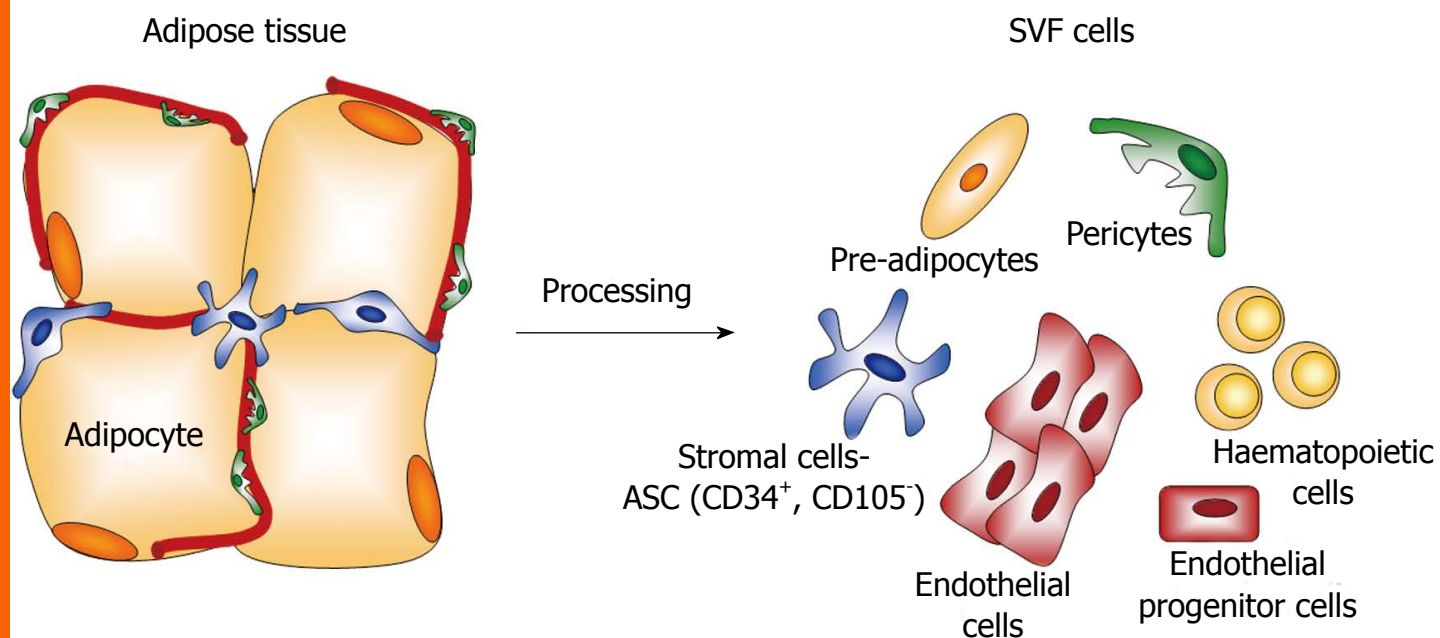


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World Journal of Stem Cells
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-85381891
Fax: +86-10-85381893
E-mail: wjsc@wjgnet.com
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A familiar stranger: CD34 expression and putative functions in SVF cells of adipose tissue

Arnaud Scherberich, Nunzia Di Maggio, Kelly M McNagny

Arnaud Scherberich, Nunzia Di Maggio, Department of Biomedicine, University and University Hospital of Basel, CH-4031 Basel, Switzerland

Kelly M McNagny, The Biomedical Research Centre, University of British Columbia, Vancouver, BC V6T 1Z3, Canada

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Correspondence to: Arnaud Scherberich, PhD, Department of Biomedicine, University and University Hospital of Basel, Hebelstrasse 20, CH-4031 Basel,

Switzerland. arnaud.scherberich@usb.ch

Telephone: +41-61-2652330 Fax: +41-61-2653990

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a short introduction to the CD34 family of sialomucins and reviews the data from the literature concerning expression and function of these proteins in SVF cells and their *in vitro* expanded progeny.

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Key words: Human adipose tissue; CD34; Sialomucins; Mesenchymal stromal cells; Endothelial progenitors

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Abstract

Human adipose tissue obtained by liposuction is easily accessible and an abundant potential source of autologous cells for regenerative medicine applications. After digestion of the tissue and removal of differentiated adipocytes, the so-called stromal vascular fraction (SVF) of adipose, a mix of various cell types, is obtained. SVF contains mesenchymal fibroblastic cells, able to adhere to culture plastic and to generate large colonies *in vitro*, that closely resemble bone marrow-derived colony forming units-fibroblastic, and whose expanded progeny, adipose mesenchymal stem/stromal cells (ASC), show strong similarities with bone marrow mesenchymal stem cells. The sialomucin CD34, which is well known as a hematopoietic stem cell marker, is also expressed by ASC in native adipose tissue but its expression is gradually lost upon standard ASC expansion *in vitro*. Surprisingly little is known about the functional role of CD34 in the biology and tissue forming capacity of SVF cells and ASC. The present editorial provides

INTRODUCTION

Multipotent stromal cells and various progenitor cells derived from adipose tissue have raised interest for regenerative medicine applications, especially because adipose tissue can be harvested in large quantities (several hundred mL) by a minimally invasive liposuction procedure. This was the topic of several recent reviews^[1-3]. In humans and mice, the stromal vascular fraction (SVF) is a heterogeneous mixture of cells isolated by enzymatic dissociation of adipose tissue followed by gradient centrifugation in order to remove the differentiated adipocytes, which float over the aqueous layer. The pellet of SVF cells contains multipotent mesenchymal cells, which are typically referred to as adipose derived stem/stromal/progenitor cells (ASC). Human ASC (hASC) have biological capacities highly comparable to bone marrow-derived mesenchymal stem/stromal cells (BMSC) and therefore are considered to be a promising alternative source of cells for clinical use in pathological contexts as diverse as cardiovascular disorders, pulmonary diseases, musculoskeletal disorders, soft tissue reconstruction/augmentation, liver dysfunction, gastrointestinal, urogenital or neuronal disorder.

