytotoxic chemotherapeutic agents. Similarly, other Bcl-2 family members including Bcl-XL, Bax, and BH3-only proteins such as P53 Upregulated Modulator of Apoptosis and NOXA also play regulatory roles in determining the sensitivity of cancer cells to therapeutic agents^[48,49,53]. In addition to the defects in the cell apoptotic and survival machineries, there are still some other mechanisms that contribute to cancer drug resistance including the rates of drug efflux, alterations in drug metabolism and drug targets, DNA damage repair capacity, and the changes of the local tumor microenvironment. Drug resistance can also be acquired during treatment of tumors that were initially sensitive as well as the adaptive responses such as activation of other compensatory pathways. Further investigation of the molecular basis of drug resistance will help to overcome the hurdle that limits the clinical usage of cancer chemotherapeutic drugs and design rational drug combinations to restore or enhance the sensitivity of cancer drugs and improve the tumor selectivity and efficacy of cancer therapeutics^[70,71].

STRATEGIES FOR CANCER DRUG SCREENING

The initial identification of a chemical compound or small molecule inhibitors to be translated into medicine generally occurred by screening molecules in the animal models and cellular assay systems with functional biomarkers or molecular targets which are specifically mutated in cancers. Therefore, the level of mechanistic understanding of the alterations in cancers and the suitable targets used determine the success of a design for cancer drug discovery. The emergence of the high-throughput and high-content technologies has driven modern cancer therapy to the molecular era that new generations of molecularly targeted cancer drugs were continuously discovered. In the past decades, new technologies have been developed for dissecting the mechanisms of cancer development and metastasis and the molecular targets have been identified for designing new cancer selective therapeutic drugs. Large scale profiling approaches such as transcriptomics and proteomics and the high-throughput sequencing techniques which comparatively quantify the expression levels of transcripts and proteins have uncovered numerous genes, proteins and biomarkers that correlate with tumorigenesis. Some of these genes and proteins have been used as good targets for developing cancer

therapeutics. The most prominent examples are EGFR, BCR-ABL, VEGF, BRAF, PDGFR, p53, BCL2, MDM2, and ERBB2^[24,58-65,72-78].

Target-based screening strategy focused on the rational molecular targets that are hypothesized to have a role in cancers and represents the predominant approach for cancer drug discovery. Alternatively, there is another cancer drug discovery strategy called phenotypic screening approach which is referred to as phenotypic measurement of responses upon drug treatments in the animals or cellular models and is based on the cellular phenotype or functional endpoints rather than target potency alone. Much of the early pharmaceutical and drug discovery is based on the phenotypic screening. A recent report showed that phenotypic screening achieved more success in first-in-class medicine than target-based screening. One of the successful examples of cell based phenotypic drug screening was the discovery of vorinostat, an HDAC inhibitor, for inducing differentiation of cancer cells^[79].

The integration of advanced high-content imaging system into the drug screening field has aided the rapid development of cancer drug discovery^[80-82]. Light microscope imaging methods evolving in the mid-90s have provided multiple cellular measurements from living cells but require continuous user intervention. The development of fluorescent proteins permitted the tracking of proteins in living cells and the use of green fluorescent protein to tag functional proteins allowed many different fluorescent analogs to be created quickly and be used as markers to study the functions, subcellular localization, trafficking and activities of particular proteins. The automation of light microscope imaging, particularly the laser-directed multicolor fluorescence of arrays of cells, formed the high content screening system. Image-based screening techniques provide invaluable readouts, for example, the changes in the morphology, proliferation, cell cycle progression, cell death, differentiation, cell migration and invasion in the cellular models before and after drug treatment. Multiplexed high content screening assays integrated measurements of multiple cellular targets in a single assay, can be applied in numerous cell types which were subjected to libraries of chemical compounds or other experimental treatments such as RNAi, and emerged as an important cancer target and drug discovery platform^[83,84]. In addition, intravital imaging has gained the cellular details by tagging single cells, tissues and subcellular compartments with fluorescent proteins, through direct labelling of cells before exogenous inoculations *in vivo* or by using the specific promoters to drive the expression of genes of interest. These methods have been applied to monitor cancer drug response *in vivo* and determine the effect of cancer drugs on particular targets in animal models. Whole-body imaging techniques such as *in vivo* bioluminescence system have been used to monitor tumor progression and regression rates from multiple tissue sites during drug treatment^[82,85].

Other fluorescence-based assays are also the important platforms for cancer drug screening. Examples of these dynamic technologies include the fluorescence resonance energy transfer which can be used to measure protein-protein interactions and is suitable for lead compound identification. This approach can provide a precise measurement of drug activity and insights of drug mechanism by determining the biomolecular interactions or activations^[86,87]. Surface plasmon resonance imaging (SPRi) is an optical technology which allows the label-free and real-time detection of biomolecule interaction. It offers the possibility of monitoring hundreds of biological interactions simultaneously and from the binding profiles, allows the estimation of the kinetic parameters of the interactions between immobilized probes and the ligands in solution. SPRi has been applied in a variety of affinity systems, including protein/protein, protein/DNA, antibody/antigen, ligand/receptor, DNA/DNA, carbohydrate/protein, and cell/cell interactions. SPRi imaging as an affinity-based biosensor technology has been adopted for cancer drug screening, food and environment evaluation, and clinical diagnostics^[88-91]. Enzymatic activation of caspases, which are a class of cysteine protease, was determined by using the peptide arrays based on the SPR imaging. This strategy used streptavidin to amplify the SPR signals of the surface-immobilized substrate peptides labeled with biotin at the C-terminus and the cleavage of the substrate peptides by caspases was detected by the increased SPRi signal. This method allowed the examination of the activities of purified caspases and caspase in cell lysate and therefore can be applicable to cell-based drug screening^[92].

CONCLUSION

Enhancing or restoring apoptotic pathways in cancers are important strategies for developing cancer drugs. Some of the aberrant components in the apoptotic pathways in cancers have been identified as suitable targets for cancer therapy. These targets can be the cell surface death receptors, Bcl-2 family members, and some other intracellular molecules like p53, MDM2, IAP, and Smac. Although some of the chemical compounds or small molecule inhibitors have been discovered to modulate the activity of these targets and achieve varying degrees of success in killing cancers, drug resistance, limited patient responses and the toxic side effects still remained problematic and limited their clinic application. Further investigation of the mechanisms of apoptosis evasion in cancers and designing the rational drug combinations are still the challenges for cancer researchers in the world.

A number of high-throughput approaches including the genomic, proteomic and multiplexed imaging technologies have been applied to identify new targets in the signaling networks that are involved in cancer pathogenesis and led to discovery of new generations of target-directed chemical compounds and small molecule

drugs. The natural products from plants, Chinese herbal medicine and marine bioproducts are invaluable sources for cancer drug development and many studies need to be done to discover more druggable natural products, study the mechanism of action and cure cancer patients more efficiently and less costly.

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Basic Study

Role of nestin in glioma invasion

Alex Lin, Luigi Marchionni, Jeffrey Sosnowski, David Berman, Charles G Eberhart, Eli E Bar

Alex Lin, David Berman, Charles G Eberhart, Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD 21205, United States

Luigi Marchionni, David Berman, Charles G Eberhart, Department of Oncology, Johns Hopkins University School of Medicine, Baltimore, MD 21205, United States

Jeffrey Sosnowski, Department of Orthopedic Surgery, Johns Hopkins University School of Medicine, Baltimore, MD 21205, United States

Charles G Eberhart, Department of Ophthalmology, Johns Hopkins University School of Medicine, Baltimore, MD 21205, United States

Eli E Bar, Department of Neurological Surgery, School of Medicine, Case Western Reserve University, Cleveland, OH 44106, United States

Eli E Bar, Case Comprehensive Cancer Center, Case Western Reserve University School of Medicine, Cleveland, OH 44106, United States

Author contributions: Lin A and Bar EE performed the majority of the experiments; Sosnowski J performed immunohistochemical analyses; Marchionni L performed all statistical analyses; Berman D provided shRNA constructs; Eberhart CG and Bar EE analyzed all the data; Bar EE wrote the paper.

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Correspondence to: Eli E Bar, PhD, Assistant Professor, Department of Neurological Surgery, School of Medicine, Case Western Reserve University, Robbins Building Room E750A, 2210 Circle Drive, Cleveland, OH 44106, United States. eli.bar@case.edu
Telephone: +1-216-3680933
Fax: +1-216-3681144

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Abstract

AIM: To determine the role for the intermediate filament protein nestin in glioma invasion.

METHODS: We examined the expression and function of nestin in gliomas (Grades II-IV as defined by the World Health Organization). We determined nestin expression using Immunohistochemical methods. To elucidate nestin's biological function(s), we reduced mRNA levels by 61% and 87% in two glioblastoma-derived neurosphere lines using short hairpin RNAs and determined the effect of reduced nestin expression on glioma cell proliferation and invasion using MTS and matrigel migration assays, respectively. We also utilized quantitative real time polymerase chain reaction assays

to determine the effect of reduced nestin expression on the expression of other markers associated with glioma stem cells and their differentiated progenies.

RESULTS: We found a significant correlation between nestin immunoreactivity and astrocytoma tumor grade, with 36% of grade II, 75% of grade III, and 100% of grade IV tumors expressing significant levels of the protein when assessed using immunohistochemistry. Reduction in nestin expression had no effect on cell growth in culture, but did retard the capacity of one line to migrate *in-vitro* on matrigel. Interestingly, in the line whose migration was not affected, mRNA levels of a second intermediate filament, synemin (also known as desmuslin), were elevated following introduction of shRNA targeting nestin. As synemin was not induced in the line which required nestin for migration, it is a possibility that synemin may compensate for the loss of nestin in this process.

CONCLUSION: Nestin expression is prominent in high-grade astrocytomas. Nestin is not required for cell growth but it may, however, be required for cell motility.

Key words: Nestin; Stem cells; Migration; Glioma; Neurosphere

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Core tip: Despite its common use as a marker of poorly differentiated stem and progenitor cells, the functional role of nestin in normal and neoplastic cells is poorly understood. Here we show that in gliomas, there is a significant positive correlation between nestin protein expression and increasing pathological grade. However, when nestin expression was inhibited, we found no significant effects on cell growth, expression of stem-cell markers, and the ability to initiate intracranial xenografts. Our data suggest that the functional role of nestin is limited, even though the migratory potential of some glioblastoma neurospheres is reduced by nestin knockdown.

Lin A, Marchionni L, Sosnowski J, Berman D, Eberhart CG, Bar EE. Role of nestin in glioma invasion. *World J Transl Med* 2015; 4(3): 78-87 Available from: URL: <http://www.wjgnet.com/2220-6132/full/v4/i3/78.htm> DOI: <http://dx.doi.org/10.5528/wjtm.v4.i3.78>

INTRODUCTION

Glioblastoma is the most common malignant primary brain tumor in adults. Despite some therapeutic advances in recent years, the prognosis for patients with glioblastoma remains dismal, and most patients die within 2 years of diagnosis. Several reports have suggested that the inability to cure glioblastoma is due to persistence of cancer stem cells, which have been

shown to sustain the growth of many human tumors (reviewed in^[1]). Cancer stem cells in brain tumors are thought to share many characteristics with other neural stem cells. For example, they contain proteins preferentially expressed in neural stem and progenitor cells such as CD133^[2], OLIG2^[3] and nestin^[4,5]. The origin of stem-like cells in glioblastoma and other brain tumors is not yet clear, but they may arise from non-neoplastic adult stem cells, or from better-differentiated cells which reacquire stem-like properties *via* mutations and/or epigenetic modifications^[1].

Nestin is a class VI intermediate filament protein^[6], normally expressed in neuroepithelial stem and progenitor cells of the developing mammalian central nervous system (CNS). It is also expressed in adult neural stem cells lining the ventricular system of the subventricular zone and the dentate gyrus of the hippocampus, but in differentiated adult cells nestin is replaced by other intermediate filaments such as neurofilaments and glial-fibrillary-acidic-protein (GFAP)^[4]. Re-expression of nestin in the adult CNS usually accompanies pathological conditions such as brain injury, ischemia, inflammation, or neoplastic transformation, and its presence in these processes may indicate a role in reactive and/or regenerative processes^[7].

Nestin expression has been found in many types of brain tumors^[5,8-10]. However, its functional role in tumor formation, proliferation, and migration is still not well understood. Recently, nestin has been shown to be required for prostate cancer cell migration *in-vitro* and *in-vivo*^[11]. In the present study, we investigate the expression of nestin in astrocytic tumors and its functional role in glioblastoma. The latter experiments were performed in glioblastoma neurosphere lines containing stem-like cancer cells. Our results suggest that nestin is not required for proliferation *in-vitro* or engraftment *in-vivo*, but that it may be required for *in-vitro* migration in some glioblastoma cell lines.

MATERIALS AND METHODS

Clinical specimens and neurosphere lines

The tissue microarray, containing four 0.6mm cores per tumor from a number of grade II fibrillary astrocytomas, grade III anaplastic astrocytomas, and grade IV glioblastoma multiforme, was created as previously described using samples obtained from the Department of Pathology, Johns Hopkins University School of Medicine, with Institutional Review Board approval^[12]. A minimum of two of the four tissue cores had to be evaluable, and contain at least 10% nestin-immunopositive tumor cells, for a case to be scored as a positive. The glioblastoma neurosphere lines HSR-GBM1 and HSR040622, were a kind gift from Dr. Angelo Vescovi and were maintained as previously described^[13].

Immunostaining

Slides were deparaffinized and endogenous peroxidase activity was blocked by incubation in a hydrogen pero-

xide/methanol buffer. Unless stated otherwise, all of the following incubation steps were carried out at room temperature. First, antigen retrieval was performed by incubation of the slides in 10 mmol/L citrate buffer (pH 6.0) at 90 °C for 20 min. Incubation with the primary mouse anti-nestin antibody (1:5000; Chemicon, Billerica, MA) for 60 min was preceded by blocking with serum-free Protein Block (Dako Cytomation, Carpinteria, CA) for 20 min. Negative controls were performed by substitution of PBS for the primary antibody. After washing in PBS, HRP-conjugated anti-mouse IgG (1:500; Dako Cytomation) was applied to the slides for 30 min. For tyramide-based signal amplification the slides were subsequently treated using the TSA Biotin System according to the manufacturer's protocol (PerkinElmer Life Sciences, Waltham, MA). Finally, slides were incubated for 8 min with a DAB solution (Sigma, St. Louis, MO), counterstained with hematoxylin, dehydrated, cleared, and mounted.

For immunocytochemistry, neurospheres were spun onto Superfrost plus slides (Fisher Scientific, Pittsburgh, PA) using a Shandon Cytospin3 (Thermo Shandon, Waltham, MA) for 5 min at 1500 rpm. Cells were then washed once for 5 min with PBS and then fixed in freshly prepared 4% para-formaldehyde (in PBS, pH 7.4) at room temperature for 30 min. After three washes with PBS for 5 min each, cells were permeabilized using PBST (PBS + 0.2% Triton × 100) for 5 min. Blocking was performed for 45 min at room temperature with PBST containing 5% normal horse or goat serum. The primary antibodies used were rabbit anti-Ki67 1:1000 (Novocastra Laboratories, United Kingdom), and mouse anti-nestin MAB5326 1:2500 (Chemicon, Temecula, CA). Nuclei were stained with DAPI (Pierce, Rockford, IL) for 3 min in PBS. Quantitation of Ki-67 immunostaining was made by counting separately the positively and negatively stained nuclei. At least ten high-power fields containing a minimum of one hundred cells each were counted per slide using a 63X objective. Only moderate to strong staining intensity was scored as positive. Ki-67 index was expressed as the percentage of positively stained nuclei to all nuclei.

Lentivirus preparation and infection

Lentiviruses were generated essentially as previously described^[11]. Briefly, 5 µg of lentiviral vector (either pSicoR or pSicoR/shNestin) and 2.5 µg of each packaging vector were cotransfected in 293T cells using the FuGENE 6 reagent (Roche Diagnostics, Indianapolis, IN). Oligos targeting the human nestin RNA sequence: 5' TGCTGTTGACAGTGAGCGCGGCTAGTCCCTGCCTGAATAATAGTGAAGCCACAGATGTA-TTATTCAGGCAGGGACTAGCCATGCCTACTGCCTCGGA-3' (human nestin shRNA1). Twenty four hours after transfection, growth medium was replaced with DMEM containing 2% FBS. Supernatants were collected 48 h and 72 h post medium change, filtered through a 0.45-µm filter, and used directly to infect neurospheres.

One round of infection was usually sufficient to infect enough cells for subsequent drug selection. Virus was allowed to infect cells for 4 h and then the cells were washed once with PBS and plated back into Neurocult medium. Seventy-two hours later, positive cells were selected in puromycin (5 µg/mL). Puromycin resistant neurospheres were expanded for 3 wk before assayed for nestin expression.

RNA extraction and quantitative real time polymerase chain reaction (RT-PCR). For RNA extraction from cultured cells, 3.75×10^5 cells were plated in 75 cm² tissue culture flasks each containing 10 mL of NeuroCult medium (Stem Cell Technologies, Canada) supplemented with hEGF and hFGF-b (Peprotech, Rocky Hill, NJ). Cultures were grown in the presence of 10 ng/mL puromycin (Sigma, St. Louis, MO) and incubated for 5 d in a humidified incubator. Cells were spun at $276 \times g$ for 10 min followed by a rinse with ice-cold PBS. Cell pellets were processed for RNA extraction using the Qiagen RNeasy kits. Reverse transcription was performed as previously described^[14].

mRNA levels were analyzed by RT-PCR analysis performed in triplicate with SYBR Green reagents (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions on an I-Cycler IQ real-time detection system (BioRad, Hercules, CA). To minimize contaminating genomic DNA, a thirty minutes on-column DNase step was included during RNA extraction. The standard curve method was used to determine expression levels, and all values were normalized to actin. Oligo sequences were as follows: Nestin forward: 5'CCAGGAGCCACTGAAGACTC; nestin reverse: 5' CCTTCCAGGTTCTCTTCC; actin beta forward: 5' CCCAGCACAAATGAAGATCAAT; actin beta reverse: 5' GATCCACACGGAGTACTTG. CD133 forward: 5' CATCCACAGATGCTCCTAAGG; CD133 reverse: 5' AAGAGAATGCCAATGGGTCCA; OLIG2 forward: 5' GGACAAGCTAGGAGGCACTG; OLIG2 reverse: 5' ATGGCGATGTTGAGGTCGTG; GFAP forward: 5' AACTGAGGCACGAGCAAAGT; GFAP reverse: 5' GCAGTGCCCTGAAGATTAGC; MAP2 forward: 5' CCATCTTGGTGCCGAGTGAG; MAP2 reverse: 5' TGGGAGTCGCAGGAGATTTTG; vimentin forward: 5' TACCGGAGACAGGTGCAGTCCCTCA; vimentin reverse: 5' TCACGAAGGTGACGAGCCATTTCCT; Synemin transcript variants M/H (AJ310521.1, AJ310522.1, respectively) forward: 5' ACAGGTGCTGGAGGATGTG; Synemin transcript variants M/H reverse: 5' CGGATCGCCTTACGTTACT; Synemin transcript variants M/H/L (AJ310521.1, AJ310522.1, AJ697971.1, respectively) forward: 5' GGCCTCAGTCTGGAGGTGG; Synemin transcript variants M/H/L reverse: 5' CCCAGATCACTATCTGTGGATTACT; To ensure that measurements of gene expression changes reflected a direct effect of nestin mRNA levels, we considered a significant expression level change only in cases where un-infected and scrambled control (shC) infected cells each showed significant level change as compared with shNestin (shN)

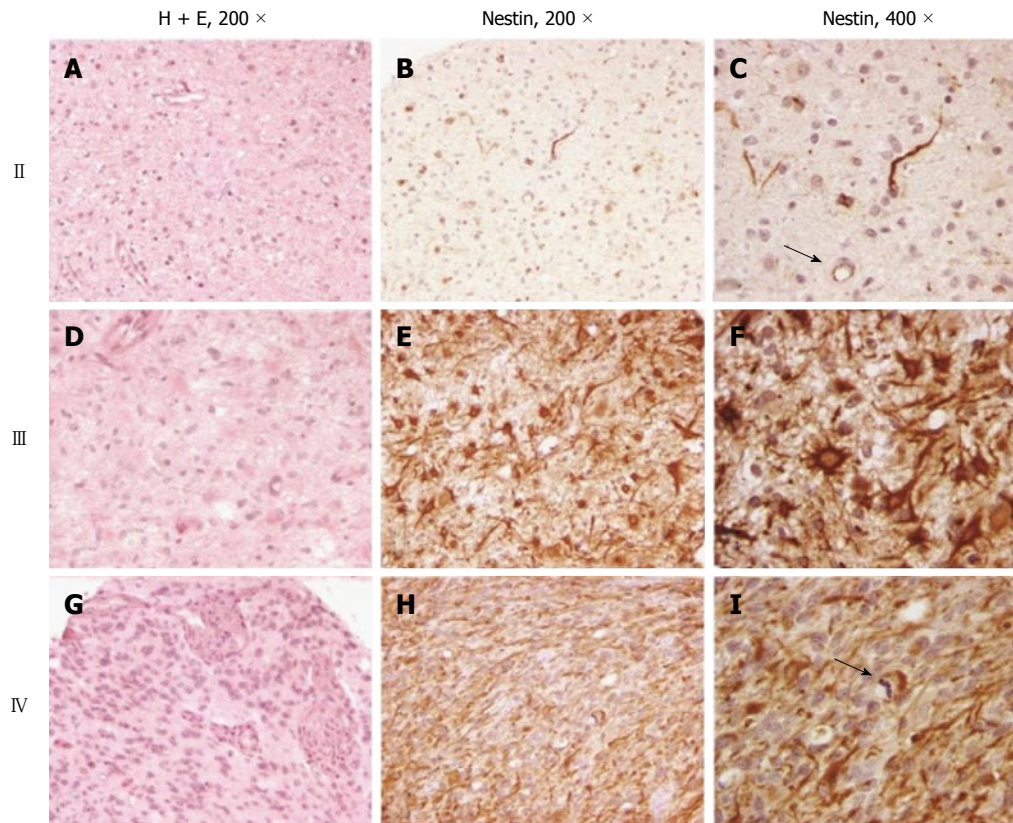


Figure 1 Nestin expression in gliomas. Diffuse astrocytomas (A) were generally either negative for nestin or contained rare positive cells (B). Endothelial cells were also often nestin positive (C, arrow). Anaplastic astrocytomas (D) generally contained moderate to frequent numbers of strongly nestin positive cells (E, F). Glioblastomas (G) were uniformly nestin positive (H, I).

infected cells.

MTS growth assays

Cells were plated in triplicates at 2500 cells in 96-well culture plates and incubated in a humidified incubator for a total period of seven days. MTS (Promega, Madison, WI) reagent was added to selected wells on the first, second, and seventh days before absorbance was read at 490 nm. Fold change in cell mass was calculated as the absorbance on day seven divided by the absorbance on day one.

Spreading/migration assays on matrigel

Forty eight hours before plating neurospheres, single cells were plated at a density of 3.75×10^5 cells/mL. To prepare the matrigel substrate, 10 cm² culture dishes were coated with low growth factor containing matrigel (BD biosciences, San Jose, CA) at a 1:100 dilution with Neurocult medium and placed in a humidified incubator over-night. The next day, plates were rinsed once with PBS before neurosphere plating. Neurospheres were plated at a very low density in which, on average, a single neurosphere could be visualized per field. Most neurospheres attached to the matrix one hour post plating at which point the medium was replaced to minimize continuous adhesion of any remaining floating neurospheres. The position of 20-25 spheres was noted on the bottom of the plate to allow monitoring of

cell spreading over time. Sphere area was calculated by multiplying the two longest perpendicular axes extending from the two furthest cells in a sphere. Each experiment was performed at least three times.

Statistical analysis

VassarStats software (<http://faculty.vassar.edu/lowry/VassarStats.html>) was used for statistical analyses. The significance of differences in nestin expression in astrocytomas of varying grades was analyzed using the Freeman-Halton extension of the Fisher exact probability test for contingency tables.

Statement from biostatistician

Statistical analyses in this study were reviewed by Dr. Luigi Marchionne, who is a trained biostatistician and a co-author.