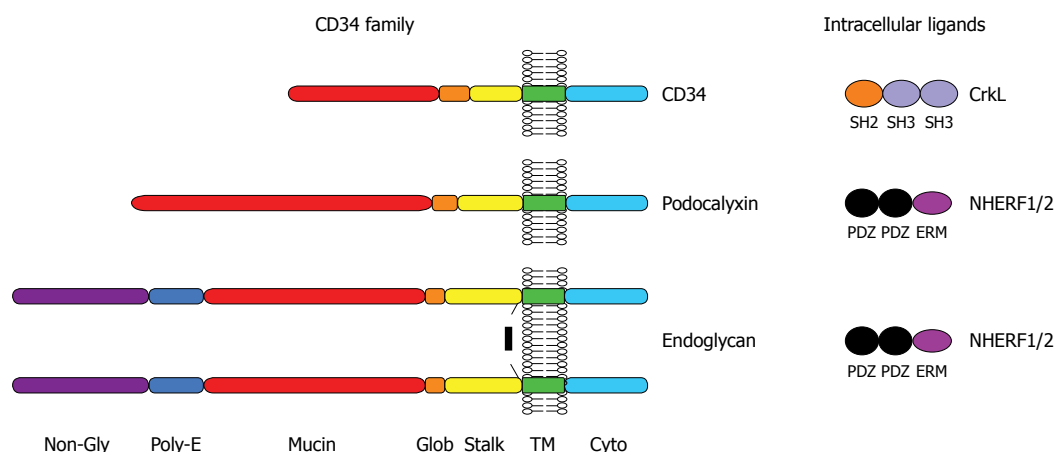


Figure 1 Protein structure of the CD34 family. CD34, podocalyxin and endoglycan are transmembrane proteins which display O-glycosylated and sialylated serine-, threonine- and proline-rich extracellular mucin domain, putative sites of N-glycosylation, a cysteine-containing globular domain and a juxtamembrane stalk region. NHERF1 and 2 bind to the C-terminal tail of podocalyxin and endoglycan but not CD34. Conversely the CrkL selectively binds to a distinct juxtamembrane sequence in CD34.

ders, skin and wound healing, bone regeneration, corneal diseases and immuno-modulation (reviewed in^[1,4]).

In addition to ASC, the SVF also contains blood-derived cells, in particular erythrocytes and leukocytes, characterized by expression of the pan-hematopoietic marker CD45 and/or the monocytic marker CD14. Interestingly, SVF was shown to also include vascular endothelial and mural/pericytic cells, harboring vasculogenic properties *in vitro* and *in vivo* (^[5,6] and recently reviewed in^[7]). The majority of SVF cells (60%-80%^[8,9]) express CD34, which was first used for the identification and isolation of hematopoietic progenitor cells. Very little is known about its possible function in adipose-derived cells, but given the pivotal role played by CD34 in the biology of several stem/progenitor cells, including hematopoietic stem cells^[10], skeletal muscle satellite cells^[11], keratinocyte stem cells^[12], hair follicle stem cells^[13,14] or adipogenic precursors^[15], a function of CD34 also in the biology of adipose-derived stem/progenitor cells can reasonably be hypothesized. The present editorial provides a concise overview of the CD34 family of sialomucins and compiles the data from the literature concerning expression and function of these proteins in SVF cells and their *in vitro* expanded progeny.

The CD34 family of sialomucins: Structure and functions

The CD34 family of cell surface proteins is a discrete subset of the large family of transmembrane sialomucins and comprises 3 members: CD34, podocalyxin (Podxl) and endoglycan (Endgl). A schematic overview of the biochemical features of CD34 family of proteins is shown in Figure 1. Although CD34 family members exhibit relatively limited linear protein sequence identity, they each contain a very similar array of biochemical domains and motifs that distinguishes them as a distinct subfamily from the much larger family of transmembrane mucins. All three contain an N-terminal signal peptide followed by a serine-, threonine- and proline-rich mucin domain that

becomes highly decorated with O-linked and, to a lesser extent, N-linked glycosylation (Endgl is somewhat unique in that it also contains an intervening N-terminal domain that lacks potential glycosylation sites but contains a site for chondroitin sulfate attachment and a poly-glutamic acid motif)^[16-18]. The mucin domain is followed by a disulfide-bonded globular domain, a juxta-membrane “stalk” domain, a transmembrane region and a charged intracellular domain of about 75 amino acids containing consensus phosphorylation sites for PKC or CKII.

At their C-termini, all three proteins contain short motifs (DTHL or DTEL) that resemble binding sites for PDZ-domain scaffolding proteins, which are known to play a role in targeting bound proteins to discrete subcellular localizations and facilitating signal transduction^[19,20]. The DTHL motifs of Podxl and Endgl have been shown to bind the PDZ-domain proteins, NHERF-1 and NHERF-2, which are well known for their ability to bind a large array of G-protein coupled receptors, tyrosine kinases, transcription factors, *etc.*^[17,21]. There is data suggesting that Podxl directs the recruitment of NHERF1, and presumably all of its signaling ligands, to discrete membrane domains within the cell^[22]. Somewhat surprisingly, CD34 does not bind NHERF1 or NHERF2 and it is therefore likely that there is a distinct, yet-to-be-discovered, PDZ domain protein that binds this family member^[21].

Relatively little is known of the intracellular ligands for CD34. In an unbiased phosphoproteomic survey of mast cells, CD34 was identified as one of the most rapidly (10 s) and highly (> 50-fold) tyrosine phosphorylated proteins in response FcεRI crosslinking^[23] providing evidence that CD34 can be modified dynamically in response to extracellular stimuli and could provide a docking site for phosphopeptide binding proteins. In addition, the membrane proximal region of CD34 has been shown to bind the SH2-SH3-SH3 containing adapter protein, CrkL^[24] through a motif that is not present in Podxl or

Endgl. CrkL is known to interact with Abl, Bcr-Abl, C3G, Sos, EPS15, and DOCK180 through its N-terminal SH3 domain whereas CD34 is the first protein known to bind through its C-terminal SH3 domain^[24]. Thus, like its relatives, Podxl and Endgl, there is reason to believe that CD34 can target signal transduction complexes to discrete cell membrane domains, possibly in response to phosphorylation.

Although the biological function of CD34 has not yet been fully clarified, several roles have been attributed to the proteins of the CD34 family. Due to its restricted expression on cycling hematopoietic progenitors, CD34 has been proposed to both promote proliferation and block differentiation (reviewed in^[25]). Indeed, ectopic expression of CD34 in cell lines^[26] and evaluation of progenitors derived from knockout mice^[27] tend to support this view. A similar study showing resistance of CD34 knockout mice to formation of skin tumors posited a similar role for CD34 in the proliferation of precursors^[14]. More recent studies have also linked Podxl and CD34 expression to enhanced trafficking and migration of hematopoietic cells^[10,28,29] (reviewed in^[17]). These studies suggest that the strong negative charge conferred by the highly glycosylated extracellular domain of CD34 family proteins serves an anti-adhesive function and enhance the mobility of cells. This may be further facilitated by a role in aiding chemotactic signaling responses as was recently suggested for CD34⁺ dendritic cell precursors^[30]. The fact that expression of a human transgene was able to rescue the disease phenotype in these mice suggests that the human gene functions in a similar fashion^[30]. While, at face value, it is difficult to reconcile the different roles for CD34 (and Podxl) observed in proliferation and cell migration, it should be noted that these may not be mutually exclusive; it is possible that CD34 plays subtle roles in both pathways. Alternatively, it is possible that by altering cell adhesion, CD34 could alter the downstream sensitivity of cells to entering cell cycle or differentiation. This would be quite compatible with a known role for integrins and other adhesion molecules in regulating cell cycle progression and differentiation (reviewed in^[17]). Finally, through their potent ability to traffic to the apical domains of cells and interact with cytoskeletal signaling proteins, CD34 and its relatives have been speculated to play a role in regulating the recruitment of cell differentiation factors to discrete cellular membranes and thereby to regulate asymmetric cell division of undifferentiated precursors (reviewed in^[17]).

Expression of CD34 in different cell types

CD34 family proteins have both unique and overlapping expression patterns. All three proteins (CD34, Podxl and Endgl) have been described as markers of hematopoietic precursors and vascular tissue (reviewed in^[17]) giving them ample opportunity to serve redundant functions as evidenced in knock-out mice. However, in addition to their overlapping expression on these cell types, these molecules are also uniquely expressed on other cells. In

the case of CD34, it is uniquely expressed by inflammatory cell precursors (mast cells, eosinophils and dendritic cells) and has been shown to be important for facilitating cell trafficking and the development of mucosal inflammatory disease, while in its absence, mice are rendered remarkably resistant to a range of disease including allergic asthma, hypersensitivity pneumonitis, colitis, Salmonella induced inflammation, and colon cancer^[30-34]. As already mentioned in the introduction, CD34 is expressed by various stem/progenitor cells such as muscle satellite cells, adipogenic precursors and by hair follicle and keratinocyte stem cells. Podxl in contrast is expressed selectively by anemic erythroid lineage cells^[28,35], kidney podocytes^[36], a subset of developing neurons^[37], and a variety of embryonic tissues including mesothelial precursors and epithelial precursors^[16,38] (and Hughes *et al*, In Tech - Advances in Cancer Management, in press). Accordingly, deletion of the Podxl gene leads to perinatal lethality due to defective morphogenesis in a number of these cell types^[16,38,39]. In BMSC, the expression and the role of CD34-related sialomucins remain unclear. However, Podxl was shown, together with $\alpha 6$ -integrin (CD49f, VLA-6), to identify early progenitor BMSC with increased clonogenicity and differentiation potential *in vitro* and highly efficient migration to infarcted heart in mice^[40]. In addition, Podxl was shown to be strikingly upregulated in the most life-threatening epithelial tumors and appears to play a role in enhancing the mobility and invasiveness of tumors^[22,38,41] (and Hughes *et al*, In Tech - Advances in Cancer Management, in press).