In-vivo xenografts

The animal protocol was designed to minimize pain or discomfort to the animals. The animals were acclimatized to laboratory conditions (23 °C, 12 h/12 h light/dark, 50% humidity, at libitum access to food and water) for at least two weeks before experimentation. For xenograft studies, 1×10^5 viable cells were diluted with fresh medium and injected over 10 min into the right striatum of athymic (nu/nu) mice (Harlan, Indianapolis, IN, <http://www.harlan.com>). Mice were

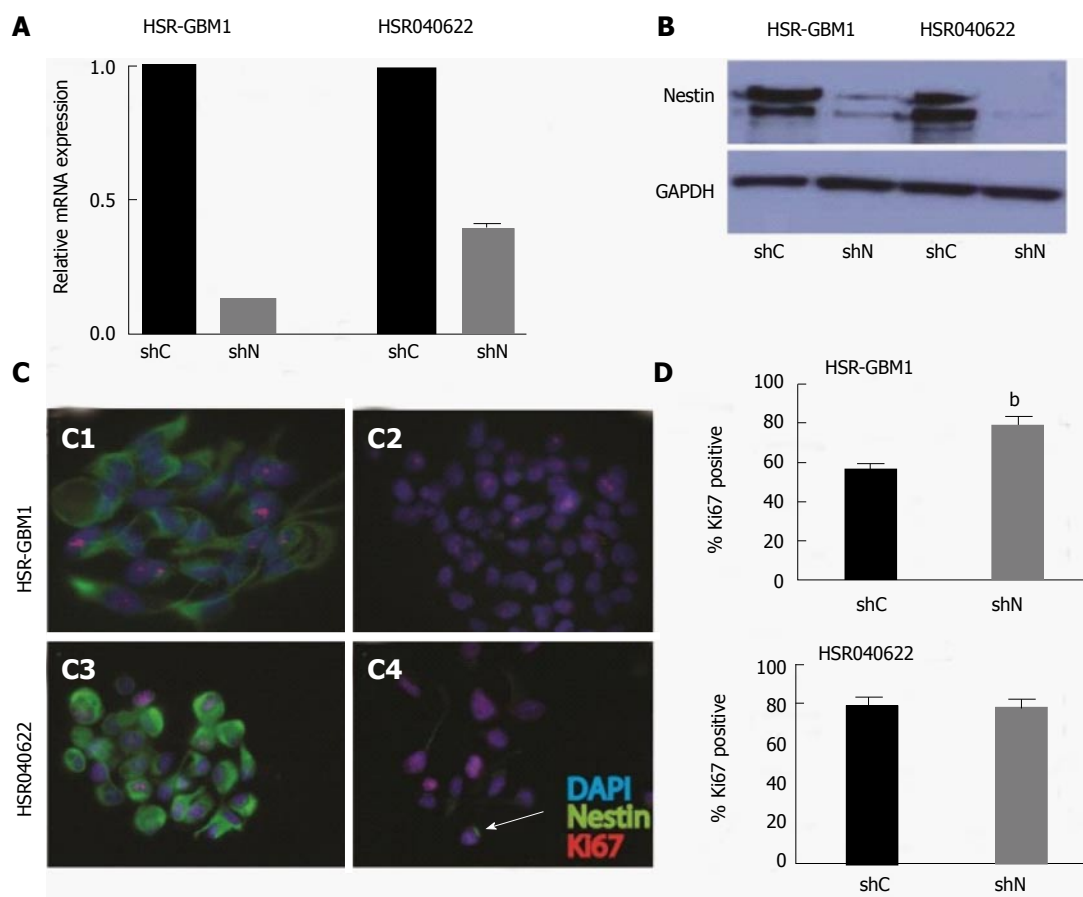


Figure 2 Nestin expression knockdown has no effect on glioblastoma cell proliferation. Quantitative real time PCR and Western Blot analyses of Nestin mRNA (A) and protein (B) expression in HSR-GBM1 and HSR040622 neurospheres confirming stable knockdown of nestin expression by short hairpin RNA. HSR-GBM1 (C1, C2) and HSR040622 (C3, C4) immuno-staining confirms nestin protein reduction in shNestin (shN) infected cells (C2, C4). Quantification of Ki67 immunoreactivity shown in D ($^bP < 0.01$, two-sided *t* tests). shC: Scrambled control.

monitored daily and sacrificed at the first indication of tumor development (ataxia, seizure, lethargy, or cachexia). Brains were surgically removed and fixed immediately in formalin before submission for histological analysis, as previously described^[15].

Animal care and use statement: Discomfort was be minimized by the use of anesthesia during potentially painful procedures (intracranial injections). The anesthetic used was Ketamine-Xylazine in sterile saline injected intraperitoneally in accordance with the Institutional Animal Care and Use Committee.

RESULTS

Nestin expression correlates with astrocytoma grade

We assessed a total of 41 astrocytic tumors for nestin immunoreactivity in tissue arrays containing evaluable cores from 11 grade II fibrillary astrocytomas, 16 grade III anaplastic astrocytomas, and 14 grade IV glioblastoma (Figure 1). Immunoreactivity for nestin was identified in all tumor types, with a positive correlation between nestin immunoreactivity and increasing grade of these infiltrating astrocytic tumors. A significant level of nestin immunoreactivity, defined as 10% or more

of tumor cells, was identified in 4/11 (36%) grade II fibrillary astrocytoma (Figure 1A-C), 12/16 (75%) grade III anaplastic astrocytoma (Figure 1D-F), and 14/14 (100%) grade IV glioblastoma, (Figure 1G-I). These differences were statistically significant ($P < 0.001$, Fisher exact test). Nestin immunoreactivity was not exclusively observed in tumor cells, and was also detected in reactive astrocytes, in microglial cells adjacent to neoplastic elements, and in endothelial cells (Figure 1C). We did not quantitate the percentage of nestin-positive cells in each tumor; but there appeared to be an increase in the extent of nestin immunoreactivity with increasing grade.

Nestin is not required for cell growth in-vitro

Proliferation and migration are important characteristics of stem and progenitor cells in the developing brain, as well as stem-like brain tumor cells^[13,16]. Both processes may be influenced by cytoskeleton dynamics. To test if nestin has a role in cellular proliferation, we stably transduced two human glioblastoma neurosphere lines with lentiviruses encoding short hairpin RNAs (shRNAs) targeting either the nestin transcript (shNestin/shN) or a nonspecific scrambled sequence (shC). Utilizing this system, we achieved 87% and 61% reduction in

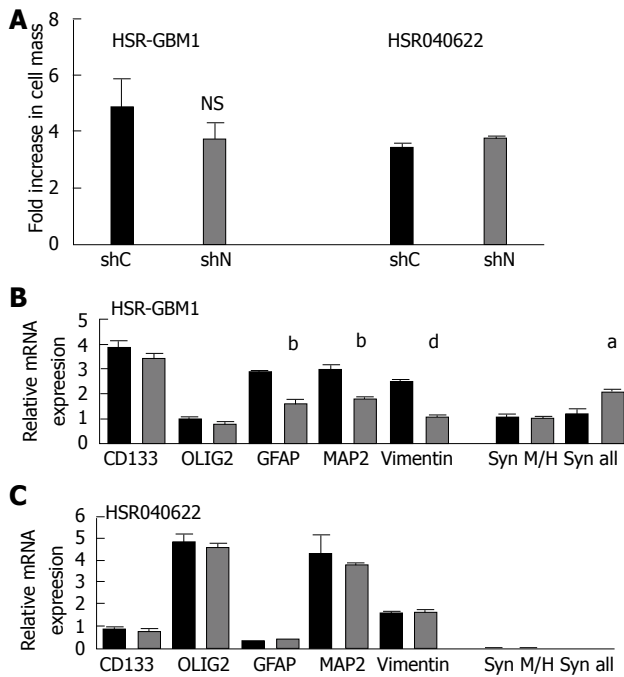


Figure 3 Effect of nestin knockdown on proliferation and the expression of stem cell and differentiation markers. **A:** HSR-GBM1 and HSR040622 cells stably expressing the lentivirus driven nestin shRNA show no significant difference in cellular proliferation over a period of 6 d as compared with shC (scrambled control) infected cells; **B:** In HSR-GBM1, lowered nestin levels has no significant effect on mRNA levels of the neural stem-cell markers CD133 and OLIG2, while statistical significant reduction of mRNA levels of GFAP (glial differentiation), MAP2 (neuronal differentiation), and the intermediate filament Vimentin was observed. The expression of the L-transcript variant of the intermediate filament synemin (syn) was significantly increased; **C:** In HSR040622, reduced nestin levels had no effect on expression of any of the tested genes mentioned above. It is important to note that the expression of the intermediate filament Synemin was barely detected. (NS = not significant, ^a $P < 0.05$, ^b $P < 0.01$, ^d $P < 0.001$; all two sided t test). shC: Scrambled control; shN: ShNestin.

nestin mRNA levels in HSR-GBM1 and HSR040622, respectively, as compared to shC expressing cells (Figure 2A and B). To further evaluate the effect of reduced nestin mRNA, we examined the expression of nestin protein on a single cell level. Immunocytochemistry analysis confirmed that nestin protein was present in the majority of cells of shC infected lines (Figure 2C1, C3) and in uninfected cells (data not shown). Transduction with shN almost completely eliminated detectable nestin staining in HSR-GBM1 (Figure 2C2). In HSR040622, sporadic residual nestin positive cells could be found (arrow in Figure 2C4). Despite the dramatic reduction in nestin expression, we found no reduction in proliferation of HSR-GBM1 cells, with 56% and 80% Ki67 positive nuclei for shC and shN infected cells, respectively (Figure 2D, top panel). Similar results were observed for HSR040622 with 79% Ki67 positive nuclei for both shC and shN infected cells, respectively (Figure 2D, lower panel). To more directly test the effect of nestin reduction on proliferation, we next performed MTS assays which allow sensitive evaluation of changes in viable cell mass over time. We found no

significant differences between the growth rates of HSR-GBM1 with high and low nestin levels, with 4.8 and 3.7 fold increases in cell mass for shC and shN infected cells, respectively. Similar results were observed for HSR040622, with 3.40 and 3.7 fold increases in total cell mass for shC and shNestin infected cells (Figure 3A).

Nestin knockdown does not significantly alter expression of stem cell markers

Nestin has been widely used as a marker of neural stem cells. We therefore investigated the possibility that nestin reduction may alter expression of other stem/progenitor or differentiation markers within glioblastoma derived neurospheres. We compared mRNA levels of the neural stem/progenitor cell markers CD133 and OLIG2, as well markers of glial (GFAP) and neuronal (MAP2) differentiation. Levels of the intermediate filaments vimentin and synemin were also measured, as changes in their expression could potentially compensate at least in part for nestin loss. The expression level of synemin was analyzed using two different primer pairs. The first (M/H) amplifies a product which corresponds to the M and H synemin transcript variants, while the second pair ("all") amplifies a product which represents the M, H, and L transcript variants. We found no significant differences in the mRNA level of CD133, OLIG2, MAP2, and vimentin in HSR040622 (Figure 3C). Interestingly, HSR-GBM1 cells with reduced nestin also express significantly lower levels of GFAP (56%, $P < 0.01$, two-tailed t -test) and MAP2 (60%, $P < 0.01$, two-tailed t -test) (Figure 3B). In contrast, we observed an 80% increase in the level of synemin when analyzed using primers which anneal to its three transcript variants (M, H, and L; $P < 0.05$, two-tailed t test), but no significant change in level of the M and H transcript variants alone. We infer from these observations that the increased levels of synemin result from a significant increase in the level of synemin L in HSR-GBM1 (Figure 3B).

Nestin is required for in-vitro motility of some glioblastoma lines

Glioma cell motility plays a key role in tumor spread, and in the current inability to cure patients with these malignancies. It has been previously shown that nestin is required for metastasis of the AT6.3 prostate cancer line, suggesting it may be directly involved in cancer cell migration^[11]. We therefore explored the possibility that nestin may play a role in glioma cell migration as well. Migration of HSR-GBM1 and HSR040622 was examined in a two-dimensional neurosphere outgrowth assay, which allowed migration from a small, defined number of cells to be assessed over time (Figure 4A). Glioma neurospheres were allowed to form over two days in suspension and then plated onto matrigel-coated plates. One hour after plating, neurospheres had tightly bound, forming small colonies with an average cross-sectional area of 196 μm^2 for HSR-GBM1 and 236

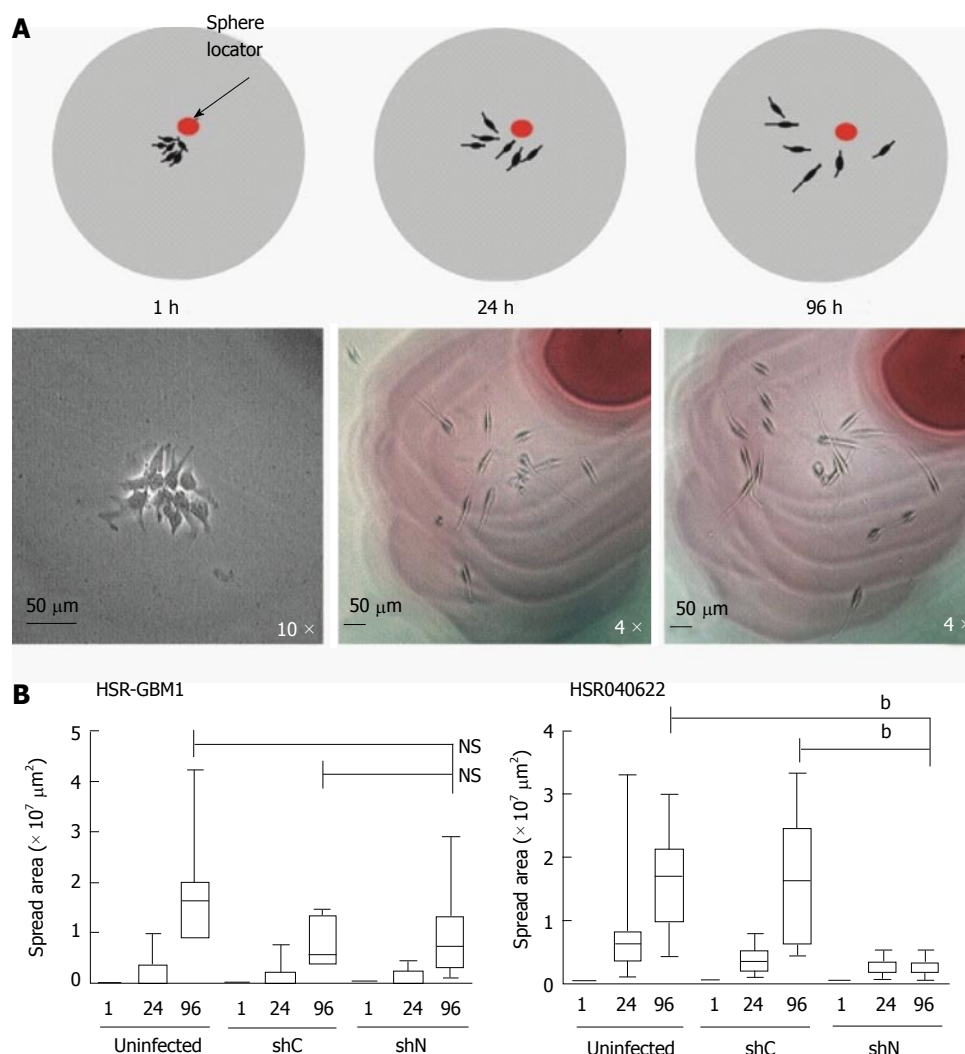


Figure 4 Effect of nestin knockdown on cell migration. A: Top, cartoon illustrating the experimental design (described in details in materials and methods). Bottom, the two-dimensional area spread of a selected HSR-GBM1 sphere is shown; B: Two dimensional area spread analysis shown no significant difference in cell migration for HSR-GBM1 (left) uninfected, shC, and shN infected cells. In contrast, a significant reduction in cell migration was observed for shN infected HSR040622 cells (right) as compared with uninfected and shC infected cells (NS: Not significant, ^bP < 0.001; two sided *t* test). shC: Scrambled control.

μm² for HSR040622. The growth in cross-sectional area of individual colonies was then measured over time. Only limited cellular proliferation occurred over the time course of the assay, and, as we found no change in proliferation with reduced nestin levels, we believe any differences in area are due to effects on migration. Nestin knockdown dramatically reduced cell spreading in HSR040622 cells (Figure 4B, right panel) from an average area of $1.66 \times 10^7 \mu\text{m}^2$ for shC infected to $2.2 \times 10^6 \times 10^5 \mu\text{m}^2$ for shN infected cells (two tailed *t* test; *P* < 0.0001). In contrast, we found no significant decrease in migration for HSR-GBM1, with an average area of $7.24 \times 10^6 \mu\text{m}^2$ for shC infected and $9.12 \times 10^6 \mu\text{m}^2$ for shNestin infected cells (Figure 4B, left panel).

Nestin is not required for intracranial xenograft growth or in-vivo migration of tumor cells

An intracranial xenograft model was employed to compare the tumorigenicity and migratory capacity of HSR-GBM1 shC and shN infected cells. We injected $1 \times$

10^5 viable tumor cells into the right striatum of athymic nude mice and monitored tumor formation for a period of 16 wk. Xenografts formed in all animals injected with either shC or shN infected HSR-GBM1 cells, but we found no significant difference between these two lines in terms of their *in-vivo* growth. Both forming large, infiltrative tumors, resulting in death as early as 63 d following injection. Immunostaining of these xenografts confirmed that tumor cells maintained reduced nestin expression over the relatively prolonged period of *in-vivo* growth (Figure 5).

DISCUSSION

We examined nestin immunoreactivity in astrocytomas of grades II to IV, and found a significant positive correlation between protein expression and increasing pathological grade. These results are similar to those previously reported in gliomas by several other groups^[4]. Dahlstrand *et al*^[4], showed higher nestin expression in

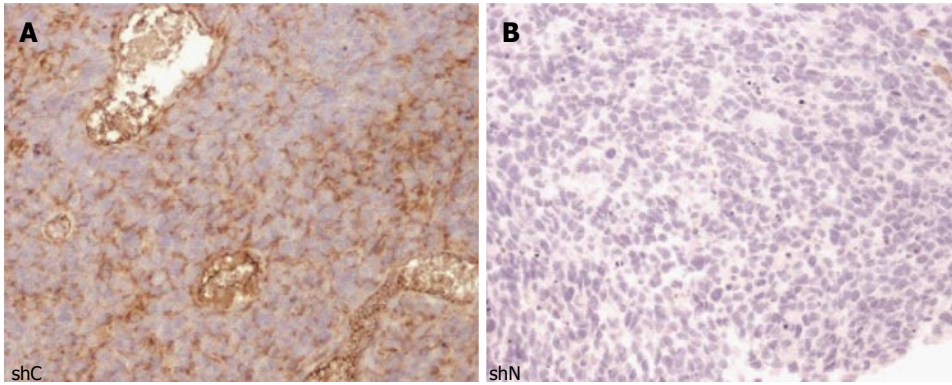


Figure 5 Nestin is not required for xenograft engraftment in HSR-GBM1. Nestin immunostaining analysis of shC (A) and shN (B) infected HSR-GBM1 cells engrafted into the striatum of athymic nude mice. No significant differences in cellular morphology or migration of tumor cells were apparent at the time the mice were sacrificed. Similar phenotype was observed when uninfected cells were injected (not shown). In all experiments, mice survived for about 63 d post injection. shC: Scrambled control.

malignant tumors such as glioblastoma when compared to lower grade glial tumors. Ehrmann *et al.*^[17] reported that in astrocytomas and malignant melanomas, nestin expression could be used as an auxiliary indicator of dedifferentiation and progression. Nestin expression has also been detected in non-glial malignancies, including hemangioblastomas^[9], melanoma^[18,19], basal epithelial breast tumors^[20], prostate cancer^[11], and in gastrointestinal stromal tumors^[21]. In many of these, immunoreactivity correlated with increasing pathological grade as well^[4,8,17,20-25]. The fact that higher grade tumors show increased nestin immunoreactivity suggests that the percentage of cells with a stem/progenitor phenotype may increase during tumor progression. In addition, the fact that nestin expression is associated with increasing grade in a very wide variety of malignancies indicates that a better functional understanding of this protein may lead to insights into how tumors progress.

Despite its common use as a marker of poorly differentiated stem and progenitor cells, the functional role of nestin in normal and neoplastic cells is poorly understood. It has recently been shown that nestin can serve as a scaffold for a number of signal transduction proteins, such as cdk5 and p35/45, thereby regulating their function^[26-28]. Intermediate filaments function as cytoskeletal scaffolds in the nucleus and cytoplasm^[29] and may be involved in cellular migration and metastatic potential^[30-32]. In addition, it has been shown that nestin is required for migration and metastasis of prostate cancer cells^[11]. Such studies highlight the need to examine potential functional roles of nestin, in addition to using it as a marker of differentiation status.

We examined the requirement for ongoing nestin expression in growth and migration of two glioblastoma-derived neurosphere lines. Growth was not significantly affected in either line when nestin levels were reduced by 60% or more using shRNA. These findings indicate that as in prostate cancer^[11], glial cells appear to grow nicely with severely reduced nestin levels. It has been suggested that nestin may be only one of several

intermediate filaments involved in proliferation, and therefore its loss may be compensated for by other members of this large family of proteins^[11]. Indeed, we detected about a two fold-increase in synemin mRNA levels following knockdown of nestin in HSR-GBM1, although it appears that only the L transcript variant is induced (Figure 3B).

We also examined the expression of several mRNA expressed in either stem/progenitor or better differentiated cells in lines with varying nestin levels. Loss of nestin did not seem to alter expression of CD133 or Olig2, although the glial marker GFAP and the neuronal marker MAP2 were decreased somewhat following nestin knockdown. The significance of this latter observation is not clear, but the fact that CD133 and Olig2 levels were unchanged, and that cultures could be passaged for over six months with low nestin levels, strongly suggest that it is not required for the maintenance of tumor-propagating stem-like cells.

Understanding the migration of glial tumor cells is of fundamental importance if we are to eventually cure malignant brain tumors. Our results suggest that nestin may be required for such migration *in-vitro* in a subset of tumors, as the spread of HSR040622 cells was almost completely abolished *in-vitro* by nestin knockdown. In contrast, the *in-vitro* spread of the second line examined was unaffected by shRNA targeting nestin, and these cells could still form invasive intracranial xenografts despite prolonged reduction of nestin. The molecular basis for the varying requirements of glioblastoma neurosphere lines for nestin is not clear. It is possible that the increase in synemin levels observed in the HSR-GBM1 cells with nestin-targeting shRNA may compensate for the reduced nestin levels, allowing cells to migrate normally. Indeed, the intermediate filament synemin has previously been shown to contribute to the migratory properties of astrocytoma cells by influencing the dynamics of the actin cytoskeleton^[33].

In summary, our studies support the concept that nestin expression is a common feature of astrocytic brain tumors, and that protein levels correlate with

tumor grade. However, the functional role of nestin appears to be limited, although the migratory potential of some glioblastoma neurospheres is reduced by nestin knockdown. Further studies will be needed to fully understand the role of nestin in migration, and the heterogeneity between different glioblastoma lines.

COMMENTS

Background

The challenges in curing glioblastoma have been partially attributed to the persistence of cancer stem cells following treatment. This subpopulation of cells have been shown to express proteins preferentially expressed in neural stem and progenitor cells such as CD133, OLIG2, and nestin. Nestin is a class VI intermediate filament protein, normally expressed in neuroepithelial stem and progenitor cells of the developing mammalian central nervous system (CNS). In this study, the authors focus on determining if nestin plays a functional role in tumor formation, proliferation, and migration as this is still not well understood.

Research frontiers

Previous work has established that nestin is required for prostate cancer cell migration *in-vitro* and *in-vivo*. In the present study, the authors investigate the expression of nestin in astrocytic tumors and its functional role in glioblastoma.

Innovations and breakthroughs

This is the first study evaluating nestin's role in tumor formation, proliferation, and migration utilizing glioma stem cells.

Applications

The findings suggest that nestin may be involved in glioblastoma cell invasion in some tumors. Inhibition of nestin expression and/or function may represent a potential therapeutic approach to reduce or inhibit glioblastoma cell spreading throughout the CNS.

Terminology

All terms used in this paper are described in the main text.

Peer-review

The relationship of nestin and glioma was well discussed in the paper.

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Basic Study

New categorization of human vascular endothelial cells by pro- vs anti-proliferative phenotypes

Miwako Nishio, Masako Nakahara, Chikako Sato, Koichi Saeki, Hidenori Akutsu, Akihiro Umezawa, Kazuyuki Tobe, Kazuki Yasuda, Akira Yuo, Kumiko Saeki

Miwako Nishio, Masako Nakahara, Chikako Sato, Akira Yuo, Kumiko Saeki, Department of Disease Control, Research Institute, National Center for Global Health and Medicine, Tokyo 162-8655, Japan

Koichi Saeki, Section of Cell Engineering, Department of Basic Research, DNAVEC Center, ID Pharma Co., Ltd., Ibaraki 300-2611, Japan

Hidenori Akutsu, Akihiro Umezawa, Department of Reproductive Biology, National Research Institute for Child Health and Development, Tokyo 157-8535, Japan

Kazuyuki Tobe, First Department of Internal Medicine, School of Medicine, Toyama University, Toyama 930-0194, Japan

Kazuki Yasuda, Department of Metabolic Disorder, Diabetes Research Center, Research Institute, National Center for Global Health and Medicine, Tokyo 162-8655, Japan

Kumiko Saeki, PRESTO, Japan Science and Technology Agency, Saitama 332-0012, Japan

Author contributions: Nishio M, Nakahara M and Sato C performed the experiments and analyzed the data; Saeki K, Akutsu H and Umezawa A contributed to the establishment of human iPS cells, Tobe K, Yasuda K and Yuo A coordinated the research; Saeki K designed the research and wrote the paper.

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Correspondence to: Kumiko Saeki, MD, PhD, Division Chief, Department of Disease Control, Research Institute, National Center for Global Health and Medicine, 1-21-1 Toyama Shinjuku-ku, Tokyo 162-8655, Japan. saeki@ri.ncgm.go.jp
Telephone: +81-3-32027181
Fax: +81-3-32027364

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Abstract

AIM: To integrally understand the effects of human vascular endothelial cells (VECs) on the proliferation of vascular smooth muscle cells (VSMCs).

METHODS: Various kinds of human VECs of different origins were co-cultured with human aortic smooth muscle cells, a representative of human VSMCs. To exclude the irrelevant effects due to growth competition between VECs and VSMCs, the proliferation of VECs had previously been arrested *via* a low-dose gamma ray

irradiation. To discriminate the proliferation of VSMCs from that of VECs, the former cells were labeled with red fluorescent dye while the latter cells were labeled with green fluorescent dye before performing co-culture experiments. After 4 d, total cells were harvested and subjected to flow cytometric analyses. Decrements in red fluorescence intensities due to proliferation-mediated dilutions were measured and mathematically processed using a specific software to quantitatively evaluate the proliferation of VSMCs. The findings obtained from the flow cytometry-based analyses were further validated by microscopic observations.

RESULTS: Commercially available primary cultured human VECs exclusively promoted VSMC proliferation regardless of their tissue origins and we termed these pro-proliferative VECs as “type-I”. By contrast, VECs freshly generated from human bone marrow-derived endothelial progenitors cells or human pluripotent stem cells including embryonic stem cells and induced pluripotent stem cells suppressed VSMC proliferation and we termed these anti-proliferative VECs as “type-II”. Repetitive subcultures as well as oxidative stress induced “type-II VECs to type-I” conversion along with an induction of Regulator of G-protein signaling 5 (RGS5). Compatibly, anti-oxidant treatments suppressed both the subculture-dependent “type-II to type-I” conversion and an induction of RGS5 gene. Immunostaining studies of clinical specimens indicated that RGS5 protein expressions in endothelial layers were low in normal arteries but they were up-regulated in pathological arteries including hypertension, atherosclerosis and autoimmune vasculitis in a dose-dependent manner. Overexpression and knockdown of RGS5 caused that “type-II to type-I” and “type-I to type-II” phenotype conversions of VECs, respectively.