Expression of CD34 by hASC

Human adipose tissue was shown to turn over^[42] and adipocytic progenitor cells, i.e., ASC in mice have been shown to reside in the adipose vasculature^[43]. However, the precise origin of the native hASC still remains a debated question. Indeed, although it was recently proposed that ASC originate from a pericyte population lacking CD34 expression^[44], Traktuev *et al*^[6] suggested a CD34⁺ pericytic origin for ASC, which have been previously characterized as Lin⁻/CD29⁺/CD34⁺/Sca-1⁺/CD24⁺ cells^[45]. To clarify this question, the expression of CD34 by hASC in native adipose tissue was addressed by characterizing expression of CD34 in subpopulations of cells from the SVF^[46,47]. Initially, two CD34⁺ populations with a difference in the intensity of antigen expression were identified and a majority of the cells expressed CD34 at low intensity. ASC freshly isolated from human SVF were characterized as CD31⁻/CD34⁺/CD45⁻/CD90⁺/CD105⁻/CD146⁻ cells, but were shown to become CD105⁺ when plated^[48]. Upon adhesion to tissue culture plastic, cell expansion and passaging, CD34⁺/CD45⁻ cells from SVF, were shown to gradually lose CD34 expression in monolayer culture, although the kinetics of decrease in CD34 expression seemed to vary strongly with culture conditions, such as plating density or culture medium used^[48,49]. As a consequence, hASC, which are typically expanded as a monolayer on tissue culture plastic for several passages

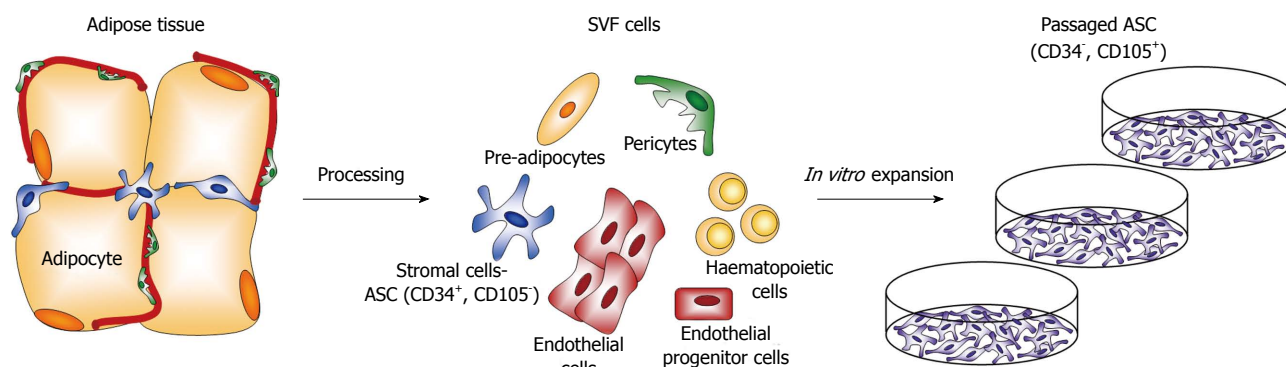


Figure 2 In the adipose tissue, adipose mesenchymal stem/stromal cells are localized perivascularly and are characterized by the expression of CD34. Adipose mesenchymal stem/stromal cells (ASC) are isolated upon collagenase digestion together with other cell types including endothelial and endothelial progenitor cells. Upon adhesion to plastic and active *in vitro* proliferation, ASC change their phenotype, lose CD34 expression and begin to express CD105.

prior to their use, have been reported to be CD34⁺ in most publications so far. This is in accordance with similar reports made on freshly cultured primary human vascular endothelial cells, where endothelial cells initially express CD34, which is later down-regulated with proliferation in continuous culture and is predominantly absent after only nine population doublings^[50]. This was confirmed by the fact that CD34 seems to be especially expressed by endothelial “tip cells”, which are endothelial cells actively participating in the angiogenic process but which do not proliferate^[51]. A follow up study on adipose-derived cells from the group of Yoshimura correlated CD34 expression by freshly isolated hASCs in SVF with a higher clonogenicity and proliferation capacity, reduced differentiation potentials into mesenchymal lineages, namely adipocytic and osteoblastic, increased expression of angiogenesis-related genes^[52]. One limitation of the latter study resides in the fact that CD34⁺ cells were considered as a homogenous population. Our group investigated which cell population in SVF gives rise to hASC in monolayer culture^[5] by sorting SVF cells according to the expression of CD34 and the endothelial marker CD31. We showed that the CD34⁺/CD31⁻ fraction contains progenitors able to differentiate into the osteogenic lineage and colony-forming cells. This indicates that hASC are CD34⁺/CD31⁻ cells in the SVF, which upon adhesion give rise to expanded hASC in culture. A study by another group concomitantly compared freshly isolated hASC and hASC expanded as monolayers on tissue culture plastic with serial passaging and also evaluated hASC morphology in their niche to define their tissue localization within intact human adipose samples^[53]. This study showed that native human ASC (1) are CD34 positive and that its expression is dramatically decreased with *in vitro* proliferation; (2) display both stromal and perivascular positions but (3) do not express *in situ* pericytic markers such as NG2 and CD140b; and (4) exhibit a peculiar cell morphology with long protrusions *in situ*^[53]. In conclusion, hASC are CD34⁺ in the native adipose tissue and in SVF, but rapidly change their phenotype and lose CD34 expression when expanded as monolayer (Figure 2).