CONCLUSION: Human VECs are categorized into two types: pro-proliferative RGS5^{high} VECs (type-I) and anti-proliferative RGS5^{low} VECs (type-II).

Key words: Vascular endothelial cells; Vascular smooth muscle cells; Human induced pluripotent stem cells; Human embryonic stem cells; Regulator of G-protein signaling 5; Oxidative stress

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Core tip: There is a longstanding controversy over the effects of vascular endothelial cells (VECs) on the proliferation of vascular smooth muscle cells (VSMCs). Since the controversy came from lack of systematic studies, we performed an integrated analysis using various human VECs to quantitatively evaluate their effects on VSMC proliferation. Here we report that: (1) human VECs are classified into two groups: pro-proliferative (type-I) vs “anti-proliferative” (type-II); (2) oxidative stress and ageing induced “type-II to type-I” conversion; and (3) RGS5 is the responsible gene for VEC phenotype determinations. Thus, human VECs are categorized into pro-proliferative RGS5^{high} (type-I) and

anti-proliferative RGS5^{low} (type-II) VECs.

Nishio M, Nakahara M, Sato C, Saeki K, Akutsu H, Umezawa A, Tobe K, Yasuda K, Yuo A, Saeki K. New categorization of human vascular endothelial cells by pro- vs anti-proliferative phenotypes. *World J Transl Med* 2015; 4(3): 88-100 Available from: URL: <http://www.wjgnet.com/2220-6132/full/v4/i3/88.htm> DOI: <http://dx.doi.org/10.5528/wjtm.v4.i3.88>

INTRODUCTION

Ischemic diseases are caused by stenosis of the artery (*i.e.*, arteriostenosis). Although cholesterol medications have produced remarkable results by reducing the rate of atherosclerosis, there still remain problems including medication-resistant patients and cases with restenosis after stent therapies. It is well known that the pathological basis of arteriostenosis is the hyperproliferation of vascular smooth muscle cell (VSMC). However, roles for vascular endothelial cells (VEC) in the development of arteriostenosis remain elusive. Controversial ideas have been raised regarding the effects of VECs on the proliferation of VSMC. From a clinical standpoint, it has been suggested that VECs prevent the proliferation of VSMC because VSMC proliferation is usually observed under circumstances where VECs are lost or dysfunctional. In accordance with this idea, VECs of hypertensive rats, but not those of normal counterparts, reportedly promote VSMC proliferation^[1]. On the other hand, *in vitro* co-culture experiments using fetal human umbilical vein endothelial cells (HUVEC) and bovine aortic smooth muscle cells showed that VECs enhanced VSMC proliferation^[2]. Nevertheless, this finding requires detailed validation. First, it must be re-validated by co-culture experiments using the cells of the identical species (*e.g.*, human VSMCs and human VECs). Secondly, it must be re-checked by co-culture experiments using adult samples because dynamic vasculogenesis with active VSMC proliferations occurs during fetal development. In addition, physiological events during the fetal development often resemble to pathological events in the adult life such as fetal gene expression profiles in angiosarcoma^[3], coronary artery disease^[4], diabetic retinopathy^[5] and arteriostenosis^[6] in adults. Thirdly, it must be re-validated by co-culture experiments using freshly produced VECs because characters of the cells often change during the process of *ex vivo* subcultures. For example, the quality of *ex vivo*-expanded primary cultured human cells becomes considerably lower than that of freshly differentiated human embryonic stem cells (ESC) or induced pluripotent stem cells (iPSC)^[7].

To address all those issues, we performed VEC/VSMC co-culture experiments using various kinds of human VECs of diverse origins. Furthermore, we performed microarray analyses to identify the key gene that determines the phenotypes of human VECs.

MATERIALS AND METHODS

Cells, tissues and reagents

HUVEC, human neonatal dermal microvascular endothelial cells (HMVEC), human adult aortic endothelial cells (HAEC) and human adult coronary arterial endothelial cells (HCAEC) were purchased from Dainippon Sumitomo Pharma Co., Ltd. (Osaka Japan). Human endothelial progenitors cells (EPCs) were provided as follows: Two lots of human adult bone marrow mononuclear cell-derived endothelial progenitor outgrowth cells (EPOCs) at 4th passage were purchased from BioChain Institute, Inc., Hayward, CA. Two lots of human umbilical cord blood endothelial colony forming cells (ECFC) were purchased from Lonza Group Ltd., Basel, Switzerland, and one lot of human cord blood-derived EPOCs at 4th passage, were purchased from BioChain Institute, Inc. The cells were cultured on 0.1% gelatin-coated plates using EGM[®]-2 BulletKit (Lonza Group Ltd. Basel, Switzerland). Human aortic smooth muscle cells of different donors were purchased from Lonza Group Ltd. (Basel, Switzerland) and cultured using SmGM[™]-2 BulletKit[™] (Lonza Group Ltd.). Cells were re-seeded at split ratios of 1:3-1:4 twice a week. VECs within 8th passage were used in all experiments. The hESC lines (KhES-1, -3, -5) were established by the Institute for Frontier Medical Science, Kyoto University^[8]. SeV-hiPSCs were established from HUVEC^[9] and BJ fibroblast^[9] by using iPS-Tune[™] (ID Pharma Co., Ltd., Ibaraki, Japan). Ret-hiPSC lines were provided as follows: 253G1^[10] and 201B7^[11] were provided by CIR at Kyoto University; #25 was provided by National Research Institute for Child Health and Development as used elsewhere^[7]. Astaxanthin (A3236, Sigma-Aldrich Co. LLC. St. Louis, MO 63178, United States) was dissolved by DMSO at the concentration of 5 mmol/L. Frozen sections of clinical specimens were purchased from BioChain Institute, Inc.: normal human artery (Cat: T5595-4763 Lot: L11052523C11052523), arteries of hypertension patients (Cat: T1236013Hd-2 Lot: B502175), arteries of systemic lupus erythematosus (Cat: T1236013LUP Lot: A804253), arteries of arteriosclerosis (Cat: T1236013Hd-4 Lot: B502176).

Quantitative evaluations of VSMC proliferation

VECs were -irradiated (5 Gy) and stained with carboxy-fluorescein diacetate, succinimidyl ester by using CFSE Cell Division Assay Kit (Cayman Chemical Co., Ann Arbor, MI), while VSMCs were stained with PKH26 by using PKH26 Red Fluorescent Cell Linker Kit (Sigma-Aldrich Co. LLC., St. Louis, MO 63178) according to the manufacturer's guidance. For contact co-culture, irradiated and CFSE-stained VECs were seeded at the density of 2×10^5 cells/well on 0.1% gelatin-coated 24-well culture plates, and on the following day, PKH26-stained VSMCs were seeded at the density of 3.75×10^3 cells/well on VEC layers or gelatin layers as control. After 4 d, total cells were harvested and subjected to flow cytometry analyses by FACSCalibur[™] (BD Biosciences,

San Jose, CA) and FL1 and FL2 fluorescence intensities were measured by CellQuest[™] Pro software (BD Biosciences). FL2 (PKH26) fluorescence intensities were further analyzed mathematically by ModFit LT[™] software (Verity Software House Inc., Topsham, ME) to calculate the proliferation index. Regarding experiments on astaxanthin treatments, VECs were stained by PKH26 and VSMCs were treated with CFSE because of red colored-fluorescence interference by astaxanthin.

Microarray analyses

Total RNAs were isolated by using TRIzol[®] Reagent (Life Technologies, Inc., Grand Island, NY) and subjected to GeneChip[®] Gene 1.0 ST array (Affymetrix, Inc., Santa Clara, CA, United States) by Pharma Frontier Co. Ltd. (Tokyo, Japan). Alterations in gene expressions were estimated as significant if > 2.0-fold increments or < 0.5-fold decrements in signal intensities were detected. Functional analyses were performed using IPA[®] Ingenuity Pathways Analysis software ver. 14855783 (Ingenuity Systems, Inc. Redwood City, CA) and hierarchical clustering was executed using GeneSpring GX 12.0 software (Agilent Technologies, Santa Clara, CA). All analyses were performed by Chemicals Evaluation and Research Institute (CERI, Tokyo, Japan).

RT-PCR

Total RNA was extracted from VECs using TRIzol[®] Reagent (Life Technologies, Inc.). First strand DNA was synthesized by using SuperScript[™] III First-Strand Synthesis System kit (Life Technologies, Inc.). PCR was performed using GeneAmp[®] PCR system 9700 (Life Technologies, Inc) and Ex-Taq (Takara Shuzo Co. Ltd., Shiga, Japan) with a following program: the initial denaturation at 94 °C for 5 min, 24-28 cycles and 20-22 cycles of amplification process for RGS5 and -actin, respectively, renature (55 °C, 30 s), extension (72 °C, 30 s) and denature (94 °C, 30 s) with a final extension (72 °C, 10 min). Primers used for RGS5 were Fw: CTGGATTGCCTGTGAGGATT and Rv: TCAGGGCATGGATTCTTTTC and those for *b*-actin were Fw: GCAGGAGATGGCCACGGCGGC Rv: TCTCCTTCTGCATCCTGTGTCAGC. The RT-PCR products were subjected to 1.5% agarose gel electrophoresis and the amplified DNA bands were visualized by ethidium bromide staining.

Quantitative RT-PCR

Total RNA was extracted from VECs using TRIzol[®] Reagent (Life Technologies, Inc.). Complementary DNA was prepared from 1 g of RNA using SuperScript[™] III First-Strand Synthesis System kit (Life Technologies, Inc.), and used in quantitative PCR reactions with FAST SYBR[®] Green Master Mix (Applied Biosystems[®] from Life Technologies, Inc.). qRT-PCR was performed using the StepOnePlus[™] PCR machine (Applied Biosystems[®] from Life Technologies, Inc.). Primers used for RGS5 were Fw: GGAGGCTCCTAAAGAGGTGA and Rv: GGGGAAGGTTCACCAGGTTC, and primers used for

GAPDH were Fw: CCACTCCTCCACCTTTGAC and Rv: ACCCTGTTGCTGTAGCCA.

Immunostaining

Cultured cells and clinical specimens were fixed by methanol/acetone (1:1) for 10 min on ice. The 1st antibody reactions were performed by using a 1:200-diluted chicken polyclonal anti-human RGS5 antibody (ab14265, Abcam plc., Cambridge, United States) and/or a 1:50-diluted rabbit polyclonal anti-human PECAM antibody (sc-8306, Santa Cruz Biotechnology Inc.) and the 2nd antibody reactions were performed by using an Alexa Fluor® 594-conjugated goat anti-chicken IgG (A11042, Life Technologies, Inc) and/or Alexa Fluor® 488-conjugated goat anti-rabbit IgG (A11008, Life Technologies, Inc). Photomicrographs were taken by Olympus BX51 Fluorescence Phase contrast Microscope (Olympus Optical Co. Ltd.) equipped with DP-2 TWIN digital camera system (Olympus Optical Co. Ltd.) and cellSens® standard imaging software (Olympus Optical Co. Ltd.). Regarding clinical specimens, white balance was adjusted so that autofluorescence from lamina elastic became white.

Western blotting

The 1×10^5 VECs were lysed by using 20 µL sample buffer solution [(2ME+) ($\times 2$), (Cat. 196-11022) and (WAKO Pure Chemical Industries, Osaka, Japan)]. The first antibody reaction was performed by using a 1:1000-diluted anti-human RGS5 antibody (ab83230, Abcam, Cambridge, United States) or a 1:1000-diluted anti-human β -tubulin antibody (sc-9104, Santa Cruz Biotechnology Inc., Santa Cruz, CA, United States) and the second antibody reaction was performed by using a 1:2000-diluted anti-rabbit IgG HRP-linked antibody (#7074S) (Cell Signaling Technology, Inc.).

Nucleofection

Expression vectors for shRNA against RGS5 were purchased from OriGene Technologies Inc. (Rockville, MD, United States). A Homo sapiens cDNA, FLJ96402, which corresponds to Homo sapiens regulator of G-protein signaling 5 (RGS5), transcript variant 1, mRNA (NM_003617.3), with two nucleotide substitutions, was purchased from National Institute of Technology and Evaluation (Tokyo, Japan), and the two substituted nucleotides were corrected by using KOD-Plus-Mutagenesis Kit (Toyobo Co. Ltd., Osaka, Japan) to become identical to the nucleotide sequences in NM_003617.3. The RGS5 cDNA was inserted into pmaxCloning™ expression vector (Lonza Group Ltd. Basel, Switzerland). Transfection was performed by using a Nucleofector™ (Lonza Group Ltd., Basel, Switzerland) according the manufacturer's guidance. The Amaxa HUVEC Nucleofector Kit (#VPB-1002, Lonza Group Ltd.) was used for HUVEC and the Amaxa Basic Nucleofector Kit Primary Endothelial Cells (#VPI-1001, Lonza Group Ltd.) was used for EPCdECs, hESdECs and hiPSdECs.

Statistical analysis

Experiments were performed independent three experiments ($n = 3$) and the data were analyzed according Student *t* test. Results were shown as averages (AV) \pm SD.

RESULTS

Studies on commercially available primary cultured human VECs

The effects of VECs on the proliferation of VSMCs were quantitatively evaluated by a flow cytometry-based technique (Figure 1A and B). Briefly, "contact" or "non-contact" (*i.e.*, Boyden) co-culture experiments were performed using various kinds of human VECs, whose growths were previously arrested by a low dose gamma ray irradiation, and adult human VSMCs. After 4 d, VSMC proliferation was assessed by mathematically processing the reduction degree of the red fluorescence intensity using ModFit LT™ software.

First, the effects of commercially available primary cultured VECs were examined. As previously reported^[2], the proliferation of VSMCs was up-regulated by both "contact" and "non-contact" co-cultures with HUVEC (Figure 1C and D), indicating that HUVEC enhanced VSMC proliferation *via* a soluble factor(s). These findings were confirmed by microscopic observations (Figure 1E). Similar results were obtained from HAEC, HCAEC and HMVEC (Figure 1F). Thus, commercially available primary cultured human VECs exclusively enhance VSMC proliferation *via* a soluble factor(s).

Studies on VECs generated from human endothelial progenitor cells

We next evaluated the effects of VECs that were produced from commercially available human (EPCs). Adult bone marrow mononuclear cell-derived EPCs at passage 4 were purchased. Then, EPC-derived mature VECs (EPCdECs) were prepared after additional three passages using a specialized medium (totally at passage 7) and subjected to co-culture experiments. Surprisingly, EPCdECs of the first donor (EPC1dEC) suppressed the proliferation of VSMCs under contact co-culture (Figure 2A and C). By contrast, EPC1dEC enhanced VSMC proliferation under non-contact culture (Figure 2A, right panel; Figure 2B, gray column), indicating that a potent growth-inhibitory activity, which was stronger than the growth-promoting activity of the soluble factor, was transmitted *via* cell-cell interactions. We confirmed that EPC1dEC purchased as an independent package after a half year provided similar findings (data not shown), guaranteeing the high reproducibility of our assay system. Interestingly, anti-proliferative potentials of EPC1dEC were considerably attenuated at passage 8 (Figure 2D, middle column) and finally nullified at passage 12 (Figure 2D, right column), indicating that the anti-proliferative capacity is susceptible to subculture-dependent stresses. In

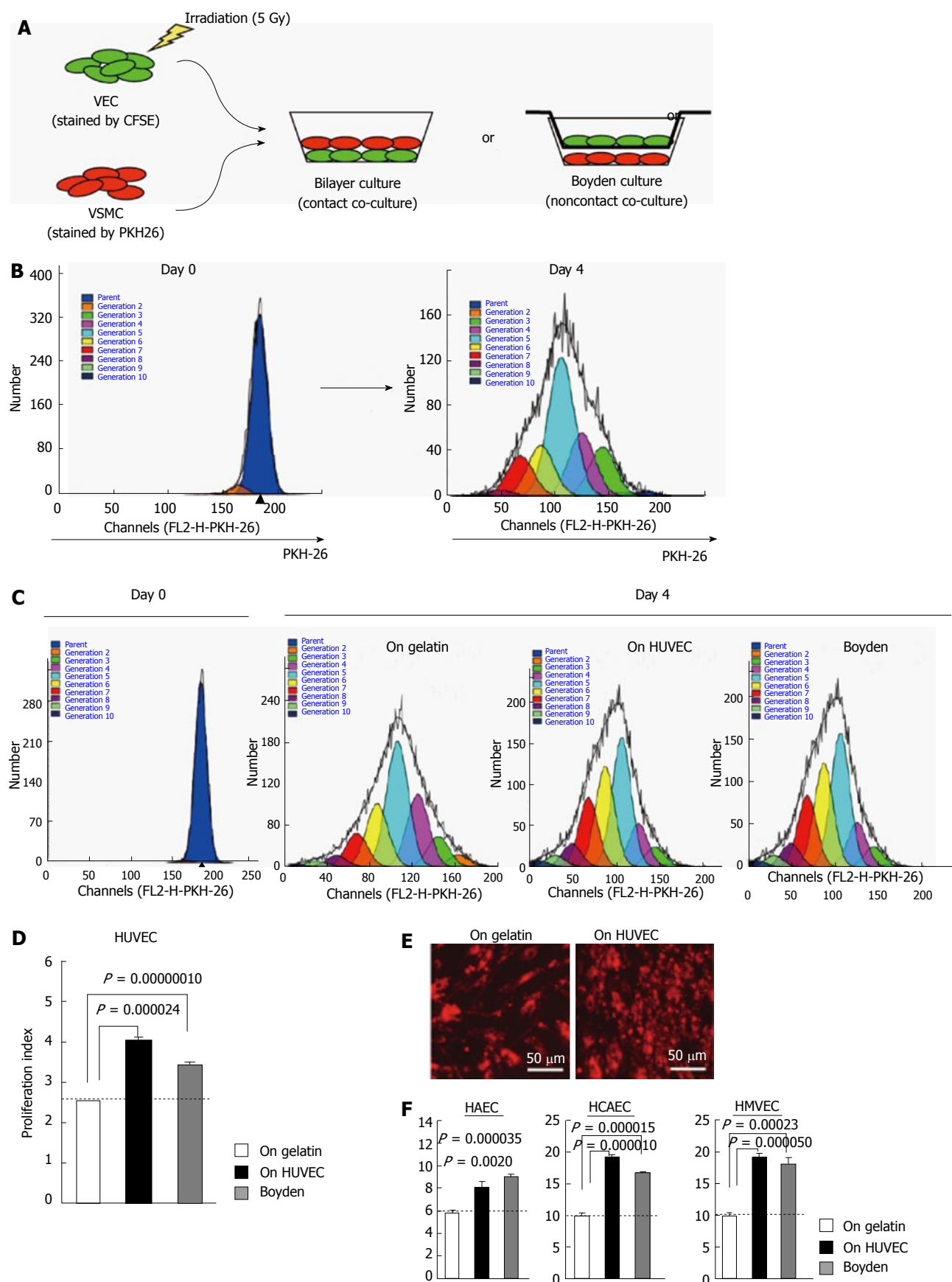


Figure 1 Phenotype determination of primary cultured human vascular endothelial cells. A and B: Illustrations of the method to calculate proliferation indexes of VEC-co-cultured VSMC via ModFit LT™ software-based analyses; C: Results of flow cytometric analyses of HUVEC-co-cultured VSMC with ModFit LT™ analyses; D: Results in C were statistically evaluated ($n = 3$); E: Fluorescent microscopy of PKH-26-stained VSMC subjected to contact co-culture with HUVEC; F: Results of VSMC-co-culture experiments using HAEC, HCAEC and HMVEC ($n = 3$). VEC: Vascular endothelial cells; HAEC: Human umbilical artery endothelial cells; HCAEC: Human coronary artery endothelial cells; HUVEC: Human umbilical vein endothelial cells; HMVEC: Human microvascular endothelial cell; VSMC: Vascular smooth muscle cell.

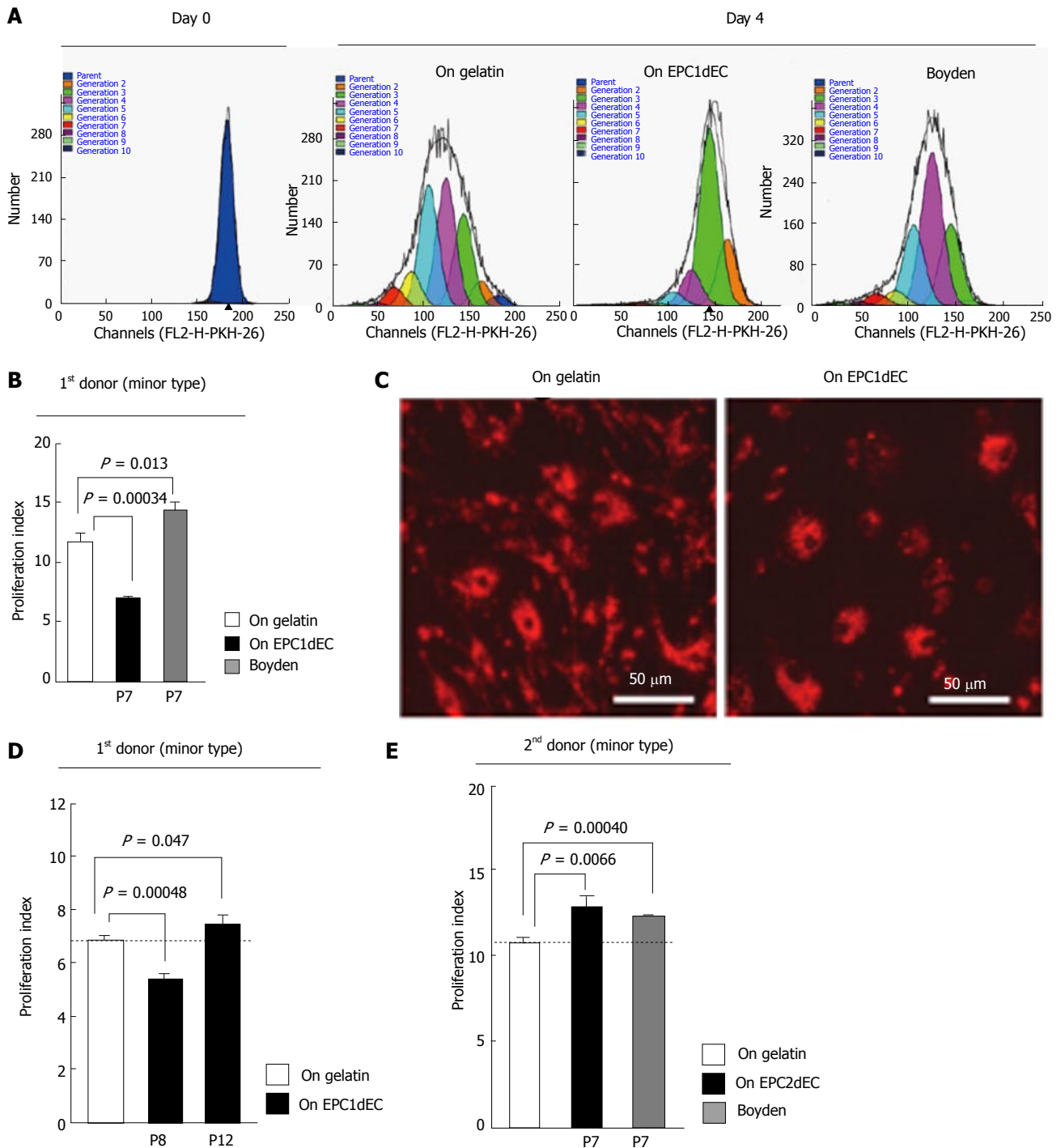


Figure 2 Phenotype determination of human embryonic stem cell-derived vascular endothelial cells. A: Results of flow cytometric analyses of hEPC1dEC[P7]-co-cultured VSMC with ModFit LT™ analyses; B: Results in (A) were statistically evaluated ($n = 3$); C: Fluorescent microscopy of PKH-26-stained VSMC subjected to contact co-culture with hEPC1dEC[P7]; D: Results of VSMC-co-culture experiments on the layer of hEPC1dEC at passage 8 and on that of hEPC1dEC passage 12; E: Results of VSMC-co-culture experiments using hEPC2dEC[P7] ($n = 3$). VSMC: Vascular smooth muscle cell; VEC: Vascular endothelial cell.

addition, anti-proliferative potentials could not be detected when fixed VECs were used (data not shown), indicating that living VECs were required for transmitting the anti-proliferative capacity. We termed VECs with anti-proliferative capacities as “type-II”, whereas we termed VECs with pro-proliferative capacities as “type-I”.