Expression of CD34 by vascular cells in human SVF

The presence of vasculogenic cells within the SVF was first proposed in 2004^[54] and thereafter confirmed by different groups^[8,55,56]. The CD34⁺/CD31⁻ cell population from adipose tissue differentiates into endothelial cells and exhibits the capacity to rescue hindlimb ischemia in animal models^[8,57]. Also, both CD34⁺ and CD34⁻ primitive mesodermal progenitors within the SVF of human adipose tissue were shown to exhibit hematopoietic and hemangioblastic activities *in vitro*^[58]. In a recent study, our group investigated the vasculogenic potential of different subpopulations of SVF cells. We showed that neither CD34⁺CD31⁻ nor CD34⁺CD31⁺ from SVF were able to form vascular structures alone, but that a combination of the two resulted in robust vascular structure formation *in vitro*^[5], extending a preliminary report showing tubule formation on Matrigel® by CD90⁺/CD34⁺ cells^[59]. We thereby showed that CD34⁺CD31⁻ cells not only include hASC but also cells with a pericytic phenotype necessary for the stabilization of endothelial capillaries formed by CD34⁺CD31⁺ adipose endothelial cells (hAEC, Figure 1), as previously suggested^[6]. However, despite a clear potential of these vasculogenic cells derived from adipose tissue to initiate vascularization in regenerative medicine applications (reviewed in^[2] or to treat ischemic disorders^[8], their safety for clinical applications should still be considered with caution. For example, the adipose tissue used for autologous lipotransfer procedures, e.g., for breast reconstruction, in some cancer patients (which is rich in CD34⁺ progenitors) was shown to promote tumor growth, angiogenesis and metastases in several orthotopic models of human breast cancer^[60]. This possibly reflects a side-effect due to mast cells, which are present in the adipose transplants, release VEGF and thereby induce tumor angiogenesis^[61].

In summary, it is important to make a distinction between the CD34⁺CD31⁺ hAEC constitutively present in the native adipose tissue and in the SVF and CD34⁺CD31⁺ endothelial-like cells obtained by differentiation of CD34⁺/CD31⁻ expanded hASC^[62,63]. Interestingly, this indicates that, like primary vascular endothelial cells^[50,51], cells

derived from adipose tissue, that lose the expression of CD34 in monolayer culture retain the ability to re-express this marker if stimulated appropriately.

Function of CD34 in SVF cells and hASC

Despite the loss of CD34 expression during *in vitro* culture, expanded hASC retain proliferative capacity and multipotency even after several passages. However, little is known about how CD34 affects the biological features and the functionality of SVF cells, i.e., hASC or endothelial/vasculogenic cells.

With regard to the role of CD34 in the biology of endothelial cells from SVF, it is likely to involve mechanisms mostly similar to the ones demonstrated for lumen formation in the developing mouse aorta^[64], as suggested by our preliminary data showing similar tubes as well as lumen formation by SVF cells *in vitro*^[5]. Moreover, the endothelial CD34⁺/CD31⁺ fraction within SVF cells exhibit the ability to form functional blood vessels *in vivo* and connect to the vasculature of the recipient mice, both after perfusion culture^[9] or if implanted directly after isolation from adipose tissue^[65]. We also find that vasculogenic cells from the SVF can generate highly organized vascular structures *in vitro*, which by anastomosis with the vasculature of the host greatly enhance both the amount and depth of bone tissue formation inside tissue engineered osteogenic constructs *in vivo*^[5]. The functional role of CD34 in vasculogenic cells from SVF, and the mechanisms involved will be further investigated by us in the future.

With regard to CD34 expression in ASC, few studies have addressed the role of CD34 in hASC function, *in vitro*. Suga *et al.*^[52] used SVF cells after a short initial expansion phase on tissue culture plastic and showed that, in sorted SVF-derived cells, both CD34⁺ and CD34⁻ cells are clonogenic and that the impact of CD34 expression on hASC function was limited. Indeed, CD34⁺ cells showed a slightly decreased proliferation and a limited increase in differentiation capacity towards mesodermal lineages. More recently, in 8-d expanded hASC, Maumus *et al.*^[53] demonstrated that CD34⁺ hASC are the only subpopulation of hASC containing clonogenic cells, and the only one able to differentiate into adipogenic and osteogenic lineages. We simultaneously confirmed and extended this finding to unexpanded, SVF cells by showing that it is more specifically the CD34⁺/CD31⁻ cell population which contains the cells with osteoblastic differentiation capacity and also clonogenic, colony-forming cells^[5].

In the context of bone tissue engineering, several reports suggest that, despite the loss of CD34 upon expansion of hASC, they can maintain an actual capacity to form bone tissue *in vivo*, if cultured adequately (reviewed in^[2]). SVF cells were used by our group to generate osteogenic grafts with intrinsic vasculogenic capacity, both if implanted immediately^[65] or when SVF cells were seeded and cultured for 5 d within hydroxyapatite porous scaffolds inside a perfusion-based bioreactor system^[9].

In both cases, it is important to mention that more than 65% of the cells, including hASC, still expressed CD34 in the experimental conditions used. These studies critically established that human SVF cells, similarly to expanded hASC, are capable of generating frank bone tissue *in vivo*. This was possible even in the absence of exogenous osteoinductive signals, when SVF cells were cultured under perfusion^[9], whereas direct implantation of SVF cells required an osteogenic trigger such as bone morphogenetic protein 2 to support ectopic osteogenesis *in vivo* (Mehrkens *et al.*, In press). The potential importance of CD34 in bone formation by hASC is currently being investigated by our group by ectopic implantation of the CD34⁺ and CD34⁻ fractions.