We further examined the effects of EPCdECs of different donors. In contrast to EPC1dECs, donor 2 EPC-derived VEC (EPC2dEC) showed type-I phenotype

from the earliest phase (*i.e.*, at passage 7) (Figure 2E). Moreover, all the other commercially available EPCdECs were exclusively type-I VECs (data not shown). Thus, EPCs that could produce type-II VECs belonged to a rather rare population among commercially available EPC sources. We also examined the phenotypes of VECs generated from fetal umbilical cord-derived EPCs (UCEPCdECs) and found that they were exclusively “type-I” VECs (data not shown), reflecting dynamic

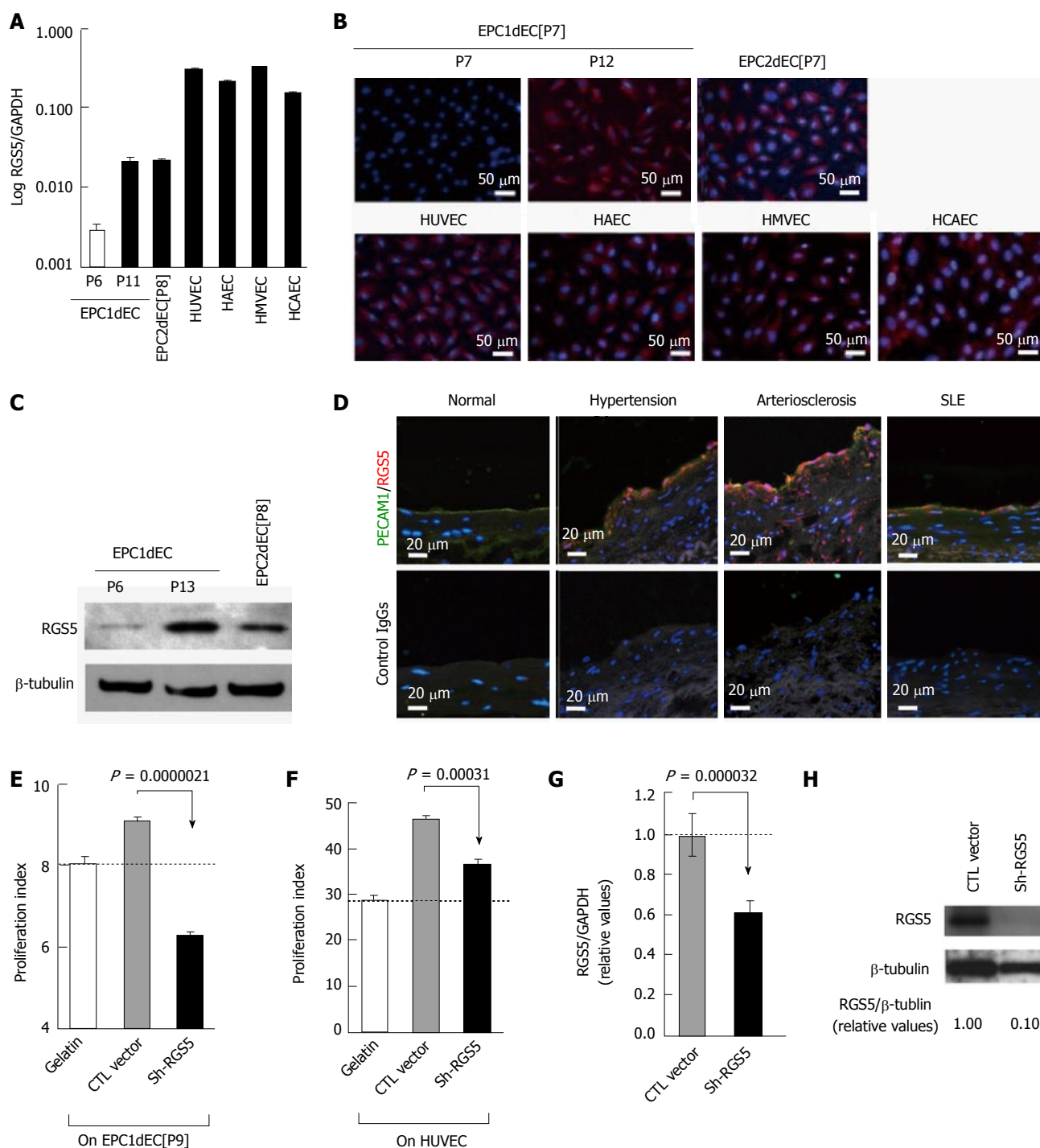


Figure 3 Regulator of G-protein signaling 5 is involved in the phenotype conversion of vascular endothelial cells. A: qRT-PCR results of RGS5 message expression were shown with normalization by GAPDH. The horizontal axis was demonstrated as logarithm; B: Immunostaining of indicated cells by using an anti-RGS5 antibody (red) with DAPI counterstaining (blue); C: Western blotting of RGS5 protein; D: Clinical specimens of the artery were subjected to immunostaining studies by using an anti-RGS5 antibody (red) and an anti-human PECAM1 antibody (green) with nuclear counterstaining with DAPI (blue); E-G: RGS5 knockdown experiments. An expression vector for shRNA against RGS5 (Sh-RGS5) or control RNA (CTL RNA) were transfected into EPC1dEC[P9] (E) or HUVEC (F). The results of VSMC co-cultured experiments (E and F) along with qRT-PCR (RGS5/GAPDH) (G) and Western blotting (H) regarding HUVEC were shown. GAPDH: Glyceraldehyde-phosphate dehydrogenase; VSMC: Vascular smooth muscle cell; VEC: Vascular endothelial cell; iPSC: Induced pluripotent stem cell; PCR: Polymerase chain reaction; RGS5: Regulator of G-protein signaling 5; HUVEC: Human umbilical vein endothelial cell; HAEC: Human adult aortic endothelial cells; HMVEC: Human neonatal dermal microvascular endothelial cells.

vasculogenesis during fetal development.

RGS5 is the causative gene for phenotype conversion of human VECs

To determine the responsible gene for "type-I vs type-

II" phenotyping of human VECs, microarray analyses were performed using "type-II" VECs, which were EPC1dEC[P7] of the two independent packages, and "type-I" VECs, which were HUVEC, HAEC, HMVEC, EPC1dEC[P12], EPC2dEC[P7] and UCEPC1dEC (GEO

Accession ID: GSE60999). We found that regulator of G-protein signaling 5 (RGS5) was the only gene that showed a discriminating expression pattern between “type-II” and “type-I” VECs. The results of the microarray were confirmed by quantitative RT-PCR (qRT-PCR): About one-order up-regulations of *RGS5* expressions in “type-I” EPC1dEC[P11] and EPC2dEC[P8] and almost two-order up-regulations of *RGS5* expression in HUVEC, HAEC and HMVEC compared to EPC1dEC[P6] (Figure 3A). Immunostaining (Figure 3B) and Western blotting (Figure 3C) further confirmed the findings. In addition, clinical relevance of *RGS5* induction was evidenced by immunostaining studies using human specimens: Undetectable expression in normal subjects, mild inductions in patients suffering from hypertension or systemic lupus erythematosus and high-level inductions in patients with arteriosclerosis (Figure 3D). In the specimen of arteriosclerosis, *RGS5* protein was even detected in neointima especially at perimeters of vasa vasorum, which were recognized as hollow spaces. The distribution pattern of *RGS5* was highly analogous to that of oxidative stress-damaged cells^[12], suggesting that oxidative stress is one of the major triggers of the “type-II to type-I” conversion of VECs.

The involvement of *RGS5* in the phenotype conversion of VECs was verified by gene knockdown studies. As shown in Figure 3E, “type-I” EPC1dEC[P9] was converted into “type-II” VECs by an introduction of shRNA-*RGS5* expression vector (Figure 3E). Regarding HUVEC, pro-proliferative capacities were lowered by *RGS5* knockdown (Figure 3F). Although a shRNA-*RGS5* introduction into HUVEC effectively lowered the levels of *RGS5* message expression (Figure 3G) and protein expression (Figure 3H), its effect on phenotype alteration was rather mild probably due to particularly high *RGS5* expression in HUVEC (Figure 3A). Nevertheless, the results were reproducible when distinct shRNA-*RGS5* with different nucleotide sequences were introduced (data not shown). Thus, *RGS5* is the causative gene for the “type-II to type-I” conversion of VECs.

Studies on VECs generated from human pluripotent stem cells

Although we discovered the existence of “type-II” VECs for the first time in the world, their applications were highly limited because EPC1dEC[P7] could not be expanded without losing type-II phenotype and because commercially available EPCdECs of other donors were exclusively type-I VECs. To find an alternative source for the production of type-II VECs, we examined the phenotypes of VECs that were produced from human embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs). High-purity subculturable VECs were generated without a contamination by mural cells based on our previously reported method^[13,14]. We found that human ESC-derived VECs (ESdECs) showed exclusively “type-II” phenotypes at their early passages; however, they were converted into “type-I” VECs after a few rounds of subcultures (Figure 4A) with up-regulated

expressions of *RGS5* message (Figure 4B) and protein (Figure 4C and D) although the timing of conversion differed depending on lines. Because an involvement of oxidative stress in *RGS5* induction was suggested by the similarity in tissue-distributing profiles between *RGS5*-positive cells (Figure 3D) and oxidative stress-damaged cells^[12], we examined the effect of hydrogen peroxide treatments on *RGS5* gene expression. We found that hydrogen peroxide treatments induced *RGS5* expressions in type-II ESdECs (Figure 4E) and a treatment with astaxanthin, which is the most potent anti-oxidant whose singlet oxygen-quenching activity^[15] and its anti-lipid peroxidation activity are reportedly superior to vitamin E by two-order^[16], suppressed *RGS5* inductions (Figure 4F). Moreover, astaxanthin treatment significantly delayed the timing of “type-II to type-I” conversion (Figure 4G), suggesting that oxidative stress is one of the major causes of *RGS5* induction.

Because astaxanthin treatment could not completely block *RGS5* induction in type-II ESdECs after repetitive subcultures (Figure 4F), we searched for still other candidates for type-II VECs. Since the timing of the “type-II to type-I” conversion was latest in the VECs produced from KhES-5, which was established latest among the three lines of human ESCs, we hypothesized that “the more recently human iPSC lines are established, the more stably type-II phenotype will be maintained”. Since we recently established two lines of Sendai virus vector-based iPSCs (SeV-iPSCs) from HUVEC and BJ fibroblast^[9], we produced VECs from these SeV-iPSCs (SeV-iPSdECs) and examined their characters. At the same time, we generated VECs from widely distributed conventional retrovirus vector-based iPSCs (Ret-iPSCs) including #25^[7,17], 253G1^[7,17,18] and 201B7^[7,17,19,20] and examined the phenotypes of these Ret-iPSC-derived VECs (Ret-iPSdECs). Regarding #25 and 253G1, anti-proliferative potentials were undetectable even at early passages (Figure 5A, upper and middle); nevertheless, VSMC proliferations were suppressed under contact co-culture conditions compared to non-contact co-culture conditions. Therefore, weak anti-proliferative potentials were transmitted from these Ret-iPSdECs. Regarding 201B7, anti-proliferative potentials were clearly detected at early passages (Figure 5A, lower) as in the case of ESdECs (Figure 4A). In all three lines of Ret-iPSdECs, pro-proliferative capacities were augmented after repetitive subcultures (Figure 5A). Thus, comparable results were obtained from Ret-iPSdECs to ESdECs as a whole. On the other hand, SeV-iPSdECs well preserved anti-proliferative capacities until later passages (Figure 5B) without an induction of *RGS5* gene (Figure 5C and D). They also showed high resistance to oxidative stress-induced *RGS5* induction (Figure 5E), supporting the idea that the most recently established human pluripotent stem cells provide the most effectual type-II VECs.

Collectively, human pluripotent stem cells provide an excellent source for the production of anti-proliferative type-II VECs.

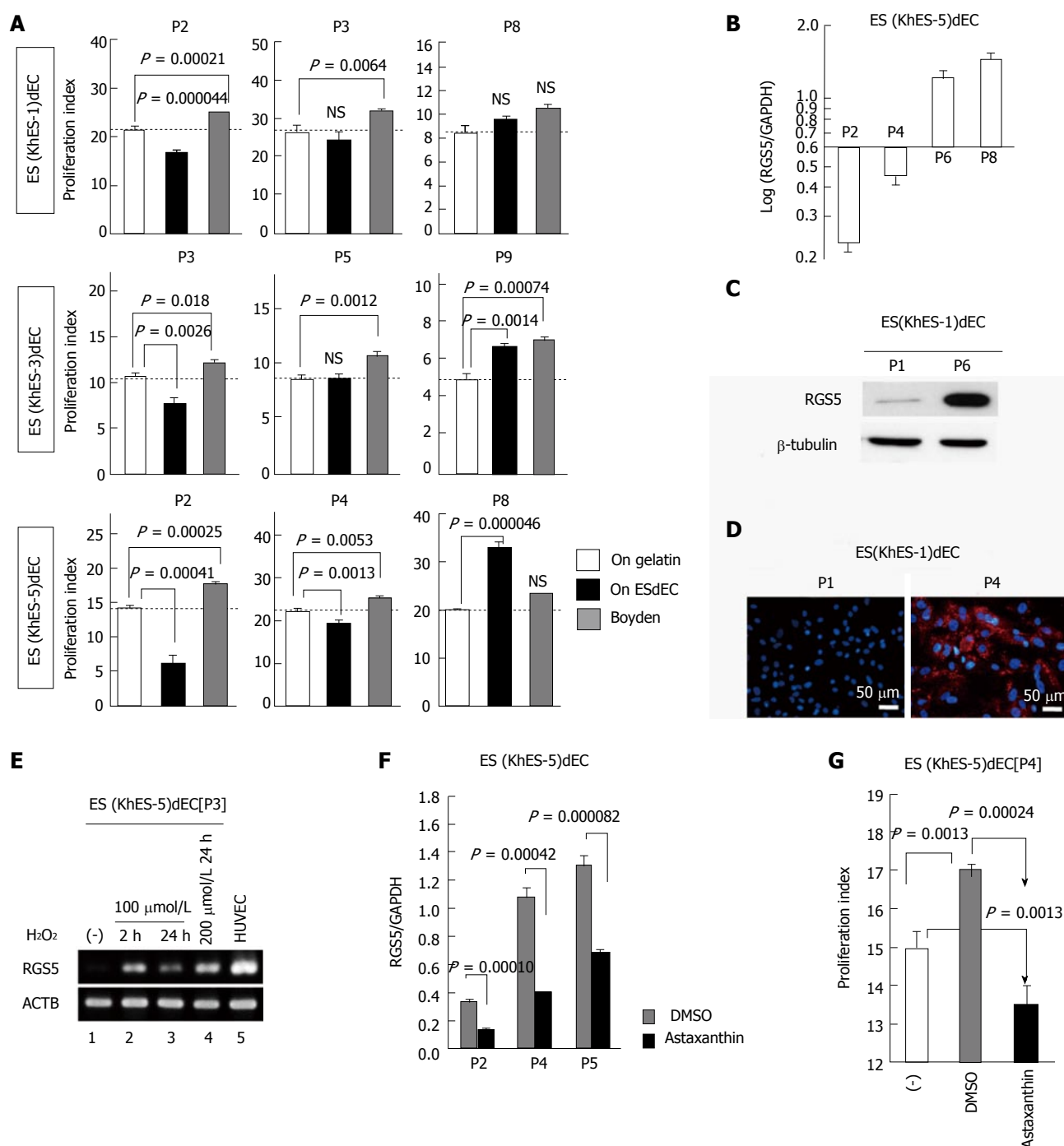


Figure 4 Phenotype evaluation of human embryonic stem cell-derived vascular endothelial cells. A: VSMC-co-culture experiments of VECs generated from three lines of hESCs (KhES-1, KhES-3, KhES-5) at indicated passage number ($n = 3$); B: qRT-PCR of RGS5 message of KhES-5-derived VECs [ES (KhES-5) dEC] at indicated passage numbers; C: Western blotting of RGS5 protein in KhES-1-derived VECs [ES (KhES-1) dEC] at passage 1 and passage 6; D: Immunostaining of RGS5 protein in ES (KhES-1) dEC at passage 1 (upper) and passage 4 (lower); E: ES (KhES-5) dECs were treated with hydrogen peroxide and RGS5 message expressions were examined by RT-PCR. Lane 1: no treatment; lane 2: 100 μ mol/L H_2O_2 treatment for 2 h; lane 3: 100 μ mol/L H_2O_2 treatment for 24 h; lane 4: 200 μ mol/L H_2O_2 treatment for 24 h; lane 5: HUVEC; F: qRT-PCR of RGS5 message of ES (KhES-5) dECs subcultured in the presence of DMSO (gray column) or 10 μ mol/L astaxanthin (closed columns); G: VSMC-co-culture experiments of ES (KhES-5) dECs maintained with culture medium (open column), in the presence of DMSO (gray column) or 10 μ mol/L astaxanthin (closed column). VSMC: Vascular smooth muscle cell; VEC: Vascular endothelial cell; PCR: Polymerase chain reaction; DMSO: Dimethylsulfoxide; RGS5: Regulator of G-protein signaling 5; HUVEC: Human umbilical vein endothelial cell.

DISCUSSION

In the current study, we presented a new concept for the categorization of human VECs based on their effects on VSMC proliferation: *pro-proliferative* RGS5^{high} VEC

(type-I) and anti-proliferative RGS5^{low} VECs (type-II) (Figure 6). Since oxidative stresses and subculture-dependent mechanochemical stresses induced "type-II to type-I" conversion along with an induction of RGS5 expression, "type-I" RGS5^{high} VECs may well be regarded

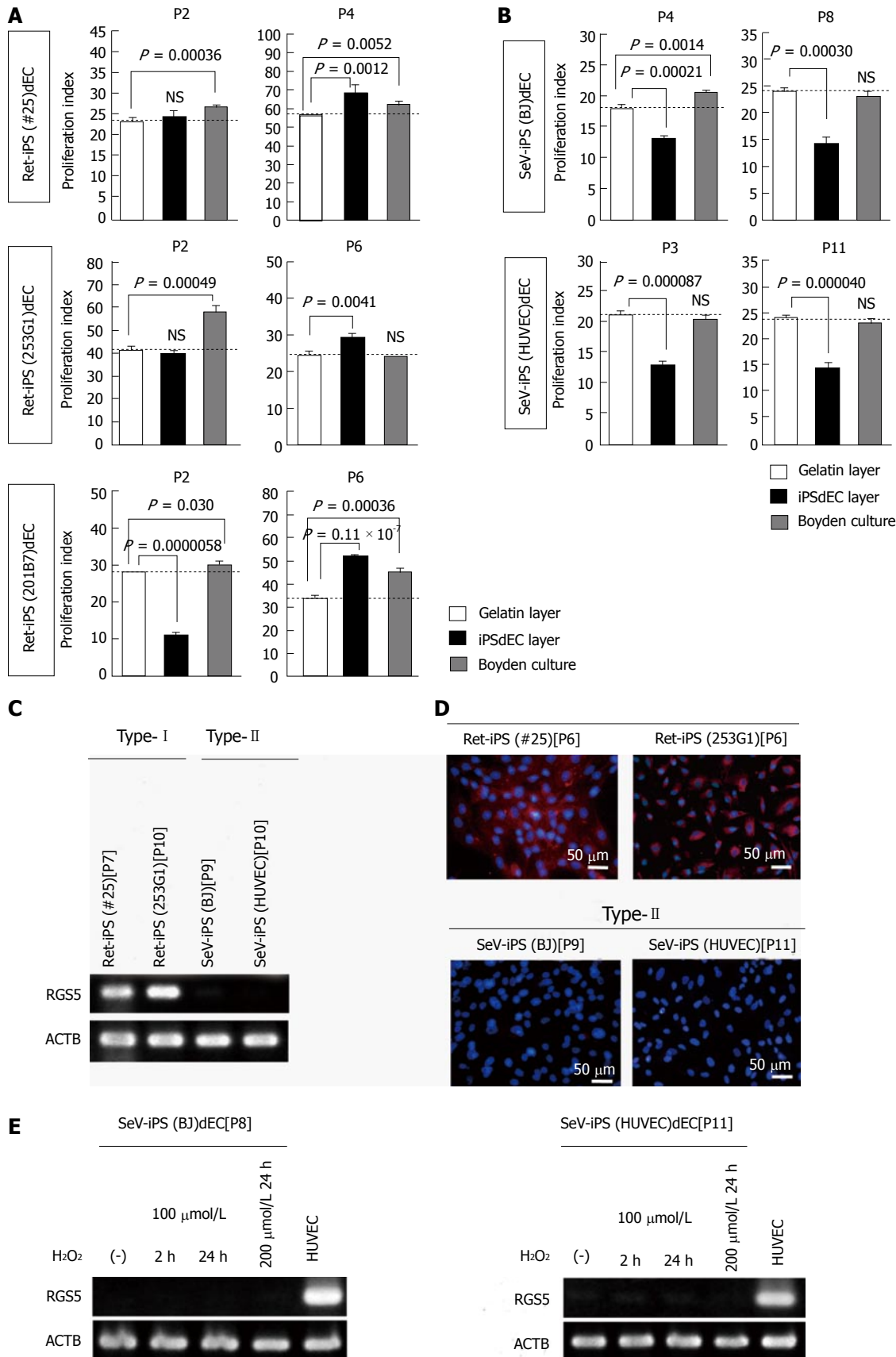


Figure 5 Phenotype evaluation of human induced pluripotent stem cells-derived vascular endothelial cells. A and B: VSMC-co-culture experiments of VECs generated from three lines of Ret-iPSCs (#25, 253G1 and 201B7); A and those from two lines of SeV-iPSCs(SeV-iPS(BJ) and [SeV-iPS(HUVEC)]); B: At indicated passage number ($n = 3$); C: RT-PCR of RGS5 message of Ret-iPSdECs (type-I) and SeV-iPSdECs (type-II); D: RGS5 immunostaining studies of Ret-iPSdECs (upper) and SeV-iPSdECs (lower); E: H_2O_2 -treating experiments were performed in type-II SeV-iPSdECs as in Figure 4E. VSMC: Vascular smooth muscle cell; VEC: Vascular endothelial cell; iPSC: Induced pluripotent stem cell; PCR: Polymerase chain reaction; RGS5: Regulator of G-protein signaling 5; HUVEC: Human umbilical vein endothelial cell.

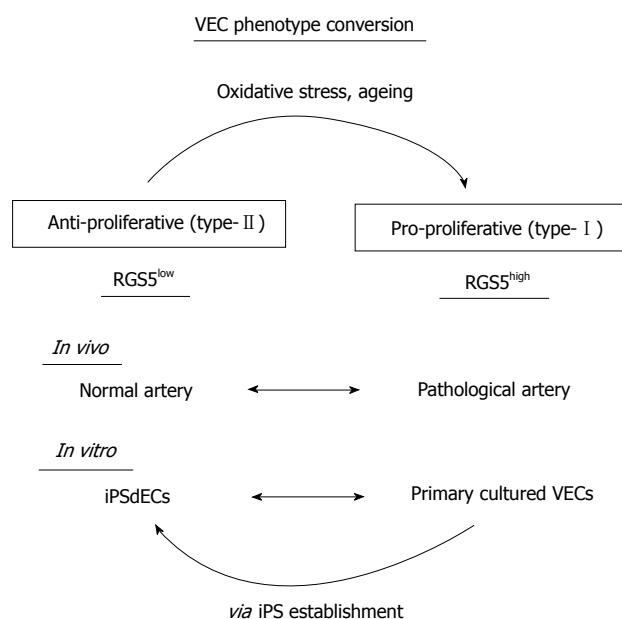


Figure 6 A model for the type-I vs type-II phenotype regulation of human vascular endothelial cells. RGS5: Regulator of G-protein signaling 5; VEC: Vascular endothelial cell; iPSC: Induced pluripotent stem cell.

as degenerative VECs. It might be shocking that *widely used* commercially available primary cultured human VECs exclusively belong to type-I VECs. However, there is a rational reason for this idea because primary cultured cells have inevitably received multiple kinds of stresses during the process of their preparations including tissue removal, cell dissociation and cell expansion. It is almost impossible for them to preserve every character that they had in *in vivo* environments. Indeed, commercially available primary cultured human VECs preserve fundamental functions of VECs such as cord-forming activities and acetylated low density lipoprotein (Ac-LDL)-up-taking capacities; however, they may have possibly lost certain sophisticated functions before they are on the distribution routes. The type-II anti-proliferative capacity may be one of such refined functions.

We have also shown that RGS5 is a causative gene for "type-II to type-I" conversion. Because EPC2dEC at passage 7 (EPC2dEC[P7]) was larger in size and showed longer doubling time than EPC1dEC passage 7 (EPC1dEC[P7]) (data not shown), EPC2dEC might be in ageing states. This idea was supported by the cluster analysis of the microarray data (GEO Accession ID: GSE61000): EPC2dEC[P7] was located closer to EPC1dEC[P12] than EPC1dEC[P7] (data not shown) and the gene function item "senescence" marked the highest value in the matching rate (data not shown). Thus, the "type-II to type-I" phenotype conversion may well be considered as an ageing-associated degeneration. Nevertheless, "type-II to type-I" conversion is not an identical concept to senescence because type-I primary cultured human VECs show no signs of senescence. Rather, it should be considered as a sign of degeneration. A clinical study supported this idea, showing that VECs

of the subcutaneous vessels of normal individuals are negative for RGS5 expression while those of scleroderma patients are positive for RGS5 expression along with an induction of interferon alpha gene^[21,22]. Similar to the case of human VECs, murine VECs are reportedly negative for RGS5 expression^[23]. Thus, RGS5 induction in VECs may provide a useful marker for degenerative vessels.

RGS5 plays beneficial roles depending on the kinds of cells. Lack of RGS5 expression in VECs is advantageous as reported in RGS5-deficient mice, which show an advantageous phenotype with normalization of tumor vasculatures^[24]. However, up-regulated expressions of RGS5 in VSMCs reportedly bring about favorable outcomes such as angiogenesis promotion^[25] and atherosclerosis improvements^[26,27]. It seems that opposite effects are exerted by RGS5 between VECs and VSMCs. Thus, we have to be careful enough in performing RGS5-targeted drug discovery to avoid the side effects due to reduced RGS5 expression in VSMCs.

Although "type-II to type-I" conversion is usually a one-way process, "type-I" primary cultured human VECs can be converted into "type-II" VECs *via* "iPSC establishment and subsequent VEC differentiation" (Figure 5B). We showed that VECs generated from *freshly* established SeV-iPSCs bare high resistance to stress-induced "type-II to type-I" conversion (Figure 5E), and thus, they may provide an excellent tool for the transplantation therapy for the treatments of refractory arteriosclerosis.

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COMMENTS

Background

Ischemia leads to the development of life-threatening diseases including ischemic heart disease and stroke. It is caused by narrowing of arteries (*i.e.*, arteriosclerosis), whose pathological basis is hyperproliferation of vascular smooth muscle cells. Although roles for vascular smooth muscle cells (VSMCs) and macrophages in the development of arteriosclerosis are well understood, those for vascular endothelial cells (VECs) remain controversial. Toward the development of new therapeutics, however, involvements of VECs in the progression of arteriosclerosis should be elucidated.

Research frontiers

There is a longstanding controversy over the effect of VECs on the proliferation of VSMCs: Clinical observations suggest that VECs prevent the proliferation of VSMCs while *in vitro* co-culture experiments showed that human umbilical cord VECs enhanced the proliferation of bovine VSMCs.

Innovations and breakthroughs

The controversy came from lack of systematic studies, and thus, the authors performed an integrated analysis to quantitatively evaluate the effects various kinds of adult human VECs on the proliferation of adult human VSMCs. The authors have discovered for the first time that human VECs are categorized into two groups by their effects on VSMC proliferation and expression levels of Regulator of G-protein signaling 5 (RGS5): pro-proliferative RGS5^{high} VECs (type-I) and anti-proliferative RGS5^{low} VECs (type-II). Clinical relevance of our finding was supported by the fact that VECs of pathological arteries with tunica media thickening were RGS5^{high} while VECs of normal arteries were RGS5^{low}.

Applications

RGS5 expression in VECs provides a useful indicator for the drug discovery for the treatment of ischemic diseases. Furthermore, human pluripotent stem cell-derived type-II VECs will provide a useful tool for transplantation therapy of arteriosclerosis.

Terminology

Regulator of RGS5 is known as an inhibitory molecules against the signaling from G protein-coupled receptor. It is reportedly involved in the regulation of VEC-VEC interaction via VE-cadherin and VEC-VSMC interaction via N-cadherin.

Peer-review

Authors tried to solve the controversy in human VECs to the proliferation of human vascular smooth muscle cells by characterize the human VECs from various sources in two groups as either pro-proliferative or antiproliferative. The studies presented herein implicate regulator of RGS5 as a modulator of VEC phenotype, where VECs expressing high RGS5 are pro-proliferative and VECs expressing low RGS5 are anti-proliferative. Oxidative stress induces RGS5 expression and shifts VECs into a pro-proliferative phenotype. The studies are novel and the manuscript is well written.

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Basic Study

p38 mitogen-activated protein kinase regulates type-I vs type-II phenotyping of human vascular endothelial cells

Masako Nakahara, Miwako Nishio, Koichi Saeki, Akira Yuo, Kumiko Saeki

Masako Nakahara, Miwako Nishio, Akira Yuo, Kumiko Saeki, Department of Disease Control, Research Institute, National Center for Global Health and Medicine, Tokyo 162-8655, Japan

Koichi Saeki, Section of Cell Engineering, Department of Basic Research, DNAVEC Center, ID Pharma Co., Ltd., Ibaraki 300-2611, Japan

Kumiko Saeki, PRESTO, Japan Science and Technology Agency, Saitama 332-0012, Japan

Author contributions: Nakahara M and Nishio M performed the experiments and analyzed the data; Saeki K constructed viral vectors; Yuo A designed and coordinated the research; Saeki K designed the research, constructed viral vectors and wrote the paper.

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Correspondence to: Kumiko Saeki, MD, PhD, Division Chief, Department of Disease Control, Research Institute, National Center for Global Health and Medicine, 1-21-1 Toyama Shinjuku-ku,

Tokyo 162-8655, Tokyo 162-8655, Japan. saeki@ri.ncgm.go.jp
Telephone: +81-3-32027181
Fax: +81-3-32027364

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Abstract

AIM: To identify kinases involved in phenotype regulation of vascular endothelial cells (VECs): Pro-proliferative G-protein signaling 5 (RGS5)^{high} (type-I) vs anti-proliferative RGS5^{low} (type-II) VECs.

METHODS: Proteomic kinase assays were performed to identify the crucial kinase involved in the phenotype regulation of human VECs using type-I VECs, which promotes the proliferation of human vascular smooth muscle cells (VSMCs), and type-II VECs, which suppress the proliferation of human VSMCs. The assays were performed using multiple pairs of type-I and type-II VECs to obtain the least number of candidates. The involvement of the candidate kinases was verified by evaluating the effects of their specific inhibitors on the phenotype regulation of human VECs as well as the expression levels of regulator of RGS5, which is the causative gene for the "type-II to type-I" phenotype conversion of human VECs.

RESULTS: p38 α mitogen-activated protein kinase (p38 α MAPK) was the only kinase that showed distinctive activities between type-I and type-II VECs: p38 α MAPK activities were low and high in type-I and type-II VECs, respectively. We found that an enforced expression of RGS5 indeed lowered p38 α MAPK activities

in type-II VECs. Furthermore, treatments with a p38 α MAPK inhibitor nullified the anti-proliferative potential in type-II VECs. Interestingly, MAPK inhibitor treatments enhanced the induction of *RGS5* gene. Thus, there is a vicious cycle between "RGS5 induction" and "p38 α MAPK inhibition", which can explain the unidirectional process in the stress-induced "type-II to type-I" conversions of human VECs. To understand the upstream signaling of RGS5, which is known as an inhibitory molecule against the G protein-coupled receptor (GPCR)-mediated signaling, we examined the effects of RGS5 overexpression on the signaling events from sphingosine-1-phosphate (S1P) to N-cadherin, because S1P receptors belong to the GPCR family gene and N-cadherin, one of their downstream effectors, is reportedly involved in the regulation of VEC-VSMC interactions. We found that RGS5 specifically bound with S1P $_1$. Moreover, N-cadherin localization at intercellular junctions in type-II VECs was abolished by "RGS5 overexpression" and "p38 α MAPK inhibition".

CONCLUSION: p38 α MAPK plays crucial roles in "type-I vs type-II" phenotype regulations of human VECs at the downstream of RGS5.

Key words: Vascular endothelial cells; Vascular smooth muscle cells; Proteomic kinase assay; p38 α mitogen-activated protein kinase; Regulator of G-protein signaling 5; Sphingosine-1-phosphate; N-cadherin

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Core tip: We previously reported that human vascular endothelial cells (VECs) are categorized into two types by their effects on the proliferation of vascular smooth muscle cells and the expressions of regulator of G-protein signaling 5 (RGS5): Pro-proliferative RGS5^{high} (type-I) and anti-proliferative RGS5^{low} (type-II) VECs. Performing proteomic kinase assays and inhibitor studies, we show here that p38 mitogen-activated protein kinase (p38 MAPK) is the crucial kinase that determines VEC phenotyping at the downstream of RGS5. Not only RGS5 overexpression suppressed p38 MAPK activities but also p38 MAPK inhibitions up-regulated RGS5 expression, indicating that "RGS5 induction" and "p38 MAPK inhibition" creates a vicious cycle in "type-II to type-I" conversions of human VECs.

Nakahara M, Nishio M, Saeki K, Yuo A, Saeki K. p38 mitogen-activated protein kinase regulates type-I vs type-II phenotyping of human vascular endothelial cells. *World J Transl Med* 2015; 4(3): 101-112 Available from: URL: <http://www.wjgnet.com/2220-6132/full/v4/i3/101.htm> DOI: <http://dx.doi.org/10.5528/wjtm.v4.i3.101>

INTRODUCTION

We previously reported that human vascular endothelial cells (VECs) are classified into two groups by their

effects on the proliferation of vascular smooth muscle cells (VSMCs) in *in vitro* co-culture experiments along with the expression levels of regulator of G-protein signaling 5 (RGS5): Pro-proliferative RGS5^{high} VEC (type-I) vs anti-proliferative RGS5^{low} VECs (type-II)^[1]. We also demonstrated that commercially available primary cultured human VECs exclusively belong to *pro-proliferative* RGS5^{high} VECs (type-I). On the other hand, human bone marrow-derived endothelial progenitor cells (EPCs) and human embryonic stem cells (ESCs) produced *anti-proliferative* RGS5^{low} VECs (type-II)^[1]. Because RGS5 expression was hardly detectable in normal human VECs^[1,2] but significantly induced under pathological conditions^[1,3,4] and because oxidative stress and subculture-dependent stress induced "type-II to type-I" conversion along with RGS5 gene induction^[1], pro-proliferative RGS5^{high} VECs (type-I) should be considered as degenerative VECs. The reason why widely used commercially available primary cultured human VECs belong to type-I degenerative VECs may be attributed to their histories: They have survived the drastic environmental changes from *in vivo* to *in vitro* by overcoming multiple stresses during the process of their preparations such as tissue removals, cell dissociations and cell expansions. Therefore, their characters may have changed in such a manner that they lose type-II *anti-proliferative* capacities. By contrast, EPC/ESC-derived VECs do not have such stressful histories, and thus, it is possible that they preserve type-II *anti-proliferative* phenotypes. Nevertheless, EPC/ESC-derived type-II RGS5^{low} VECs were inevitably converted into type-I RGS5^{high} VECs after a few rounds of subcultures^[1].

In the case of induced pluripotent stem cells (iPSCs), the situations are rather complicated. Regarding VECs produced from retroviral vector-based iPSCs (Ret-iPSCs), type-II *anti-proliferative* capacities were often deteriorated from early phases and subculture-dependent "type-II to type-I" conversions were often accelerated^[1]. By contrast, in the case of Sendai virus vector-based iPSC (SeV-iPSCs), "type-II to type-I" conversion was highly repressed: SeV-iPSC-derived VECs (SeV-iPSdECs) showed high resistance to subculture-dependent and oxidative stress-induced "type-II to type-I" conversions^[1]. The phenotype differences between Ret-iPSC-derived VECs (Ret-iPSdECs) and SeV-iPSdECs can be explained, at least in part, by the differences in the degree of stresses. Ret-iPSCs have multiple copies of viral vectors in their chromosomes, and thus, they suffer from genome stresses. On the other hand, SeV-iPSCs completely get rid of such genome stresses because the Sendai virus vector is an RNA virus-based vector and do not integrate into the host chromosomes.

In our previous study, the signaling pathway involved in the "type-II to type-I" conversion at the downstream of RGS5 remained elusive. Because RGS5 was the only gene that showed a discriminative expression pattern between type-I and type-II VECs in microarray analyses^[1], we thought that an alternative strategy

other than transcriptomic approaches was required to identify the downstream signaling events of RGS5. In the current study, we performed proteomic kinase assays to identify the crucial kinase that regulates the RGS5-mediated “type-I vs type-II” phenotyping of human VECs.

MATERIALS AND METHODS

Cells and reagents

Human umbilical vein endothelial cells (HUVEC), human neonatal dermal microvascular endothelial cells (HMVEC), human adult aortic endothelial cells (HAEC) and human adult coronary arterial endothelial cells (HCAEC) were purchased from Dainippon Sumitomo Pharma Co., Ltd. (Osaka, Japan). The cells were cultured on 0.1% gelatin-coated plates using EGM[®]-2 BulletKit (Lonza Group Ltd. Basel, Switzerland). Human aortic smooth muscle cells of different donors were purchased from Lonza Group Ltd. (Basel, Switzerland) and cultured using SmGM[™]-2 BulletKit[™] (Lonza Group Ltd.). Cells were re-seeded at split ratios of 1:3-1:4 twice a week. VECs within 8th passage were used in all experiments. The hESC lines (KhES-1, -3, -5) were established by the Institute for Frontier Medical Science, Kyoto University^[2]. SeV-hiPSCs were established from HUVEC^[3] and BJ fibroblast^[3] by using iPS-Tune[™] (ID Pharma Co., Ltd., Ibaraki, Japan). The Ret-hiPSC line of 253G1^[4] was provided by CiRA at Kyoto University. Sphingosine-1-phosphate (S1P) (#62570, Cayman Chemical Co., Ann Arbor, MI, United States) was dissolved by 0.3 mol/L NaOH at the concentration of 4 mg/mL as a stock. A p38 MAP Kinase inhibitor (#506126, Calbiochem Co., La Jolla, CA, United States) was dissolved by DMSO at the concentration of 10 mmol/L as a stock. A JNK inhibitor SP600125 (#BML-EI305, Enzo Life Sciences, Inc., Farmingdale, NY, United States) was dissolved by DMSO at the concentration of 10 mmol/L as a stock. An extracellular signal-regulated kinase (ERK) inhibitor PD98059 (#513001, Calbiochem Co.) was dissolved by DMSO at the concentration of 50 mmol/L stock. FTY720 Phosphate (#10008639, Cayman Chemical Co.) was dissolved by chloroform at the concentration of 0.5 mg/mL as a stock. All reagents were kept at -20 °C.

Quantitative evaluations of VSMC proliferation

VECs were g-irradiated (5 Gy) and stained with carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE) by using CFSE Cell Division Assay Kit (Cayman Chemical Co., Ann Arbor, MI), while VSMCs were stained with PKH26 by using PKH26 Red Fluorescent Cell Linker Kit (Sigma-Aldrich Co. LLC., St. Louis, MO, United States) according to the manufacturer's guidance. For contact co-culture, irradiated and CFSE-stained VECs were seeded at the density of 2×10^5 cells/well on 0.1% gelatin-coated 24-well culture plates, and on the following day, PKH26-stained VSMCs were seeded at the density of 3.75×10^3 cells/well on VEC layers or gelatin layers as control. After 4 d, total cells were

harvested and subjected to flow cytometry analyses by FACSCalibur[™] (BD Biosciences, San Jose, CA, United States) and FL1 and FL2 fluorescence intensities were measured by CellQuest[™] Pro software (BD Biosciences). FL2 (PKH26) fluorescence intensities were further analyzed mathematically by ModFit LT[™] software (Verity Software House Inc., Topsham, ME, United States) to calculate the proliferation index.

Quantitative real-time polymerase chain reaction

Total RNA was extracted from VECs using TRIzol[®] Reagent (Life Technologies, Inc.). Complementary DNA was prepared from 1 µg of RNA using SuperScript[™] III First-Strand Synthesis System kit (Life Technologies, Inc.), and used in quantitative polymerase chain reaction (PCR) reactions with FAST SYBR[®] Green Master Mix (Applied Biosystems[®] from Life Technologies, Inc.). Quantitative real-time PCR was performed using the StepOnePlus[™] PCR machine (Applied Biosystems[®] from Life Technologies, Inc.). Primers used for RGS5 were Forward: 5'-GGAGGCTCCTAAAGAGGTGA-3' and Reverse: 5'-GGGAAGGTTCCACCAGGTTC-3', and primers used for GAPDH were Forward: 5'-CCACT-CCTCCACCTTTGAC-3' and Reverse: 5'-ACCCTG-TTGCTGTAGCCA-3'.

Immunostaining of N-cadherin

Human ESC/iPSC-derived cells were fixed by 4% paraformaldehyde for 15 min at room temperature. The 1st antibody reaction was performed by using a 1:100-diluted rabbit polyclonal anti-human N-cadherin antibody (ab12221, Abcam plc, Cambridge, MA, United States). The 2nd antibody reaction was performed by using an Alexa Fluor[®] 488-conjugated goat anti-rabbit IgG (A11008, Life Technologies, Inc.). Photomicrographs were taken by Olympus BX51 Fluorescence Phase contrast Microscope (Olympus Optical Co. Ltd.) equipped with DP-2 TWIN digital camera system (Olympus Optical Co. Ltd.) and cellSens[®] standard imaging software (Olympus Optical Co. Ltd.).

Western blotting

The 1×10^5 VECs were lysed by using 20 µL sample buffer solution (2ME+) ($\times 2$) (Cat. 196-11022) (WAKO Pure Chemical Industries, Osaka, Japan). The first antibody reaction was performed by using a 1:1000-diluted anti-human RGS5 antibody (ab83230, Abcam, Cambridge, MA, United States), a rabbit polyclonal anti-p38 α antibody (C-20) (sc-535, Santa Cruz Biotechnology Inc., Santa Cruz, CA, United States), a rabbit polyclonal anti-p38 (phospho T180) antibody (ab51050, Abcam plc.), a rabbit monoclonal anti-c-Jun (60A8) (#9165, Cell Signaling Technology, Inc., Beverly, MA, United States), a rabbit monoclonal anti-Phospho-c-Jun (Ser73) (D47G9) (#3270, Cell Signaling Technology, Inc.), a rabbit monoclonal anti-Phospho-c-Jun (Ser63) (54B3) (#2361, Cell Signaling Technology, Inc.), a rabbit polyclonal anti-Phospho-c-Jun (Ser243) Antibody (#2994, Cell Signaling Technology, Inc.), a rabbit polyclonal anti-c-

Jun (phospho T93) antibody (ab28854, Abcam plc.) or a 1:1000-diluted anti-human β -tubulin antibody (sc-9104, Santa Cruz Biotechnology Inc., Santa Cruz, CA, United States) and the second antibody reaction was performed by using a 1:2000-diluted anti-rabbit IgG HRP-linked antibody (#7074S) (Cell Signaling Technology, Inc.).

Immunoprecipitation

The 3×10^6 HUVECs were lysed by 500 μ L of RIPA buffer. Immunoprecipitation was performed using a mouse monoclonal anti-human RGS5 antibody (sc-390245, Santa Cruz Biotechnology Inc.) or a goat polyclonal anti-human S1P1 antibody (sc-16070, Santa Cruz Biotechnology Inc.), anti-human S1P2 antibody (sc-31577, Santa Cruz Biotechnology Inc.), anti-human S1P3 antibody (sc-16076, Santa Cruz Biotechnology Inc.). For control, normal mouse IgG (sc-2025, Santa Cruz Biotechnology Inc.) or normal goat IgG (sc-2028, Santa Cruz Biotechnology Inc.). Precipitated samples were subjected to western blotting using a goat polyclonal anti-human S1P1 antibody (sc-16070, Santa Cruz Biotechnology Inc.) and a rabbit polyclonal anti-human RGS5 antibody (ab-83230, Abcam plc.).

Proteomic kinase assays

Proteome Profiler Antibody Arrays were performed using Human Phospho-Kinase Antibody Kit (#ARY003B, R&D Systems Inc., Minneapolis, MN, United States) according to manufacturer's guidance. In brief, 5×10^6 cells were lysed by using 500 μ L lysis buffer and, after centrifugation, 334 μ L of the lysate was diluted by 2 mL blocking buffer and used for the assay. The intensity of each spot was measured by using a free Image J software and its percentage to that of reference spot was plotted in a bar chart.

Gene transfer

A *Homo sapiens* cDNA, FLJ96402, which corresponds to *Homo sapiens* RGS5, transcript variant 1, mRNA (NM_003617.3), with two nucleotide substitutions, was purchased from National Institute of Technology and Evaluation (Tokyo, Japan), and the two substituted nucleotides were corrected by using KOD-Plus-Mutagenesis Kit (Toyobo Co. Ltd., Osaka, Japan) to become identical to the nucleotide sequences in NM_003617.3. The cDNA was inserted into pmax-Cloning™ expression vector (Lonza Group Ltd. Basel, Switzerland). The 2×10^5 human ESC-derived VECs (ESdECs) were transfected with 3 μ g vectors using a Nucleofector™ (Lonza Group Ltd.) as reported elsewhere^[1] and subjected to Western blotting. Alternatively, RGS5 cDNA was inserted into the simian immunodeficiency virus (SIV) vector (ID Pharma Co., Ltd., Ibaraki, Japan) in either forward (SIV-RGS5) or reverse (SIV-control) direction. The 2×10^5 ESdECs were infected with SIV vectors at MOI = 80. After 6 d, during which the cells were subcultured twice, immunostaining studies were performed.

Statistical analysis

Experiments were performed independent three or four experiments ($n = 3$ or 4) and the data were analyzed according to Student *t*-test. Results were shown as averages \pm standard deviations (AV \pm SD).

RESULTS

Proteomic kinase assays using multiple pairs of type-I and type-II VECs

During our trials to identify the downstream signaling target of RGS5, we found by coincidence that there was a clear difference in the phosphorylation state of c-JUN protein between type-I and type-II VECs. c-Jun protein was hyper-phosphorylation at multiples sites such as Ser63, Ser73, Ser243, Thr93 and Thr170 in type-II human VECs including ES(KhES-5)-derived VECs [ESdEC(KhES-5)] at early passages (Figure 1, blue rectangles). By contrast, c-Jun protein was hypo-phosphorylated in type-I VECs including ESdEC (KhES-5) at late passages and commercially available primary cultured human VECs (Figure 1, red rectangles). Therefore, we hypothesized that certain kinases would be working at the downstream of RGS5 for the "type-I vs type-II" phenotyping of human VECs. It is known that c-Jun protein is phosphorylated by multiple kinases including c-Jun N-terminal kinase (JNK), p38 α MAPK, ERK, glycogen synthase kinase 3 (GSK) and casein kinase 2. To identify the pivotal kinase involved in the determination of VEC phenotypes, a systemic analysis to quantitatively evaluate the activation states of various kinases using multiple pairs of type-I and type-II VECs is of great use. For this aim, we applied a commercially available proteomic kinase assay, where cell lysates were reacted with an array of site-specific phosphorylation antibodies of 43 kinases on a nitrocellulose membrane to simultaneously detect the activation state of each kinase *via* an ordinary immunoblotting procedure.

First, we compared the profiles of protein kinase activities between ESdEC(KhES-5) at passage 2 [ESdEC(KhES-5)[P2]], which showed type-II phenotype^[1], and those at passages 10 [ESdEC(KhES-5)[P10]], which showed type-I phenotype^[1]. As shown in Figure 2A, protein kinase activities were generally higher in ESdEC(KhES-5)[P2] (type-II) than type-I ESdEC(KhES-5)[P10] (type-I) in accordance with the result in Figure 1. To narrow the list of candidate kinases involved in type-I/type-II phenotyping, we performed proteomic kinase assays using VECs generated from KhES-3 line of ESC at passage 2 [ESdEC(KhES-3)[P2]], which showed type-II phenotype^[1], and those at passages 8 [ESdEC(KhES-3)[P8]], which showed type-I phenotype^[1]. ESdEC(KhES-3) provided similar results to ESdEC(KhES-5) regarding several kinases including p38 α MAPK, GSK3, AMPK and AKT (Figure 2B, Panel A) and kinase that phosphorylated p53 and HSP60 (Figure 2B, Panel B). To further narrow the list of candidate kinases, we performed proteomic kinase assays

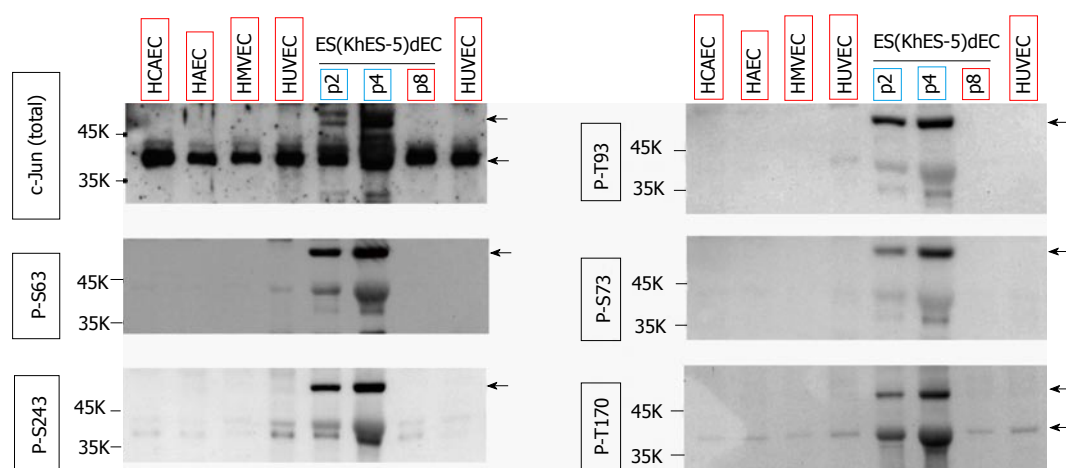


Figure 1 Western blotting assays. HCAEC, HAEC, HMVEC, HUVEC and ESdECs were subjected to western blotting using indicated antibodies. Arrows indicate the specific bands recognized by the antibodies. HCAEC: Human adult coronary arterial endothelial cells; HAEC: Human adult aortic endothelial cells; HMVEC: Human neonatal dermal microvascular endothelial cells; HUVEC: Human umbilical vein endothelial cells; ESdECs: Embryonic stem cells-derived vascular endothelial cells.

using VECs generated from KhES-1 line of ESC at passage 2 [ESdEC(KhES-1)[P2]], which showed type-II phenotype^[1], and those at passages 9 [ESdEC(KhES-1)[P9]], which showed type-I phenotype^[1]. Regarding several cases including p38 α MAPK, GSK3, AMPK and AKT, consistent results were obtained among the three lines of ESdECs (Figure 2C, Panel A). We still performed proteomic kinase assays using SeV-iPS-derived VECs (SeV-iPS(HUVEC)dEC), which showed type-II phenotype^[1], and HUVEC, which showed type-I phenotype^[1]. As shown in Figure 2D, only p38 α MAPK provided consistent results: Higher p38 α MAPK activities in type-II VECs and lower p38 α MAPK activities in type-I VECs. To confirm this finding, we further performed proteomic kinase assays using Ret-iPS-derived VECs [Ret-iPS(253G1)dEC] with type-I phenotype^[1] and SeV-iPS(HUVEC)dEC with type-II phenotype^[1]. Again, p38 α MAPK activities were higher in type-II VECs than type-I (Figure 2E). We finally performed proteomic kinase assays using additional commercially available primary cultured human VECs including HAEC, HCAEC and HMVEC, all of which showed type-I phenotype^[1], and obtained compatible results (data not shown).

Thus, we obtained p38 α MAPK as the strongest candidate for the kinase that is involved in the “type-I vs type-II” phenotyping of human VECs.