Conclusion and perspectives

The use of CD34 as an adipose-derived cell marker, and the numerous reports about expression of CD34 by hASC and its progressive loss upon *in vitro* cell culture can not compensate for a major lack of references in the literature about the precise role and function of CD34 in hASC and other cells derived from the SVF. This fact should prompt the increasing number of research teams using hASC and SVF cells in regenerative medicine applications worldwide to design new research projects aimed at addressing this question more specifically. In particular, a better understanding of the membrane localization and trafficking of CD34, of the signaling pathways induced by CD34 and of the promoters controlling the expression of CD34 in SVF-derived cells would help in better investigating its role on functions such as adhesion, migration, proliferation or differentiation, and consequently the tissue formation capacities of these cells *in vivo*.

Furthermore, alternative culture methods should be developed to avoid the loss of CD34 expression and to preserve a physiological phenotype. Our group recently investigated the possibility of expanding hASC while maintaining their expression of CD34. This study is based on the hypothesis that cell-extracellular matrix and cell-cell interactions should be favored to mimic the physiological situation. The final aim would be to reconstitute a three-dimensional physiological environment in a controlled setting as it was proposed for the bone marrow niche in one of our recent reports^[66]. The system aimed at recapitulating the complex microenvironment of the niche and establishing the chemico-physical cues required for a physiological stem cell function regulation. By applying this principle to SVF cells, it was possible to maintain CD34⁺/CD31⁻/CD105⁻ hASC, i.e., a phenotype much more similar to native hASC, for up to 6 wk in culture (our preliminary, unpublished data). SVF cells from the same donors, cultured in parallel as standard monolayer with serial, weekly passaging, generated hASC with minimal expression of CD34. A deeper characterization is needed to better understand the biological features and functionality of these cells and is currently ongoing.

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Impact of the antiproliferative agent ciclopirox olamine treatment on stem cells proteome

Gry H Dihazi, Asima Bibi, Olaf Jahn, Jessica Nolte, Gerhard A Mueller, Wolfgang Engel, Hassan Dihazi

Gry H Dihazi, Asima Bibi, Gerhard A Mueller, Hassan Dihazi, Department of Nephrology and Rheumatology, Georg-August University Goettingen, D-37075 Goettingen, Germany
Olaf Jahn, Proteomics Group, Max-Planck-Institute of Experimental Medicine, D-37075 Goettingen, Germany
Olaf Jahn, Deutsche Forschungsgemeinschaft Research Center for Molecular Physiology of the Brain, D-37073 Goettingen, Germany

Jessica Nolte, Wolfgang Engel, Institute of Human Genetics, Georg-August University Goettingen, D-37073 Goettingen, Germany

Author contributions: Dihazi GH and Bibi A performed the majority of experiments, interpreted the data and wrote the article; Nolte J and Engel W provided the stem cell lines and were also involved in revising the manuscript; Jahn O was responsible for the acquisition of the mass spectrometry data and revised the article critically; Mueller GA helped by the study design and manuscript writing; Dihazi H designed and conceptualized the study and revised the article critically; Dihazi GH and Bibi A contributed equally to this work.

Correspondence to: Hassan Dihazi, PhD, Department of Nephrology and Rheumatology, Georg-August University Goettingen, Robert-Koch-Strasse 40, D-37075 Goettingen, Germany. dihazi@med.uni-goettingen.de

Telephone: +49-551-3991221 Fax: +49-551-3991039

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Abstract

AIM: To investigate the proteome changes of stem cells due to ciclopirox olamine (CPX) treatment compared to control and retinoic acid treated cells.

METHODS: Stem cells (SCs) are cells, which have the ability to continuously divide and differentiate into various other kinds of cells. Murine embryonic stem cells (ESCs) and multipotent adult germline stem cells (maGSCs) were treated with CPX, which has been shown to have an antiproliferative effect on stem cells, and compared to stem cells treated with retinoic acid (RA),

which is known to have a differentiating effect on stem cells. Classical proteomic techniques like 2-D gel electrophoresis and differential in-gel electrophoresis (DIGE) were used to generate 2D protein maps from stem cells treated with RA or CPX as well as from non-treated stem cells. The resulting 2D gels were scanned and the digitalized images were collated with the help of Delta 2D software. The differentially expressed proteins were analyzed by a MALDI-TOF-TOF mass spectrometer, and the identified proteins were investigated and categorized using bioinformatics.

RESULTS: Treatment of stem cells with CPX, a synthetic antifungal clinically used to treat superficial mycoses, resulted in an antiproliferative effect *in vitro*, without impairment of pluripotency. To understand the mechanisms induced by CPX treatments which results in arrest of cell cycle without any marked effect on pluripotency, a comparative proteomics study was conducted. The obtained data revealed that the CPX impact on cell proliferation was accompanied with a significant alteration in stem cell proteome. By peptide mass fingerprinting and tandem mass spectrometry combined with searches of protein sequence databases, a set of 316 proteins was identified, corresponding to a library of 125 non-redundant proteins. With proteomic analysis of ESCs and maGSCs treated with CPX and RA, we could identify more than 90 single proteins, which were differently expressed in both cell lines. We could highlight, that CPX treatment of stem cells, with subsequent proliferation inhibition, resulted in an alteration of the expression of 56 proteins compared to non-treated cells, and 54 proteins compared to RA treated cells. Bioinformatics analysis of the regulated proteins demonstrated their involvement in various biological processes. To our interest, a number of proteins have potential roles in the regulation of cell proliferation either directly or indirectly. Furthermore the classification of the altered polypeptides according to their main known/postulated functions revealed that the majority of these proteins are involved in molecular functions