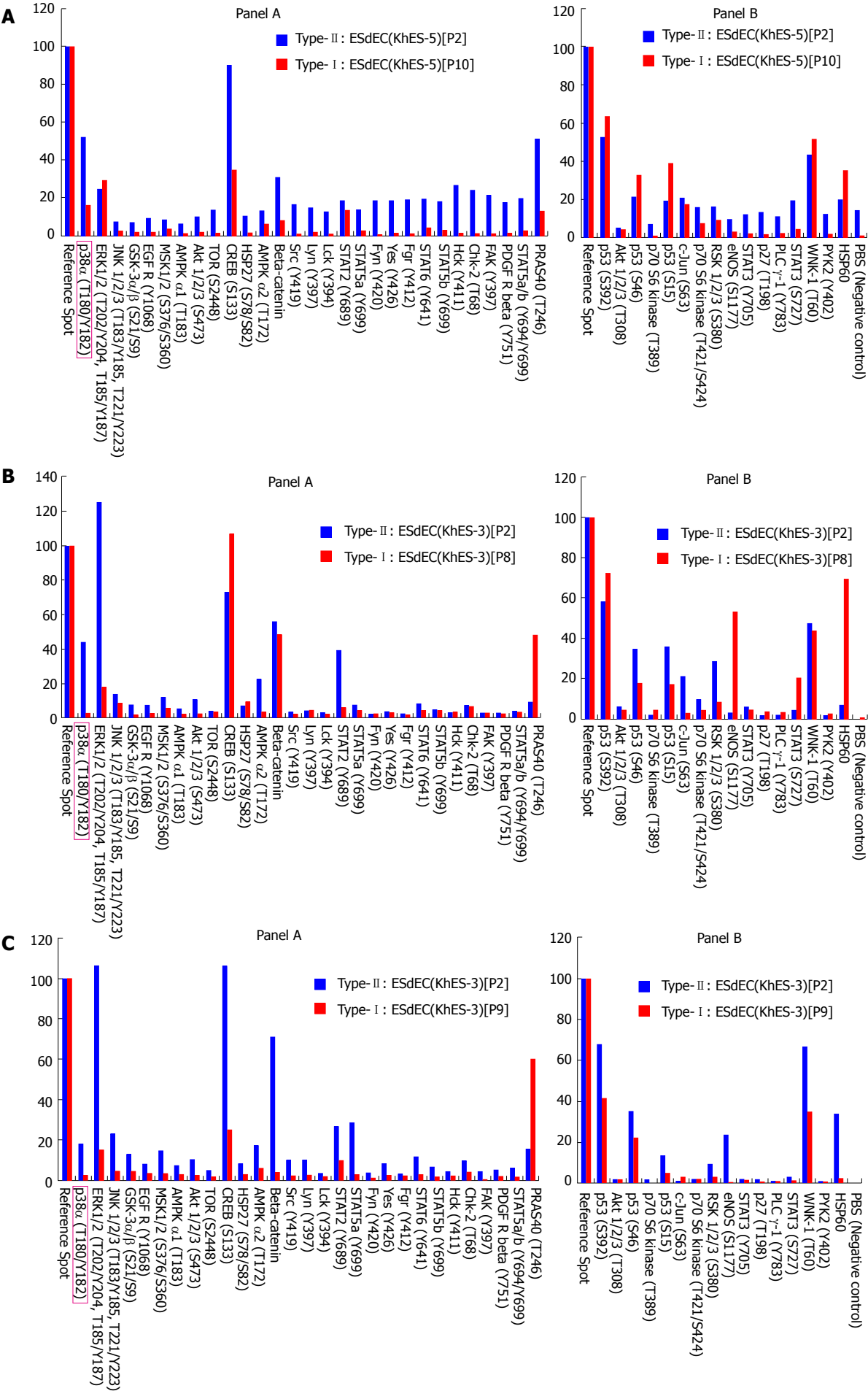
Verification of the involvement of p38 MAPK in phenotype regulation of human VECs

To verify the involvement of p38 α MAPK in RGS5-mediated phenotype regulation of human VECs, we first examined whether an enforced expression of RGS5 would affect p38 α MAPK activities. As shown in Figure 3A, transfection of the RGS5-expression vector into type-II ESdECs lowered Thr180 phosphorylation of p38 α MAPK, and thus, depressing the activity of p38 α MAPK. Next, we examined the effects of the p38 α MAPK inhibitor on the phenotype regulation of VECs. Treatment with a p38 α MAPK inhibitor (Figure 3B,

gray column), but not with a JNK inhibitor (Figure 3B, hatched column) or an ERK inhibitor (data not shown), abrogated type-II *anti-proliferative* capacities of ESdECs (Figure 3B), confirming the involvement of p38 α MAPK in RGS5-mediated “type-II vs type-I” phenotyping. Interestingly, we found that p38 α MAPK inhibitor treatments up-regulated RGS5 expression (Figure 3C). Thus, there is a positive feedback loop between “RGS5 induction” and “p38 α MAPK down-regulation”, running a vicious cycle for the unidirectional conversion from type-II VECs to type-I VECs.

RGS5 disturbed sphingosine-1-phosphate-dependent signaling events

It is known that RGS family proteins function as inhibitory molecules against G protein-coupled receptor (GPCR)-dependent signaling. It is also known that sphingosine-1-phosphate (S1P), whose receptors belong to the GPCR family, activates various kinases including p38 α MAPK, ERK and JNK and plays crucial roles in the regulation of VEC-VSMC interactions *via* N-cadherin^[5]. Therefore, we examined the effects of RGS5 expression on the signaling from S1P to N-cadherin. First, we examined the possible interactions between RGS5 and S1P receptors. As shown Figure 4, RGS5 co-precipitated with S1P receptor 1 (S1P₁) (Figure 4A), but not with S1P₂ or S1P₃ (Figure 4B), in type-I RGS5^{high} VECs. In addition, treatments with FTY720-P, an inhibitor of S1P-dependent signaling, induced “type-II to type-I” conversions (Figure 4C). Thus, RGS5 induced “type-II to type-I” conversion, at least in part, by interfering the S1P/S1P₁-dependent signaling. To further confirm the disturbance of S1P-dependent signaling by RGS5, we examined the effects of RGS5 on the subcellular localization of N-cadherin because S1P₁ receptor activation in VECs is reportedly required for N-cadherin-dependent VEC-VSMC adhesion^[5]. We found that the localization of N-cadherin at intercellular junctions was abrogated by an enforced expression of



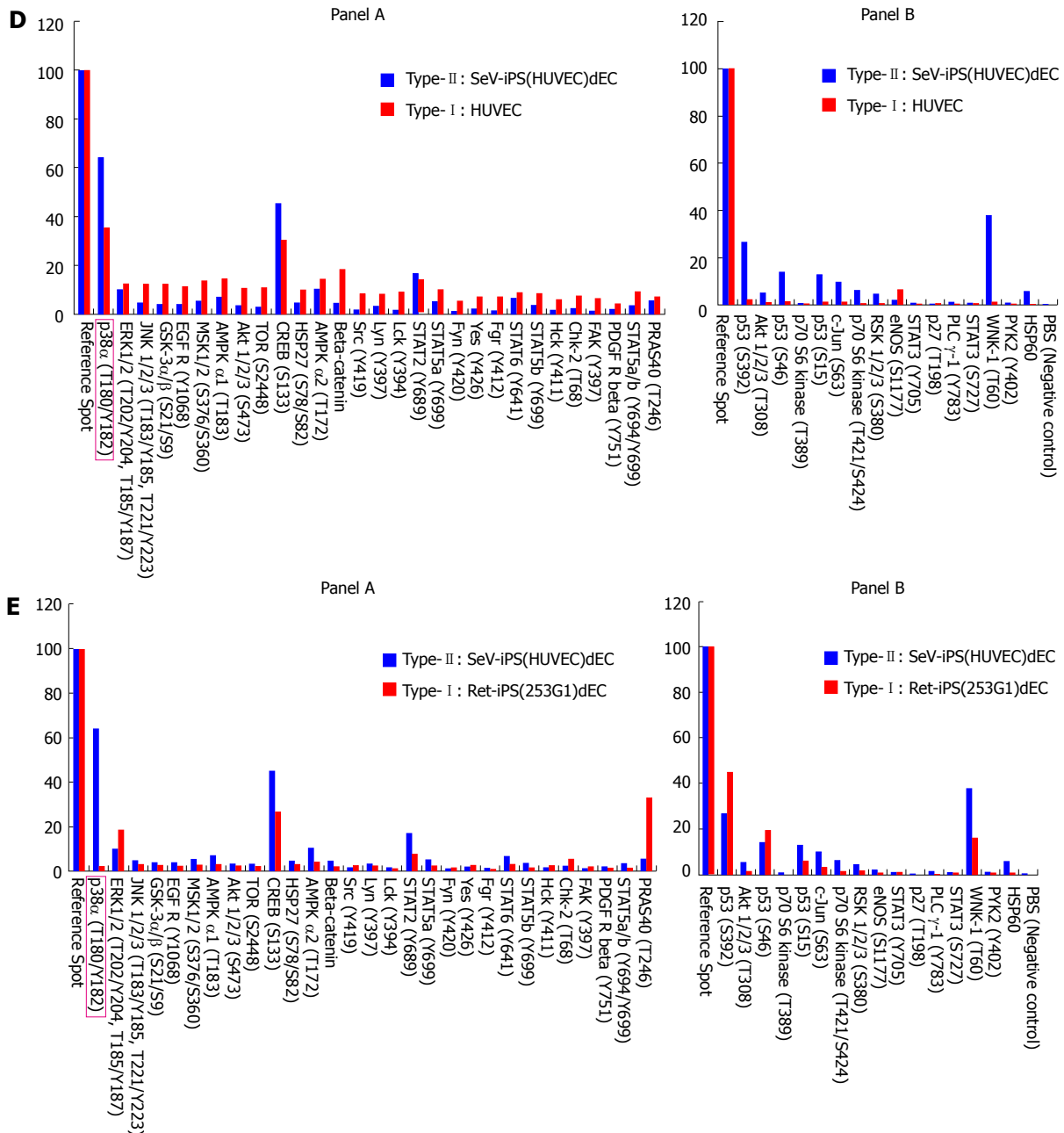


Figure 2 Proteomic kinase assays. Lysates of various pairs of type-I and type-II vascular endothelial cells were prepared as indicated and used for proteomic kinase assays. ESdECs: Embryonic stem cells-derived vascular endothelial cells; iPS: Induced pluripotent stem cells.

RGS5 in type-II VECs (Figure 5A). We also examined the intracellular localization of N-cadherin in various human VECs. In accordance with a previous report^[6], N-cadherin localization at intercellular junctions was hardly detectable in type-I commercially available primary cultured human VECs (Figure 5B). In addition, repetitive subcultures abrogated the localization of N-cadherin at intercellular junctions in type-II ESdECs (Figure 5C). We also examined the effects of an inhibitor of p38 α MAPK, which is the pivotal downstream kinase of RGS5 in the phenotype regulation of human VECs (Figures 2 and 3) and found that p38 α MAPK inhibition abrogated the localization of N-cadherin at intercellular junctions (Figure

5D). Finally, we examined N-cadherin expressions in clinical specimens of the patients with hypertension, arteriosclerosis and systemic lupus erythematoses (SLE)-associated vasculitis. Our previously report showed that RGS5 expressions in VECs were up-regulated in pathological situations in a severity-dependent manner^[1] in accordance with an earlier report on the study of scleroderma patients, which showed endothelial RGS5 overexpression in subcutaneous vessels^[3,4]. Compatible with those previous findings, N-cadherin expressions were abrogated in pathological arteries (Figure 5E). Thus, RGS5 overexpression disturbs S1P/S1P₁-dependent signaling events in human VECs.

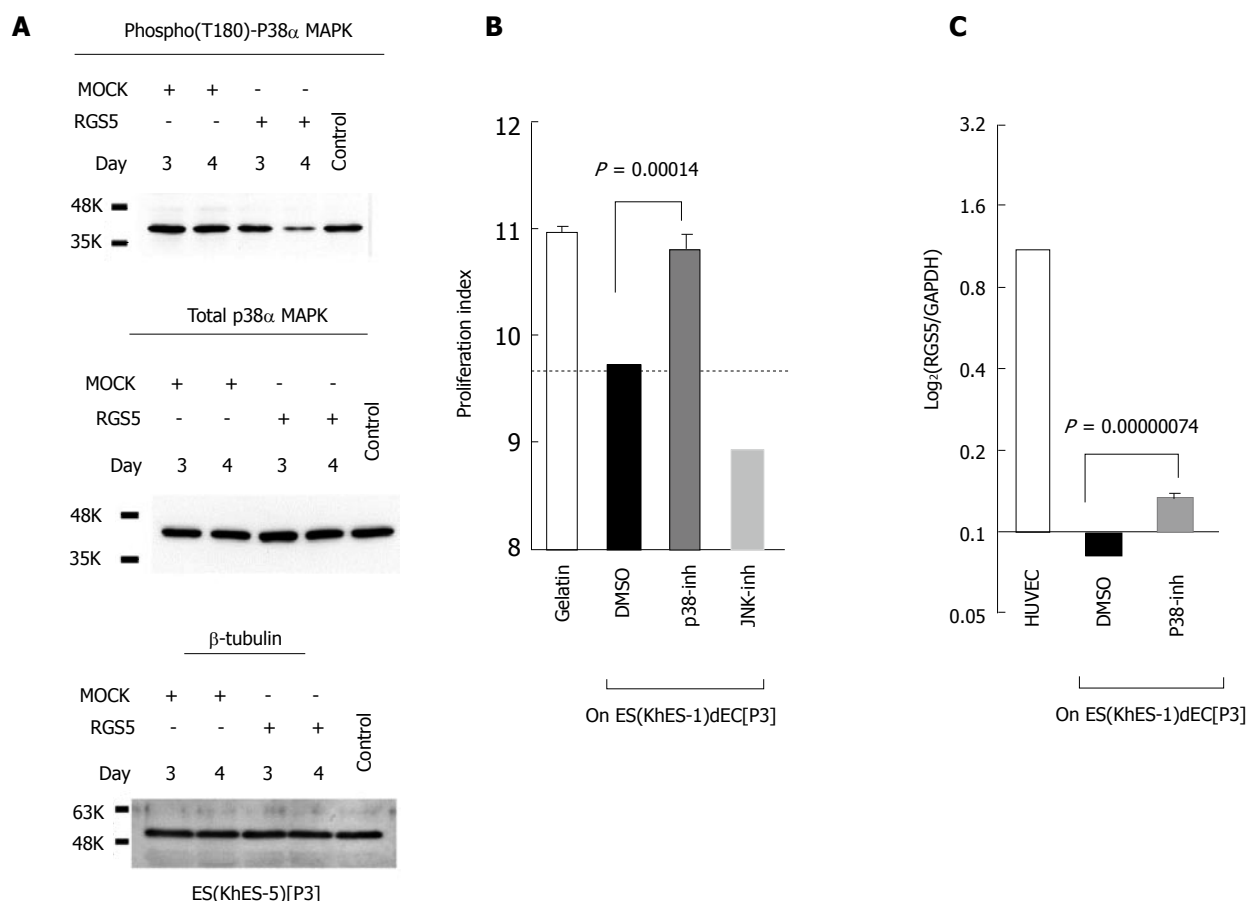


Figure 3 Involvement of p38 mitogen-activated protein kinase in the phenotype regulation of human vascular endothelial cells. A: Type-II ESdECs were transfected with either an RGS5 expression vector or an empty vector by nucleofection and subjected to Western blotting using indicated antibodies after 3 or 4 d; B: Type-II ESdECs were treated with either 0.1% DMSO, a p38 MAPK inhibitor (10 μ mol/L) or a JNK inhibitor (10 μ mol/L) for three days and subjected to co-cultures with human aortic smooth muscle cells ($n = 3$, AV \pm SD); C: Type-II ESdECs were treated by a p38 MAPK inhibitor (10 μ mol/L). Next day, RGS5 expressions were examined by qRT-PCR ($n = 4$, AV \pm SD). RGS5: Regulator of G-protein signaling 5; ESdECs: Embryonic stem cells-derived vascular endothelial cells; DMSO: Dimethylsulfoxide; JNK: Jun N-terminal kinase; MAPK: Mitogen-activated protein kinase; qRT-PCR: Quantitative real-time polymerase chain reaction; HUVEC: Human umbilical vein endothelial cells.

DISCUSSION

In the current study, we identified p38 α MAPK as a pivotal kinase that is involved in phenotype regulations of human VECs at the downstream of RGS5. Therefore, human VECs are finally categorized as follows: Pro-proliferative RGS5^{high} p38 α MAPK^{low} VECs (type-I) and anti-proliferative RGS5^{low} p38 α MAPK^{high} VECs (type-II) (Figure 6).

It is widely accepted that p38 α MAPK plays important roles in the acquisition of stress resistance. It was shown that p38 α MAPK mediated cell survival in response to oxidative stress by inducing antioxidant genes^[7]. The involvement of p38 α MAPK in the acquisition of oncogenic stress resistance was also reported^[8,9]. These findings support the idea that the activity of p38 α MAPK should be maintained at relatively high levels to prevent the "type-II to type-I" conversions by various kinds of stresses including oxidative stress and aging. It was also reported that macrophage deficiency of p38 α MAPK promoted apoptosis and plaque necrosis in advanced atherosclerotic lesions^[8]. Thus, p38 α MAPK plays indispensable roles in the amelioration of ischemic

vascular diseases in both VECs and macrophages.

We also showed that there exists a vicious cycle between "RGS5 inductions" and "p38 α MAPK inhibitions". Because this cycle induces an intensifying expression of RGS5 over time, "type-II to type-I" conversion, which is induced by oxidative stress or aging^[1], is generally an irreversible process. Our previous *in vitro* studies demonstrated that RGS5 expression in type-I VECs cannot be nullified unless they are subjected to iPSC establishment and subsequent VEC differentiation^[1]. On the other hand, the cancellation of RGS5 expression can be observed under *in vivo* conditions. It was reported that, although RGS5 expression was induced in the endothelial cells of subcutaneous vessels in scleroderma patients, it returned to normal after high dose immunosuppressive therapy followed by autologous hematopoietic cell transplant^[10,11]. Thus, it seems that there are a sufficient amount of endothelial progenitor cells (EPCs) that can produce healthy RGS5^{low} VECs (type-II) in the bone marrow of scleroderma patients and these EPCs contribute to the regeneration of healthy vessels after an intensive therapy. In this sense, not only autologous iPSC-derived VECs but also autologous

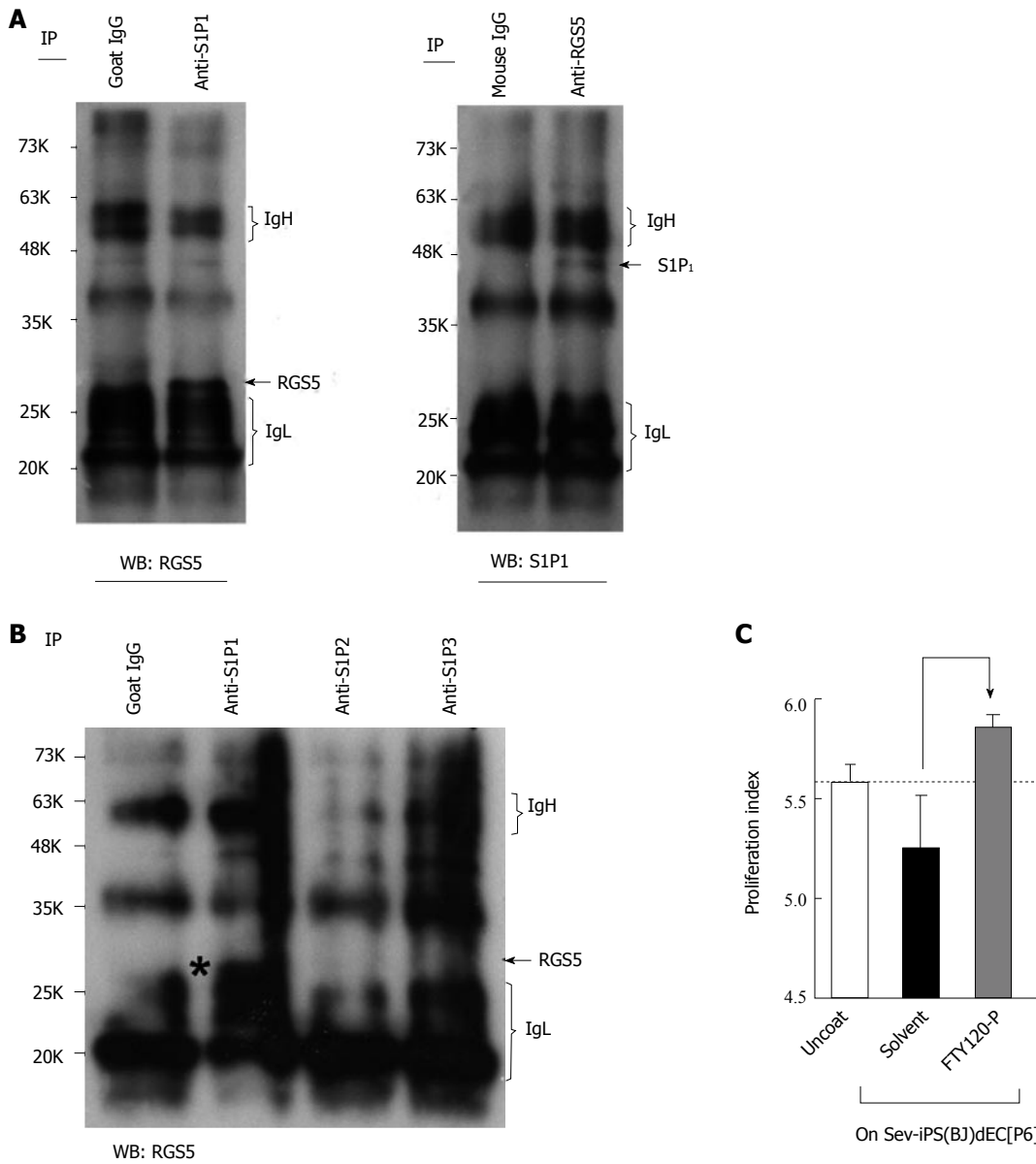


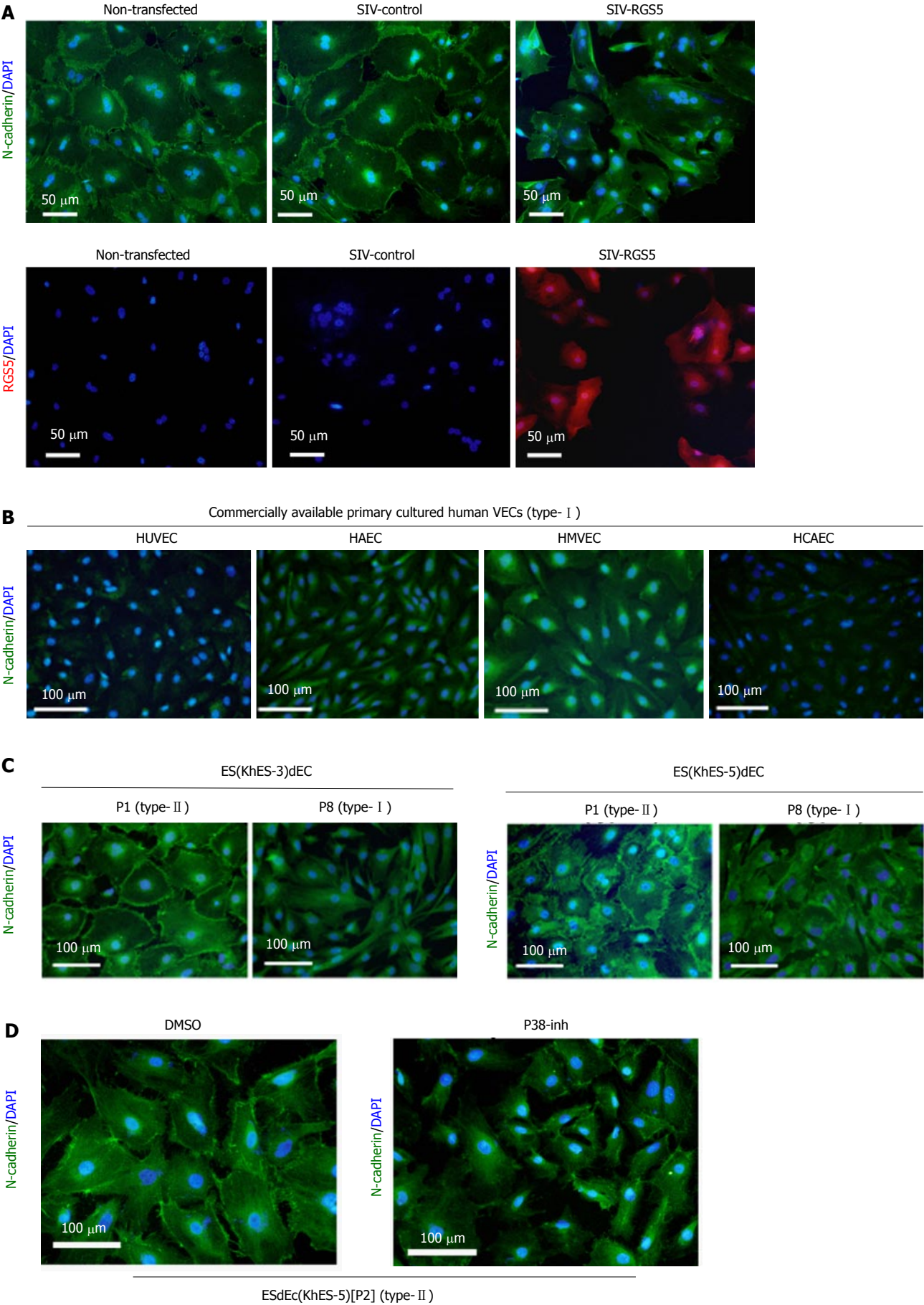
Figure 4 Interaction between sphingosine-1-phosphate receptors and regulator of G-protein signaling 5 in type-I vascular endothelial cells. A and B: Immunoprecipitation (IP) followed by Western blotting (WB) was performed using HUVEC lysates and indicated antibodies; C: Human aortic smooth muscle cells were subjected contact co-culture experiments with type-II VECs with or without 1 nmol/L FTY720-P ($n = 3$, $AV \pm SD$). HUVEC: Human umbilical vein endothelial cells; VECs: Vascular endothelial cells; Sev-iPS: Sendai virus vector-based induced pluripotent stem cells; RGS5: Regulator of G-protein signaling 5.

hematopoietic stem/progenitor cells may provide a powerful tool for the transplantation therapy for the treatment of vascular diseases.

We also demonstrated that RGS5 disturbed S1P-dependent signaling events. It was reported that the S1P₁ activation in VECs was required for N-cadherin-dependent adhesion with mural cells and that knockdown of N-cadherin expression resulted in destabilization of vascular structures^[5]. Our immunostaining studies showed that the localization of N-cadherin at intercellular junctions was abrogated in pathological arteries (Figure 5E). Thus, loss of N-cadherin localization at intercellular junctions may possibly promote the arteriosclerosis *via* the destabilization of VEC-VSMC adhesions. Our results (Figure 5B), in accordance with a previous report^[6] showed that commercially available

primary cultured human VECs lacked the localization of N-cadherin at intercellular junctions. Although the molecular basis for the lack of N-cadherin localization at intercellular junctions has not been elucidated so far, an inappropriately enhanced expression of RGS5 in type-I VECs may possibly be involved in the progression of this process, at least in part.

There may be diverse signals that induce RGS5 expressions. In addition to our previous finding that oxidative stress and subculture stress induced RGS5 expression^[1], Jin *et al.*^[12] reported that RGS5 worked as a hypoxia-inducible apoptotic stimulator in HUVEC. Therefore, it seems highly reasonable to think that RGS5 is a common downstream effector of various stressors in human VECs. Although the direct downstream signaling event remains elusive, RGS5 may provide a



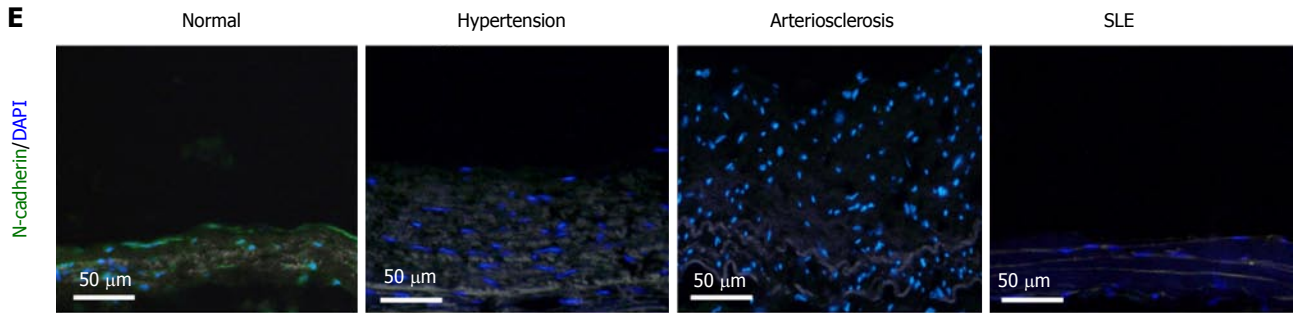


Figure 5 Effects of regulator of G-protein signaling 5 on the intracellular localization of N-cadherin. A: Type-II VECs were infected with SIV vectors carrying either human RGS5 cDNA (SIV-RGS5) or inverted human RGS5 cDNA (SIV-control) and subjected to immunostaining using an anti-N-cadherin antibody (green) or anti-RGS5 antibody (red); B-E: Immunostaining studies using an anti-N-cadherin antibody (green) with nuclear counterstaining with DAPI (blue) were performed using commercially available primary cultured human VECs (type-I) (B), ESdECs at early (type-II) and late (type-I) passages (C), ESdECs at early passages (type-II) treated with 0.1% DMSO or a p38 inhibitor (10 μ mol/L) (D) and clinical specimens (E). HCAEC: Human adult coronary arterial endothelial cells; HAEC: Human adult aortic endothelial cells; HMVEC: Human neonatal dermal microvascular endothelial cells; HUVEC: Human umbilical vein endothelial cells; ESdECs: Embryonic stem cells-derived vascular endothelial cells; RGS5: Regulator of G-protein signaling 5; DMSO: Dimethylsulfoxide; VECs: Vascular endothelial cells.

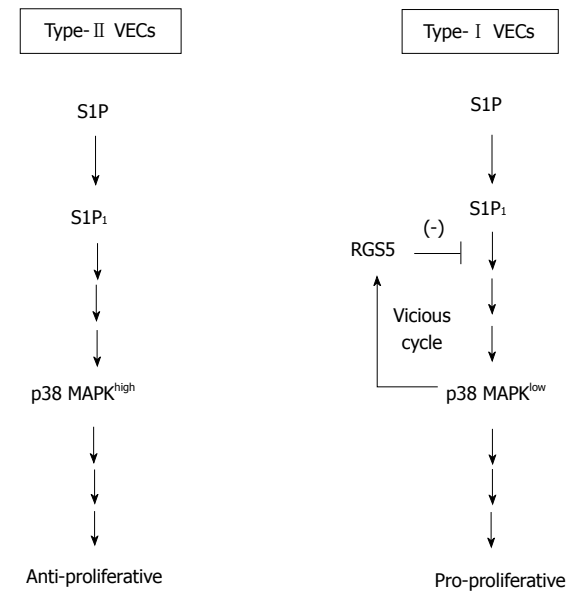


Figure 6 A model for roles of p38 mitogen-activated protein kinase in the regulation of vascular endothelial cells phenotypes. MAPK: Mitogen-activated protein kinase; RGS5: Regulator of G-protein signaling 5; S1P: Sphingosine-1-phosphate; VECs: Vascular endothelial cells.

good candidate for drug discovery in various vascular diseases.

Collectively, the preservation of p38 α MAPK activity at higher levels in VECs provides a new strategy in the drug discovery for the treatment of ischemic diseases.

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COMMENTS

Background

The pathological basis of ischemia is hyper-proliferation of vascular smooth muscle cells (VSMCs). Although there had been a longstanding controversy over the effect of vascular endothelial cells (VECs) on the proliferation of VSMCs, it

was recently clarified that human VECs are categorized into two groups by their effects on the proliferation of VSMCs: Pro-proliferative VECs (type-I) vs anti-proliferative VECs (type-II). Various stresses such as oxidative stress and aging induce "type-II to type-I" conversion of human VECs.

Research frontiers

Regulator of G-protein signaling 5 (RGS5), which is reportedly induced in endothelial cells of pathological vessels, is identified as a causative gene for "type-II to type-I" conversion.

Innovations and breakthroughs

Signaling events that are working up-stream and down-stream of RGS5 in "type-II to type-I" conversion remain elusive. By applying proteomic kinase assays, we have clarified that RGS5-mediated p38 α mitogen-activated protein kinase (p38 α MAPK) suppression is the crucial downstream signaling event for "type-II to type-I" conversion.

Applications

p38 α MAPK activity in type-II VECs even under stressful conditions may provide a useful indicator in drug discovery for ischemic diseases.

Terminology

p38 α MAPK is reportedly required for an acquisition of stress resistance in various cell types.

Peer-review

In this study, the authors have identified that p38 α MAPK is a crucial downstream effector of RGS5 in type I-type II VECs conversion. This finding provides a new strategy in the drug discovery for the treatment of ischemic disease. In general, this is a quite interesting and nice study. Experiments were well designed with appropriate controls and executed. Conclusions are significant and justified based on the high quality data. This manuscript certainly deserves to be published.

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Basic Study

Pro- vs anti-stenotic capacities of type-I vs type-II human induced pluripotent-derived endothelial cells

Miwako Nishio, Masako Nakahara, Koichi Saeki, Katsuhito Fujiu, Hiroshi Iwata, Ichiro Manabe, Akira Yuo, Kumiko Saeki

Miwako Nishio, Masako Nakahara, Akira Yuo, Kumiko Saeki, Department of Disease Control, Research Institute, National Center for Global Health and Medicine, Tokyo 162-8655, Japan

Koichi Saeki, Section of Cell Engineering, Department of Basic Research, DNAVEC Center, ID Pharma Co., Ltd., Ibaraki 300-2611, Japan

Katsuhito Fujiu, Hiroshi Iwata, Ichiro Manabe, Department of Cardiovascular Medicine, Graduate School of Medicine, the University of Tokyo, Tokyo 113-8654, Japan

Katsuhito Fujiu, Translational Systems Biology and Medicine Initiative, the University of Tokyo, Tokyo 113-8654, Japan

Katsuhito Fujiu, Kumiko Saeki, PRESTO, Japan Science and Technology Agency, Saitama 332-0012, Japan

Author contributions: Nishio M and Nakahara M performed the experiments and analyzed the data; Saeki K contributed to the establishment of human iPS cells; Fujiu K, Iwata H, Manabe I and Yuo A coordinated the research; Saeki K designed the research and wrote the paper.

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Correspondence to: Kumiko Saeki, MD, PhD, Division Chief, Department of Disease Control, Research Institute, National Center for Global Health and Medicine, 1-21-1 Toyama Shinjukuiku, Tokyo 162-8655, Japan. saeki@ri.ncgm.go.jp
Telephone: +81-3-32027181
Fax: +81-3-32027364

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Abstract

AIM: To verify *in vivo* relevance of the categorization of human vascular endothelial cells (VECs) into type-I (pro-proliferative) and type-II (anti-proliferative).

METHODS: Endothelial layers of murine femoral arteries were removed by wire injury (WI) operation, a common technique to induce arteriostenosis. Type-I and type-II VECs produced from human induced pluripotent stem cells (iPSCs), whose characters were previously determined by their effects on the proliferation of vascular smooth muscle cells in *in vitro* co-culture

experiments, were mixed with Matrigel® Matrix. The mixtures were injected into subcutaneous spaces around WI-operated femoral arteries for the transplanted human iPSC-derived VECs (iPSdECs) to take a route to the luminal surface *via* vasa vasorum, a nutrient microvessel for larger arteries. Histologies of the femoral arteries were examined over time. The presence of human iPSdECs was checked by immunostaining studies using an antibody that specifically recognizes human VECs. Degrees of stenosis of the femoral arteries were calculated after three weeks. To determine the optimal experimental condition, xenotransplantation experiments were performed under various conditions using immunocompromised mice as well as immunocompetent mice with or without administration of immunosuppressants.

RESULTS: Because immunocompromised mice showed unexpected resistance to WI-induced arteriostenosis, we performed xenotransplantation experiments using immunocompetent mice along with immunosuppressant administrations. After one week, luminal surfaces of the WI-operated arteries were completely covered by human iPSdECs, showing the efficacy of our novel transplantation technique. After three weeks, type-I-iPSdECs-transplanted arteries underwent total stenosis, while type-II-iPSdECs-transplanted arteries remained intact. However, untransplanted arteries of immunosuppressant-treated mice also remained intact by unknown reasons. We found that transplanted human VECs had already been replaced by murine endothelial cells by this time, indicating that a transient existence of human type-II-iPSdECs on arterial luminal surfaces can sufficiently prevent the development of stenosis. Thus, we re-performed xenotransplantation experiments using immunocompetent mice without administering immunosuppressants and found that arteriostenosis was accelerated or prevented by transplantation of type-I or type-II iPSdECs, respectively. Similar results were obtained from the experiments using human embryonic stem cell-derived VECs at early passages (*i.e.*, type-II) and late passages (*i.e.*, type-I).

CONCLUSION: Pro- and anti-stenosis capacities of type-I and type-II human iPSdECs were verified, respectively, promising a therapeutic application of allogenic iPSdECs.

Key words: Vascular endothelial cells; Vasa vasorum; Arteriostenosis; Wire injury; Human induced pluripotent stem cells

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Core tip: We previously reported that human vascular endothelial cells (VECs) were classified into two categories by their *in vitro* effects on the proliferation of vascular smooth muscle cells: Pro-proliferative VECs (type-I) and anti-proliferative VECs (type-II). Applying our new technique to transplant human VECs onto the luminal surface of endothelial layer-removed murine

arteries, the *in vivo* relevance of the concept for VEC categorization was validated. Transplantation of pro-proliferative VECs (type-I) resulted in total stenosis while that of anti-proliferative VECs (type-II) completely blocked the development of arteriostenosis. Thus, prostenosis (type-I) and anti-stenotic (type-II) capacities were verified *in vivo*.

Nishio M, Nakahara M, Saeki K, Fujiu K, Iwata H, Manabe I, Yuo A, Saeki K. Pro- vs anti-stenotic capacities of type-I vs type-II human induced pluripotent-derived endothelial cells. *World J Transl Med* 2015; 4(3): 113-122 Available from: URL: <http://www.wjgnet.com/2220-6132/full/v4/i3/113.htm> DOI: <http://dx.doi.org/10.5528/wjtm.v4.i3.113>

INTRODUCTION

In our previous report, we presented a new concept for the categorization of human vascular endothelial cells (VECs) based on their effects on the proliferation of human vascular smooth muscle cells (VSMCs) in *in vitro* co-culture experiments: Pro-proliferative VECs (type-I) and anti-proliferative VECs (type-II)^[1]. We also showed that commercially available primary cultured human VECs were exclusively pro-proliferative VECs (type-I)^[1] compatible with an earlier report^[2]. Although anti-proliferative (type-II) VECs were producible from commercially available human endothelial progenitor cells depending on donors^[1], they were inevitably converted into "type-I" VECs after repetitive subcultures^[1]. Human embryonic stem cells (ESCs) stably produced "type-II" VECs; however, ESC-derived type-II VECs were inevitably converted into type-I after repetitive subcultures^[1]. Molecular analyses identified that the causative gene for "type-II to type-I" conversion was Regulator of G-protein signaling 5 (RGS5)^[1], which is reportedly expressed in the endothelia of pathological vessels but not those of normal vessels^[1,3,4]. Besides repetitive subcultures, oxidative stresses also induced "type-II to type-I" conversion^[1]. Although anti-oxidant treatments lowered the degree of RGS5 induction and delayed the time of "type-II to type-I" conversion, they could not completely block RGS5 induction or "type-II to type-I" conversion^[1]. Thus, the problem of obtaining sufficient amounts of type-II VECs remained unsolved.

This problem was resolved by utilization of exogenous gene-free human induced pluripotent stem cells (iPSCs). Although type-II VECs were producible from conventional retrovirus vector-based exogenous gene-containing human iPSCs (Ret-iPSCs) depending on lines, they were inevitably converted into type-I VECs after repetitive subcultures^[1]. By contrast, type-II VECs generated from recently established Sendai virus vector-based human iPSCs (SeV-iPSCs)^[5] showed particularly high resistance to "type-II to type-I" conversion^[1]. This may be explained by an empirical rule that "the older the establishment time of ESC/iPSC is, the more prone

to phenotype conversion the ESC/iPSC-derived VECs are" due to larger cumulative stresses. Moreover, SeV-iPSCs get rid of genome stress attributed to vector insertions into chromosomes. Indeed, Ret-iPSCs often expressed RGS5 message in immature states and RGS5 expression levels were further augmented after VEC differentiation. Thus, not only the passage numbers of iPS-derived VECs but also the quality of iPSCs (inc. the time and the method of establishments) are crucial determinants of the stability of type-II characters of iPSC-derived VECs (iPSdECs).

In the current study, we validated *in vivo* relevance of the new concept for the categorization of human VECs by transplantation experiments using iPSdECs: Ret-iPSCdEC (type-I) and SeV-iPSCs-derived VECs (SeV-iPSCdEC) (type-II). For this aim, we established a new technique to transplant human VECs onto luminal surfaces of murine arteries utilizing a route *via vasa vasorum*. By applying this unique technique, we verified the *pro-stenosis* and *anti-stenosis* capacities of type-I and type-II human VECs, respectively. We also show that xenotransplantation of human VECs to murine arteries was effectively performed even under immunocompetent conditions because transplanted VECs have exerted their full effects within a short period (< 1 wk) before rejected by immune systems. Our studies not only prove the *in vivo* relevance of our new concept for the categorization of human VECs but also suggest the possible application of *allogenic* human iPS-derived VECs to therapeutic purposes.

MATERIALS AND METHODS

Cells

The hESC line (KhES-5) was generously provided by the Institute for Frontier Medical Science, Kyoto University^[5]. A SeV-hiPSC line [SeV(BJ)-hiPSC] were established by using iPS-Tune™ (DनावेC Corp., Ibaraki, Japan) from BJ fibroblast^[6]. A Ret-hiPSC line (#25) was established from human fetus lung cells (MRC-5) by infecting recombinant retroviruses expressing the four factors (Oct3/4, Sox2, Klf4 and c-Myc) at Department of Reproductive Biology, Center for Regenerative Medicine, National Research Institute for Child Health and Development as used elsewhere^[7,8]. 253G1^[9] was provided by CiRA at Kyoto University and used elsewhere^[7,8,10]. The type-I and type-II VECs were generated from Ret-hiPSC and SeV-hiPSCs, respectively^[1].

Immunostaining

The 1st antibody reactions were performed by using a 1:50-diluted rabbit polyclonal anti-human-specific PECAM antibody (sc-8306, Santa Cruz Biotechnology Inc.)^[11] or a 1:420-diluted anti-smooth muscle actin antibody (A5228, Santa Cruz Biotechnology Inc.) and the 2nd antibody reactions were performed by using an Alexa Fluor® 488-conjugated goat anti-rabbit IgG (A11008, Life Technologies, Inc.) or an Alexa Fluor® 488-conjugated goat anti-mouse IgG (A11029, Life

Technologies, Inc.), respectively. Photomicrographs were taken by either Olympus FluoView™ FV1000 Confocal Microscope (Olympus Optical Co. Ltd., Tokyo, Japan) or Olympus BX51 Fluorescence Phase contrast Microscope (Olympus Optical Co. Ltd.) equipped with DP-2 TWAIN digital camera system (Olympus Optical Co. Ltd.) and cellSens® standard imaging software (Olympus Optical Co. Ltd.).

Wire injury operation

The operation was performed as described elsewhere^[12]. Nine-week-old ICR or NOD/SCID mice were anesthetized by intraperitoneally administering the mixture of hydrochloric acid medetomidine (0.3 mg/kg), midazolam (4 mg/kg) and butorphanol tartrate (5 mg/kg) using 26 gauge needles. After depilation and disinfection, a 2 cm-long skin incision was made at the inguinal area to expose the femoral artery. After ligating the profunda femoris artery, a small incision was made on its proximal site, from which a 0.014-inch diameter guidewire (Cook Medical Inc., Bloomington, IN, United States) was inserted until it reached the bottom of the aorta descendens. Then, the guidewire was moved back and forth 10 times and rotated 5 times to evenly and completely exfoliate the endothelial layer. Finally, guidewire was removed and the profunda femoris artery was ligated at its most proximal site. After one week to three weeks from the operation, mice were anesthetized by intraperitoneally administering the mixture of hydrochloric acid medetomidine (0.3 mg/kg), midazolam (4 mg/kg) and butorphanol tartrate (5 mg/kg) using 26 gauge needles and then subjected to perfusion fixation by administering 50 mL of PBS and subsequently 100 µL of 4% paraformaldehyde solution *via* the left ventricle. Specimens of femoral artery were prepared and embedded into paraffin blocks for histological analyses.

Pervasa vasorum transplantation

An anti-asialo-GM1 antibody (300 µg) (WAKO Pure Chemical Industries, Osaka, Japan) was injected into the tail vein of 9-wk-old ICR or NOD/SCID mice, which were subsequently subjected to WI operations. On the other hand, iPSdECs/Matrigel® mixture were prepared just before the end of the WI operation as follows: 1 × 10⁶ iPSdECs were centrifuged and kept in ice-cold sterile 1.5 mL tube, to which 20 µL of ice-cold Matrigel® Matrix was promptly added and mixed. The iPSdECs/Matrigel® mixture was injected into the subcutaneous space around the outer membrane of the femoral artery by using 200 µL pipet tip. An anti-asialo-GM1 antibody (300 µg) was injected into tail vein twice a week to block NK-mediated rejection in some experiments.

All procedures involving animals, including WI operations and pervasa vasorum transplantation (PVVT), were reviewed and approved by the Institutional Animal Care and Use Committee of National Center for Global Health and Medicine, Tokyo, Japan (Authorization No. 15014). The animals were acclimatized to laboratory

conditions ($23^{\circ}\text{C} \pm 3^{\circ}\text{C}$, 12 h/12 h light/dark, $55\% \pm 15\%$ humidity, ad libitum access to food and water) prior to experimentation. The animal protocol was designed to minimize pain or discomfort to the animals by using mixed anesthetics as described above.

Statistical analysis

Experiments were performed independent three experiments using three mice ($n = 3$) and the data were analyzed using student *t* test. Results were shown as averages \pm standard deviations (AV \pm SD).

RESULTS

A new method to transplant human VECs on luminal surfaces of murine arteries.

The luminal surfaces of the arteries are continuously exposed to high-pressured and pulsatile blood flows, and thus, it is difficult for the transplanted human VECs to safely land and stably reside on the luminal surfaces if they are intravenously or intra-arterially injected into the blood stream. Thus, as alternative approach, we applied a strategy to take a route *via* vasa vasorum, which is a nutrient vessel that supply or drain the walls of the larger arteries and veins. We hypothesized that the vasa vasorum would effectively guide the transplanted human VECs at least to the middle portion of tunica media and the human VECs would then migrate to the luminal surface, which is the natural site for them to reside.

For an effective replacement of the endothelial cells of the murine femoral artery by the transplanted human VECs, the host endothelial layer was mechanically removed by wire injury (WI) operation, which is a very common technique to induce experimental arteriosclerosis. Then, human iPSCs were transplanted by our unique technique termed "PVVT", where human iPSCs were mixed with Matrigel® Matrix and the mixtures were simply injected into subcutaneous spaces around WI-operated femoral arteries (Figure 1). First, we performed experiments using immunodeficient NOD/SCID mice, which lack functional lymphocytes and permit the engraftment of a wide range of human cells. However, these mice did not develop arteriosclerosis even after WI operations by unknown reasons (data not shown). Similar results were obtained from nude mice, which suffer from deficient T cell function (data not shown). Therefore, we performed experiments using immunocompetent ICR mice along with an administration of anti- α GM1 monoclonal antibody (α AGM1)^[13] to prevent immunorejections by natural killer (NK) cells. The reason why we used α AGM1 is as follows. It is known that endothelial cells of the venous graft are replaced by arterial endothelial cells within several days from the coronary artery bypass surgery. Thus, an inhibition of the long-term immunorejections by T/B cells, which are usually induced a couple of weeks later, is not required for our purpose. On the other hand, short-term immunoreactions by NK cells,

which are usually induced after several days from transplantation, might possibly affect the efficiency of the transplantations. Therefore, we performed xenotransplantation under the condition where NK cell activities were blocked by an administration of α AGM1.

After one week from the transplantation, WI-operated arteries underwent entire loss of endothelial layers with fibrin clot-like substances on the luminal surfaces of the arteries of non-transplanted mice (Figure 2A and B, open arrows). By contrast, the luminal surfaces of the arteries of human iPSC-transplanted mice were thoroughly covered by the endothelial cells (Figure 2B, closed arrows). Immunostaining studies using an antibody that specifically recognizes human PECAM1 but not murine PECAM1^[11] confirmed that the endothelial cells that covered the luminal surfaces of the arteries were indeed human VECs (Figure 2C and D).

Thus, our new PVVT technique guaranteed the effective transplantation of human VECs onto the luminal surfaces of murine arteries.

Type-I and type-II iPSCs exacerbated and prevented the development of arteriosclerosis, respectively

Next, we analyzed the histologies of the arteries after three weeks from the transplantation. We found that type-I iPSC-transplanted arteries underwent almost complete stenosis (Figure 3A, lower left). By contrast, "type-II" iPSC-transplanted arteries showed intact morphologies (Figure 3A, lower right). For quantitative evaluations, we calculated the ratio of the square measure of the lumen and that of the area surrounded by external elastic membranes in cross sections as an indicative of stenosis. We found that transplantation of "type-I" iPSCs exacerbated the development of arteriosclerosis (Figure 3B). Histological analyses (Figure 3C, left panels) and immunostaining studies using anti-smooth muscle actin (Figure 3C, right panels) confirmed the presence of hyper-proliferated VSMCs within the neointima. Unexpectedly, AGM1 administration significantly inhibited the development of arteriosclerosis (Figure 3A, upper right), and thus, we could not detect significant differences between "WI + α AGM1 + transplantation (-)" and "WI + α AGM1 + iPSC (type-II)" groups (Figure 3B). Therefore, although we could successfully demonstrate the pro-stenosis capacity of type-II iPSC, we failed in showing the anti-stenosis activity of type-II iPSC in these experiments. We hypothesized that immunosuppressive states inhibited the development of arteriosclerosis as observed in the case of immunodeficient NOD/SCID mice and nude mice (data not shown).