like nucleotide binding and metal ion binding, and biological processes like nucleotide biosynthetic processes, gene expression, embryonic development, regulation of transcription, cell cycle processes, RNA and mRNA processing. Proteins, which are involved in nucleotide biosynthetic process and proteolysis, were downregulated in CPX treated cells compared to control, as well as in RA treated cells, which may explain the cell cycle arrest. Moreover, proteins which were involved in cell death, positive regulation of biosynthetic process, response to organic substance, glycolysis, anti-apoptosis, and phosphorylation were downregulated in RA treated cells compared to control and CPX treated cells.

CONCLUSION: The CPX treatment of SCs results in downregulation of nucleotide binding proteins and leads to cell cycle stop without impairment of pluripotency.

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Key words: Stem cells; Differentiation; Hypusination; Ciclopirox olamine; Proteomics; Retinoic acid

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INTRODUCTION

Stem cells (SCs) are cells, which are found in all multicellular organisms, which can continuously divide and differentiate into various specialized cell types and can also self-renew to produce more stem cells^[1]. The therapeutic use of embryonic stem cells (ESCs) has been constrained by problems caused by immune rejection in the patient as well as ethical issues associated with the use of embryos^[2]. Spermatogonial stem cells (SSCs) are self-renewing single cells located in the periphery of the seminiferous tubules whose continuous division maintain spermatogenesis throughout the life of a male individual^[3]. SSCs were isolated from murine testis and cultured for the first time in 2006^[4]. The pluripotency and plasticity of these cultured cells, named multipotent adult germline stem cells (maGSCs), were proven to be similar to ESCs. The ESC-like nature of maGSCs was confirmed on the microRNA level^[5], on the transcriptome level^[6] and on the proteome level^[7]. In a recent study, we investigated the effects of retinoic acid (RA) treatment on the protein expression profiles of maGSCs and ESCs^[8]. The study revealed the important role of Eif5a and its hypusination for stem cell differentiation and proliferation.

Eif5a is a universal translation elongation factor which is highly conserved in all cells. Eif5a has been shown to be associated with translation, viability and proliferation processes^[9-12]. It is the only eukaryotic protein known to have the unusual amino acid hypusine. Hypusine is es-

sential to the function of Eif5a and is involved in protein biosynthesis by promoting the formation of the first peptide bond and translation elongation^[13]. The activation of Eif5a is controlled by a unique post-translational modification called hypusination. It occurs in two steps which are controlled by two different enzymes^[14,15], which inactivation can lead to hypusination inhibition. Ciclopirox olamine (CPX), the ethanolamine salt of 6-cyclohexyl-1-hydroxy-4-methylpyridin-2(1H)-one, is a hypusination inhibitor that controls the second step of the modification, which is catalyzed by deoxyhypusine hydroxylase^[14].

CPX, a synthetic antifungal agent, has been used topically to treat fungal and yeast infection of skin or mucosa for more than 20 years^[16-19]. Apart from its antimycotic activity, CPX is also effective against both gram-positive and gram-negative bacteria^[20]. CPX might also serve as an alternative to recombinant vascular endothelial growth factor (VEGF) treatment or to VEGF gene therapy for therapeutic angiogenesis^[21]. The effect of CPX on several *Saccharomyces cerevisiae* mutants has been screened and tested, and it was suggested that CPX may exert its effect by disrupting DNA repair, DNA replication, cell division signals and a defect in mitotic spindle function. Furthermore CPX can influence the regulation of many processes, including signal transduction, transcription, cell division, and development^[22]. Recent studies demonstrated CPX as a potential anti-cancer agent for the treatment of malignancies, including leukemia and myeloma^[23-25]. However, the mechanism of CPX as a drug in angiogenesis and tumor treatment is poorly understood. CPX works as an inhibitor of the iron-dependent enzymes due to its role as a chelator of intracellular iron^[22,23]. Other studies reported the inhibition of HIV-1 gene expression by CPX^[26], the importance of Eif5a in embryogenesis and cell differentiation^[27], in hepatocellular carcinoma^[28] and in diabetes^[29]. CPX has also been used as an inhibitor of hypusination.

In a recent study, the effect of CPX on the cellular viability and proliferation of ESCs and maGSCs was investigated. CPX treatment of the stem cells resulted in an antiproliferative effect on ESCs and maGSCs *in vitro*, but did not affect the cell pluripotency^[8]. The inhibitory effect of CPX on cell differentiation was reversible and was not associated to apoptosis. The ESCs were found to be more sensitive to CPX than the maGSCs.

The aim of this study was to investigate the proteome changes of ESCs and maGSCs accompanying the treatment with CPX and subsequent inhibition of hypusination using classical proteomic techniques like 2-DE, DIGE and MS. 2D protein maps were generated from control cells and cells treated either with RA or CPX. The resulting protein maps were compared to each other and the differentially expressed proteins were investigated using bioinformatics. We could highlight that a treatment with CPX, involving proliferation inhibition, resulted in an alteration of the expression of 56 proteins compared to non-treated cells, and 54 proteins compared to RA treated cells. The majority of these proteins are involved in nucleotide binding and nucleotide biosynthetic pro-

cesses, metal binding, DNA binding, and other processes which have been linked to CPX.