When we examined the presence of human iPSCs after 3 wk from transplantation, we noticed that the transplanted human VECs already became undetectable on the arterial luminal surfaces (Figure 3D), indicating that transplanted iPSCs had already been replaced by host's endothelial cells by this time as expected from the finding obtained from bypass surgeries. Our observation at the same time indicates that the time

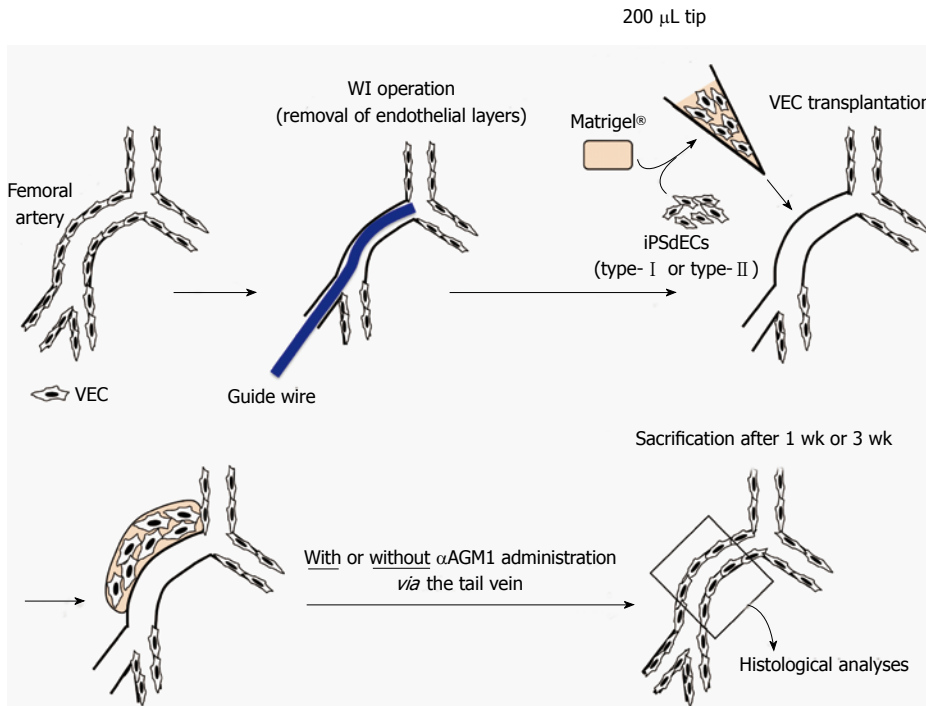
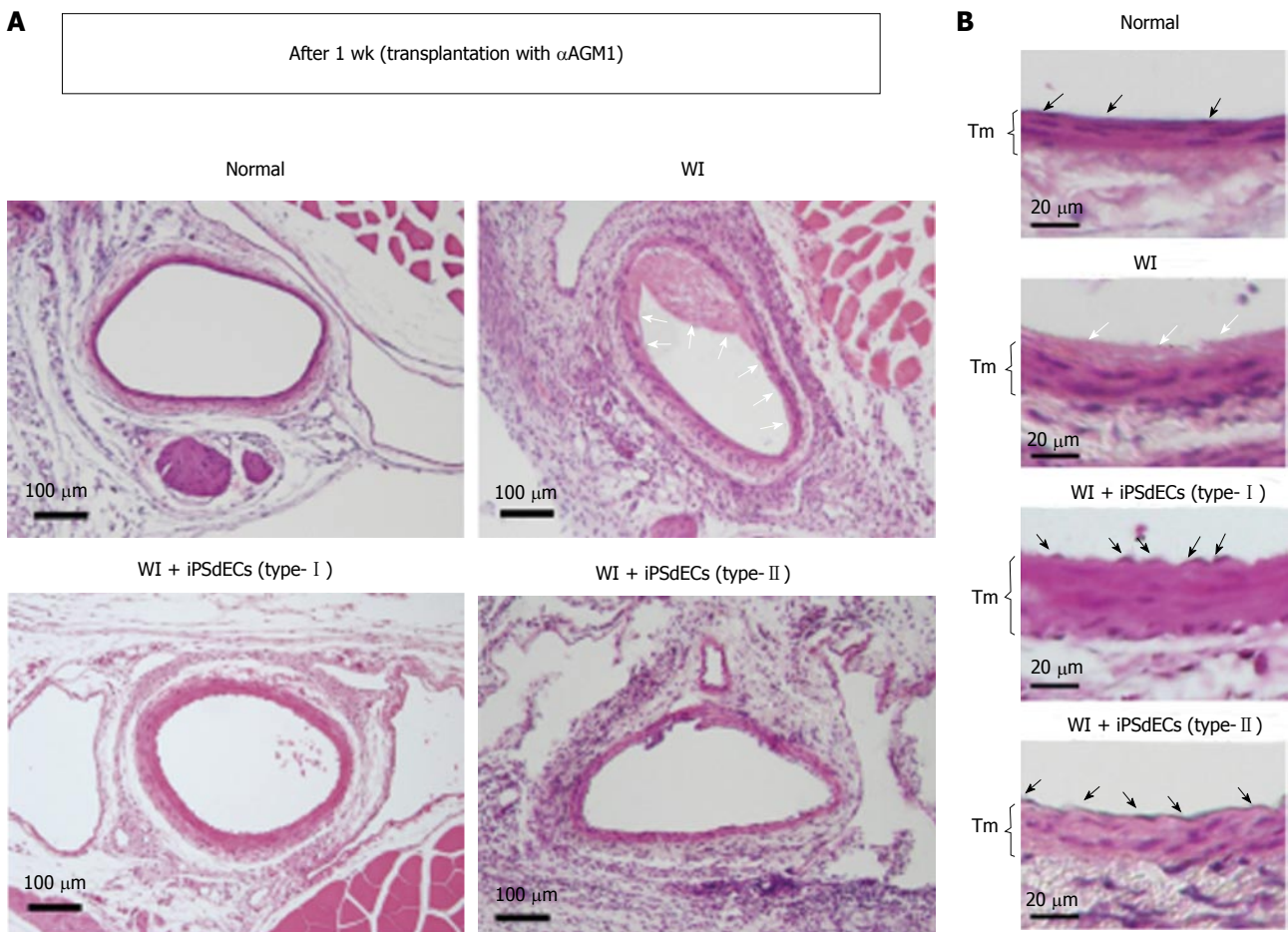


Figure 1 The procedure of pervasa vasorum transplantation. Human iPSdECs were mixed with Matrigel® Matrix and the mixtures were put into the subcutaneous regions around the WI-injured femoral arteries. WI: Wire injury; iPSC: Induced pluripotent stem cell; VEC: Vascular endothelial cell; iPSdECs: iPSC-derived VECs; α AGM1: Anti-asialo GM1 monoclonal antibody.



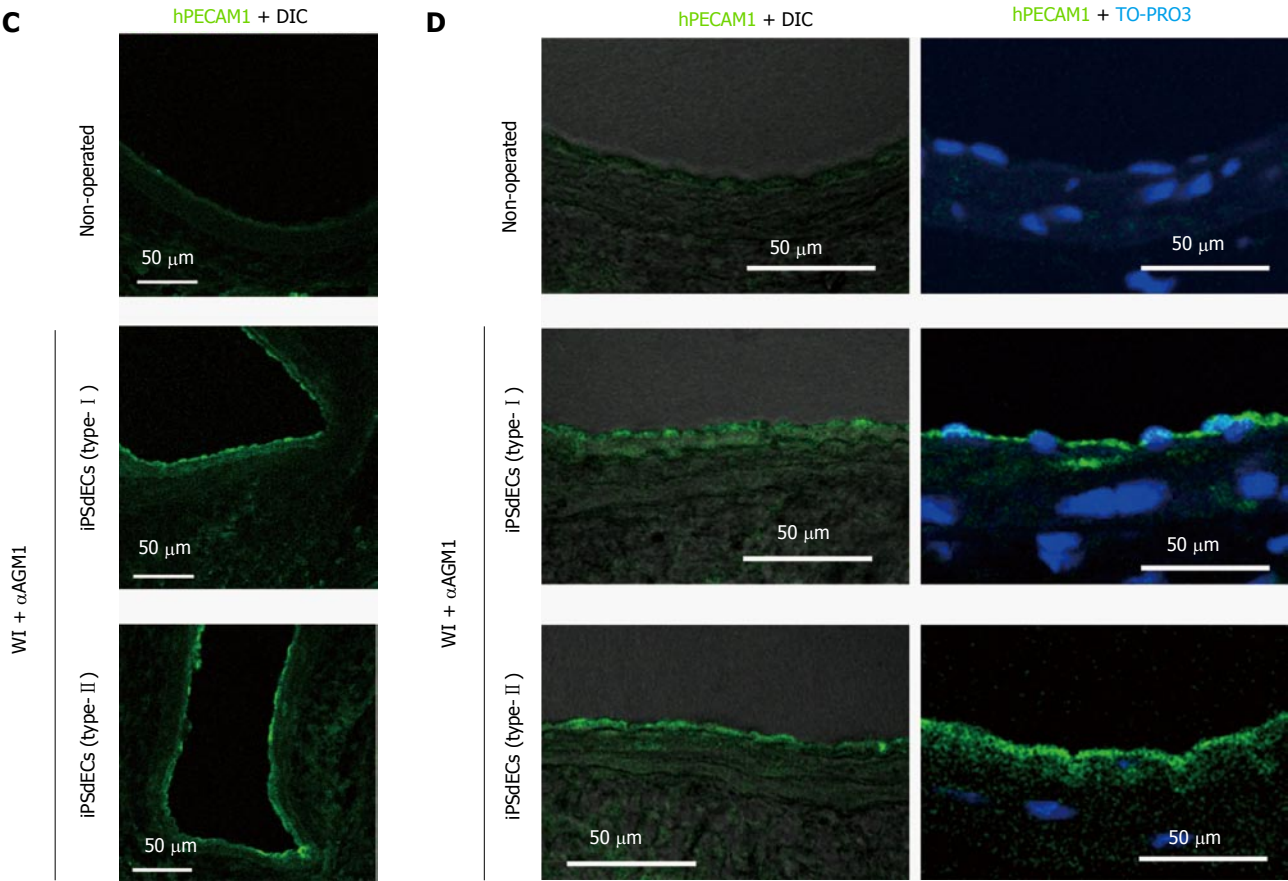
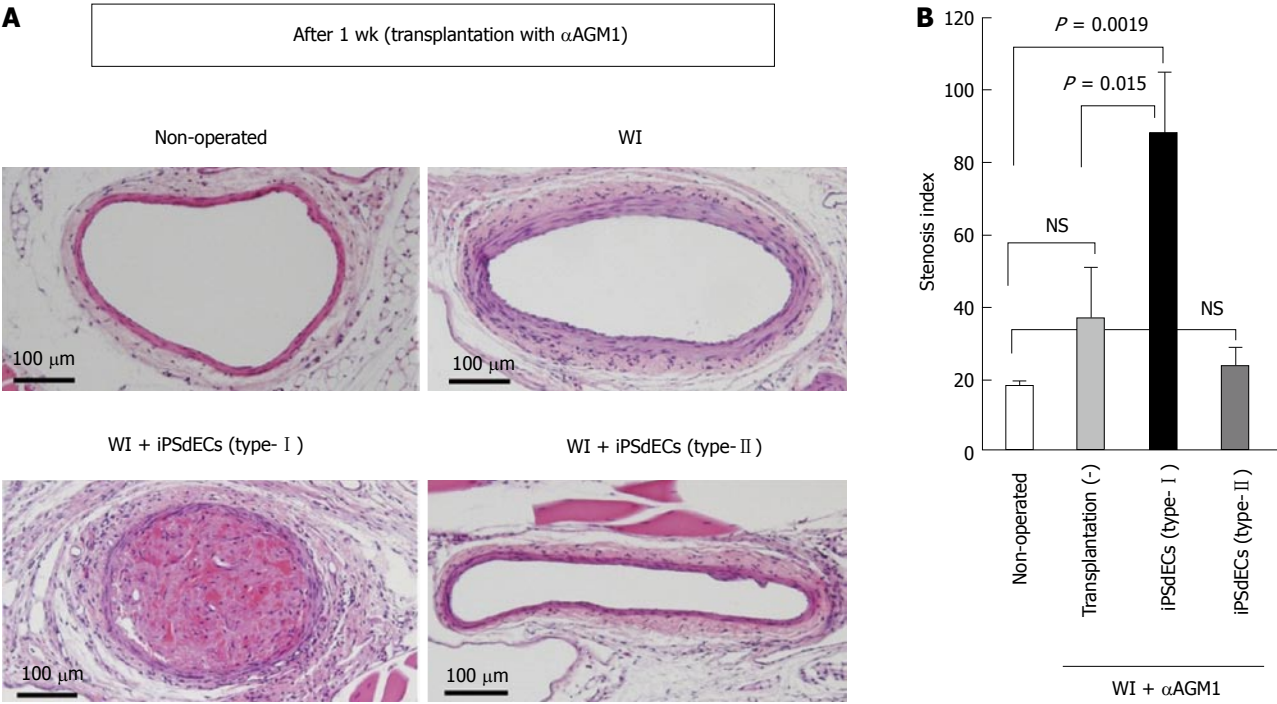


Figure 2 Histological analyses after one week. A and B: WI-operated femoral arteries that were transplanted with type-I or type-II iPScECs were examined after one week from PVVT in mice regularly administrated with asialo GM1 antibody αAGM1). Open arrows indicate fibrin deposits, closed arrows indicate nuclei of endothelial cells and Tm indicate tunica media; C and D: Con-focal microscopies of immunostained samples using anti-human PECAM1 antibody with differential interference contrast (DIC) (C) or nuclear counterstaining by TO-PRO3 (D). WI: Wire injury; iPSC: Induced pluripotent stem cell; VEC: Vascular endothelial cell; iPScECs: iPSC-derived VECs; αAGM1: Anti-asialo GM1 monoclonal antibody.



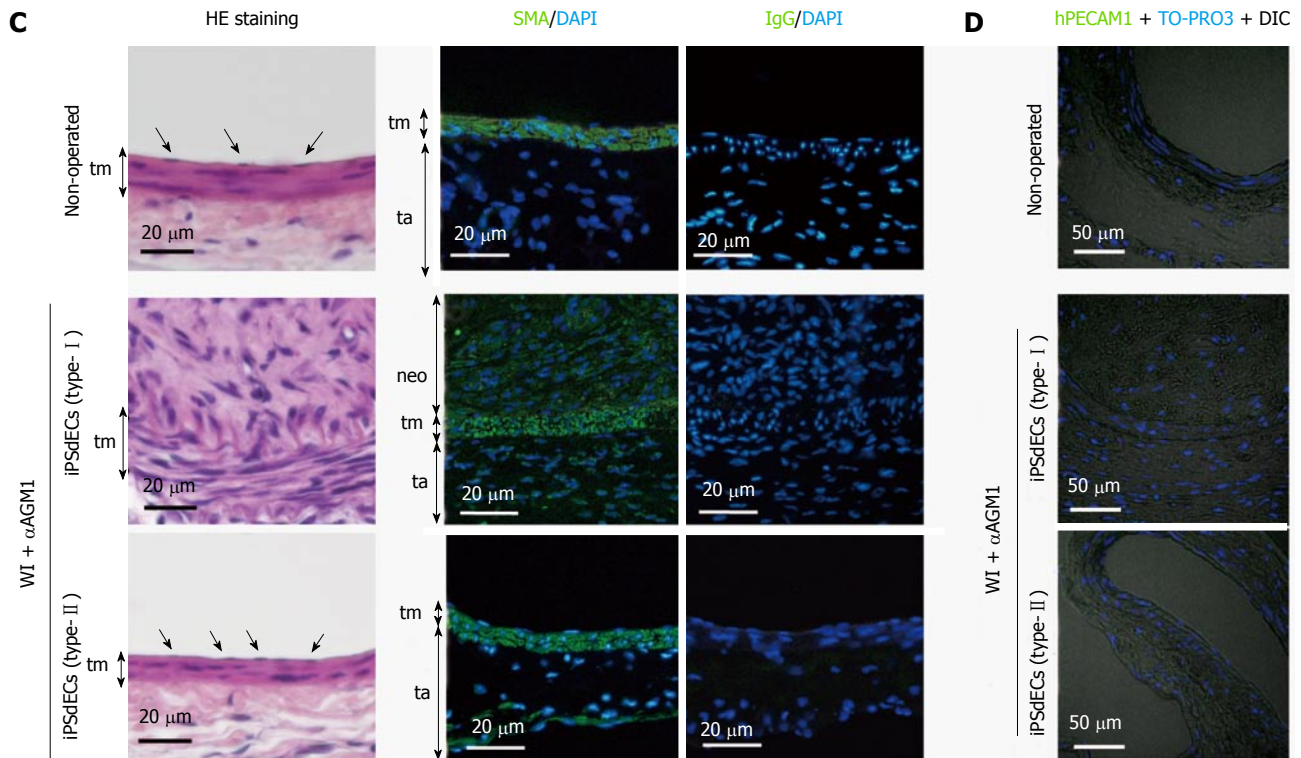


Figure 3 Histological analyses after three weeks. Histologies of WI-operated femoral arteries after three weeks from PVVT in mice regularly administrated with α AGM1 were examined. A: Photographs of HE-stained samples at low magnification; B: Stenosis indexes were calculated and statistically analyzed by student-t test. Data were presented as average (Av) \pm standard deviation (SD). Experiments were performed using three mice for each condition ($n = 3$); C and D: High magnification photographs of the samples with HE staining (C, left), immunostaining using anti-smooth muscle actin (SMA) antibody with nuclear counterstaining by DAPI (C, right) and immunostaining using anti-hPECAM1 antibody with DIC and nuclear counterstaining by DAPI (D). WI: Wire injury; iPSC: Induced pluripotent stem cell; VEC: Vascular endothelial cell; iPSdECs: iPSC-derived VECs; α AGM1: Anti-asialo GM1 monoclonal antibody.

period required for the transplanted iPSdECs to exert their full effects was rather short (about one week). Thus, we re-performed the transplantation experiments without administrating α AGM1. We confirmed that arteriostenosis was exacerbated by type-I iPSCdECs transplantation under these conditions (Figure 4A, lower left, and 4B, lower left). By contrast, the development of arteriostenosis was completely prevented in type-II iPSdECs-transplanted arteries (Figure 4A, lower right, and 4B lower right). Without an administration of α AGM1, WI-operated arteries underwent arteriostenosis (Figure 4A, upper right, and 4B upper right). Thus, *anti-stenosis* capacities of type-II iPSdECs, as well as the *pro-stenosis* capacities of type-I iPSdECs, were verified (Figure 4C). We also examined the effects of transplantation with human ESC-derived VECs (ESdECs) at early passages (type-II) and at late passages (type-I)^[1]. Although ESdECs at early passages (type-II) underwent "type-II to type-I conversion" after a few rounds of subcultures *in vitro* and thus their type-II characters were not as solid as type-II SeV-iPSdECs, we could detect clear differences in the results of transplantations between ESdECs at early passages (type-I) and those at early passages (type-II) (Figure 4D and E).

Collectively, the *in vivo* relevance of the concept for the categorization of human VECs, type-I (pro-

proliferative) and type-II (anti-proliferative), were verified *in vivo*.

DISCUSSION

In the current study, we verified the *in vivo* relevance of the new concept for the categorization of human VECs by showing that *pro-proliferative* VECs (type-I) exacerbated the stenosis of injured arteries whereas *anti-proliferative* VECs (type-II) prevented the development of arteriostenosis. Our finding highlights an unexpected importance of the endothelial cells for the maintenance of vascular structures, demonstrating that VECs serve as not only the simple cover for the luminal surface but also a crucial regulator of the proliferation of VSMCs. Therefore, an approach to the preservation of type-II VEC phenotypes provides a new strategy for the treatment of arteriostenosis. Although the major cause of arteriostenosis is currently hypercholesterolemia-based atherosclerosis and anticholesteremic agents has been exerting high therapeutic effects, new VEC-targeted drug discoveries may further contribute to the control of ischemic diseases especially in the cases of restenosis after stent therapies or resistance to cholesterol medications.

We found by chance that arteriostenosis is strongly inhibited under immunosuppressive conditions. This

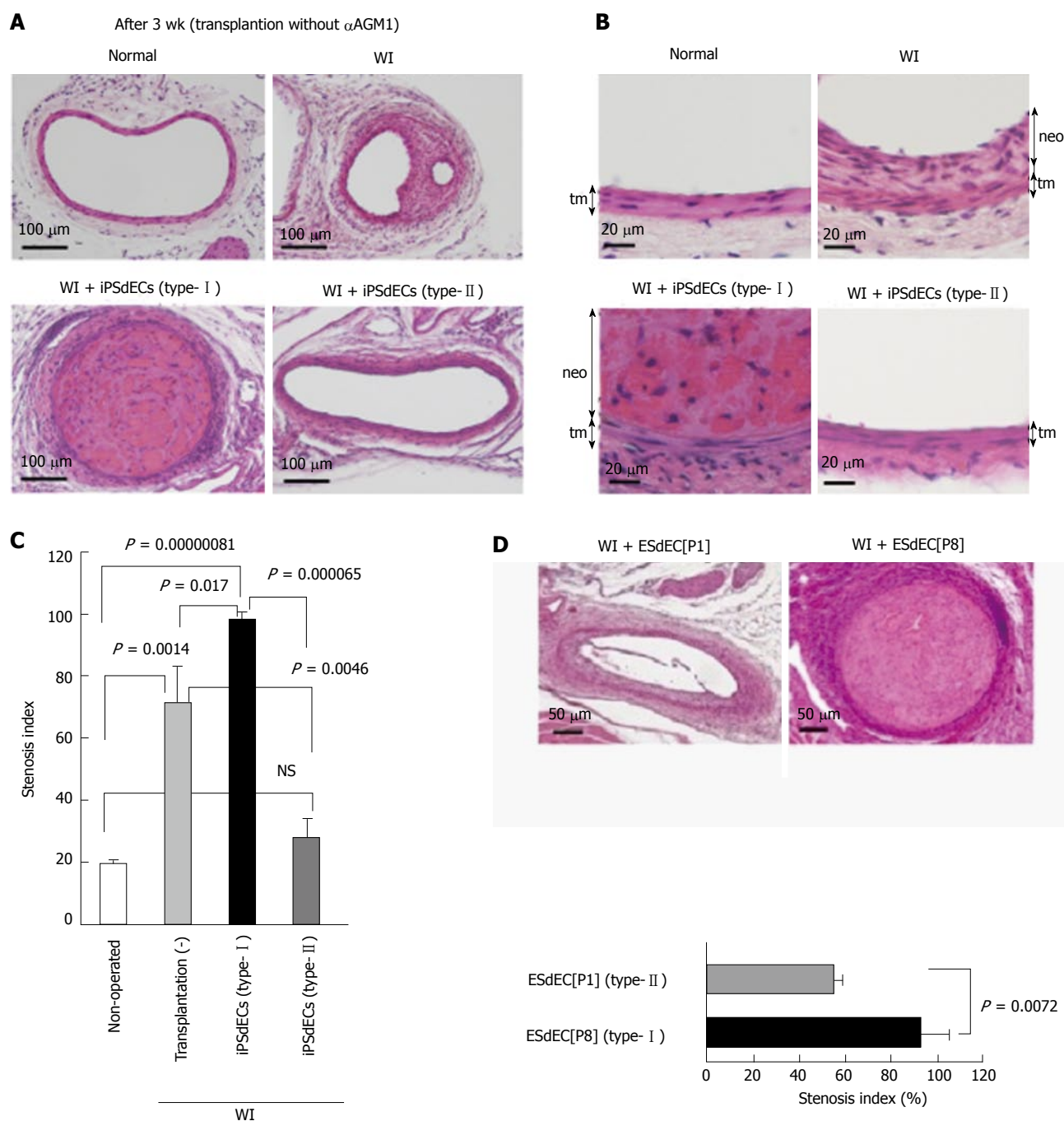


Figure 4 Transplantation without immunosuppression. A-C: WI-operated femoral arteries transplanted with type-I or type-II iPSCs were examined after 3 wk from PVVT in mice without α AGM1 administration. Photographs of HE-stained samples at high magnification, those at low magnification and calculated stenosis indexes ($Av \pm SD$, $n = 3$) (C) were shown; D and E: HE staining (D) and calculated stenosis indexes ($n = 3$) (E) of WI-operated femoral arteries after 3 wk from ESdECs transplantation without α AGM1 administration. WI: Wire injury; iPSC: Induced pluripotent stem cell; VEC: Vascular endothelial cell; iPSCs: iPSC-derived VECs; α AGM1: Anti-asialo GM1 monoclonal antibody; ESdECs: ESC-derived VECs.

finding may explain, at least in part, the high effectiveness of immunosuppressive agents used in the drug-eluting stent. The reason why immunosuppressive states prevent the development of arteriostenosis remains elusive. In WI-injured arteries of immunodeficient NOD/SCID mice, cellular compartments of tunica media were lost by unknown reasons (data not shown). Because a similar phenomenon was observed in nude mice (data not shown), a certain component of T cells

might possibly be involved in the regulation of this bizarre phenomenon. We are currently studying which components of T cells are involved in this phenomenon.

In the current study, we also established a new transplantation technique termed PVVT, which guarantees the effective transplantation of human VECs onto the luminal surface of murine arteries. PVVT is a very simple technique, which can be performed by putting human VEC-embedded gels into subcutaneous spaces

adjacent to arterial walls through a small skin incision. It can even be carried out as an ambulatory treatment if it is clinically applied in the future. Although we cannot completely exclude the possibility that there are still other paths than *vasa vasorum*, our new transplantation technique provides the easiest and safest way to transplant VECs on the luminal surfaces of injured arteries. Another merit of our PVVT technique is that it can be performed even under immunocompetent conditions, which indicates that *allogenic* iPSCs are used as effectively as *autologous* iPSCs when clinically applied. Moreover, a short-term requirement of iPSCs makes the clinical application of human iPSCs much safer. In the case of *allogenic* iPSCs, the risk of tumor formation following transplantation will be lowered to a minimum because *allogenic* iPSCs will be immunologically rejected by adaptive immune systems. Collectively, PVVT-based therapies will widen the applicability of iPSCs to clinical purposes.

Thus, our finding will shed a new light to an advanced understanding of vascular biology and contribute to the therapeutics development for the control of ischemic diseases.

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COMMENTS

Background

Human vascular endothelial cells (VECs) are categorized into two groups by their effects on the proliferation of vascular smooth muscle cells (VSMCs) in *in vitro* co-culture experiments: Pro-proliferative VECs (type-I) vs anti-proliferative VECs (type-II).

Research frontiers

It remains elusive whether pro-proliferative and anti-proliferative human VECs indeed exert pro-stenotic and anti-stenotic potentials *in vivo*, respectively. To evaluate the characters of human VECs *in vivo*, however, an innovative technique that guarantees high-efficiency transplantation of VECs onto the luminal surface of the artery, which is exposed to high-pressured blood stream, is required.

Innovations and breakthroughs

The problem to effectively transplant human VECs onto the luminal surface of the murine artery has been resolved by the novel transplantation technique, where gel-embedded VECs were injected into the substances space around arteries through a small incision opening on the skin surface. By applying this technique, *in vivo* relevance the concept for the categorization of human VECs was validated, demonstrating that transplantation of pro-proliferative VECs (type-I) and anti-proliferative VECs (type-II) resulted in deterioration and prevention of stenosis in the injured arteries, respectively.

Applications

Towards the control of ischemic diseases, transplantation of human iPSC-derived type-II VECs to the luminal surface of injured arteries via a *vasa vasorum* route may provide a new adjunct therapy with high efficacy and high safety but low risk of restenosis after revascularization.

Terminology

Vasa vasorum is a feeding microvessel that supplies the cells in tunica media of

larger blood vessels including smooth muscle cells and fibroblasts. Wire injury is a technique to mechanically remove the endothelial cells from arterial lumens by the movement of a metal wire in a longitudinal as well as vertical direction, causing the development of arteriosclerosis.

Peer-review

The manuscript is about two types of vascular endothelial cells, which have anti and pro proliferative effect on vascular smooth muscle cells. The study is well-design and written.

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