MATERIALS AND METHODS

Derivation and culture of maGSC and ESC lines

The derivation and culture of maGSCs 129/Sv was described previously^[4]. In brief, testes from adult mice were isolated and digested using collagenase. Single cell suspension was derived after trypsin digestion followed by the culture of the testis suspension cells on a mouse embryonic fibroblasts (MEFs) feeder layer in the presence of GDNF. After appearance of morphological ES-like cells, the colonies were picked and expanded in standard ES cell conditions. In this case, the maGSC line was generated without genetic selection, only by morphological criteria. The ESC R1 line was derived from the 129/Sv mouse^[30]. To maintain maGSCs and ESCs in an undifferentiated state, the cells were cultured under standard ESC culture conditions: DMEM (PAN, Aidenbach, Germany) supplemented with 20% fetal calf serum (PAN, Aidenbach, Germany), 2 mmol/L L-glutamine (PAN, Aidenbach, Germany), 50 mmol/L β -mercaptoethanol (Gibco/Invitrogen, Eggenstein, Germany), $1 \times$ non-essential amino acids (Gibco/Invitrogen), sodium pyruvate (Gibco/Invitrogen), and penicillin/streptomycin (PAN, Aidenbach, Germany). ESCs and maGSCs were cultured on a feeder layer of mitomycin C-inactivated MEFs in the presence of 1000 U/mL recombinant mouse leukemia inhibitory factor (LIF) (Chemicon, Temecula, United States). ESCs were isolated as described previously, and male ESC lines were identified and selected by PCR amplification of Sry gene-specific sequences^[31,32]. In order to differentiate maGSCs and male ESCs, the cells were plated on gelatin-coated dishes and culture medium was supplemented with 1 μ mol/L RA (Sigma-Aldrich, Steinheim, Germany) instead of LIF. Cells were cultured for 48 h before they were lysed and the proteins were extracted. For examining the effect of CPX on the proteome level, ESCs and maGSCs were treated with culture medium supplemented with 2 μ mol/L CPX for 72 h.

Protein extraction

The protein extraction for 2-DE was performed as described previously^[7]. Briefly, 75% confluent cultures were trypsinized and washed three times with PBS. The cells were harvested by centrifugation at $200 \times g$ for 10 min, the pellet was treated with 0.3–0.5 mL lysis buffer [9.5 mol/L urea, 2% CHAPS (w/v), 2% ampholytes (w/v), 1% DTT]. Ampholytes and DTT were added shortly before use. After adding the lysis buffer, the samples were incubated for 30 min at 4 °C. For removing the cell debris, sample centrifugation was carried out at $13\,000 \times g$ and 4 °C for 45 min. The supernatant was recentrifuged at $13\,000 \times g$ and 4 °C for an additional 45 min to get maximal purity. The resulting samples were used immediately or stored at -80 °C until use.

Protein precipitation

To reduce the salt contamination and to enrich the proteins, methanol-chloroform-precipitation according to Wessel *et al.*^[33] was performed. Briefly, 0.4 mL of methanol (100%) was added to 0.1 mL aliquots of protein samples and mixed together. 0.1 mL chloroform was added to the samples and the mixture was vortexed. Subsequently 0.3 mL water was added and the solution was vortexed and centrifuged at $13\,000 \times g$ for 1 min. The aqueous layer was removed, and another 0.4 mL methanol (100%) was added to the rest of the chloroform and the interphase with the precipitated proteins. The sample was mixed and centrifuged for 2 min at $13\,000 \times g$ and the supernatant was removed. The pellet was vacuum dried and dissolved in lysis buffer.

Total protein concentration was determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, United States) according to Bradford^[34]. BSA (Sigma, Steinheim, Germany) was used as a standard.

2D gel electrophoresis (2-DE)

IPG strips (11 cm, pI 5–8) were passively rehydrated in 185 μ L solution containing 150 μ g protein in a rehydration buffer (8 mol/L urea, 1% CHAPS, 1% DTT, 0.2% ampholytes, and a trace of bromophenol blue) for 12 h. The IEF step was performed on the PROTEAN® IEF Cell (Bio-Rad, Hercules, CA, United States). Temperature-controlled at 20 °C, the voltage was set to 500 V for 1 h, increased to 1000 V for 1 h, 2000 V for 1 h and left at 8000 V until a total of 50 000 Vhours was reached. Prior to SDS-PAGE, the IPG strips were reduced for 20 min at room temperature in SDS equilibration buffer containing 6 mol/L urea, 30% glycerol, 2% SDS 0.05 mol/L Tris-HCl, and 2% DTT on a rocking table. The strips were subsequently alkylated in the same solution with 2.5% iodoacetamide substituted for DTT, and a trace of bromophenol blue. For the SDS-PAGE, 12% BisTris Criterion precast gels (Bio-Rad, Hercules, CA, United States) were used according to manufacturer's instructions. The gels were run at 150 V for 10 min followed by 200 V until the bromophenol blue dye front had reached the bottom of the gel.

Gel staining

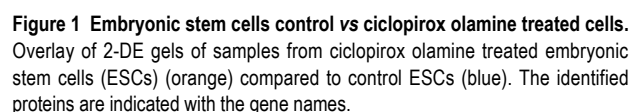
For image analysis, 2-DE gels were fixed in a solution containing 50% methanol and 12% acetic acid overnight and fluorescent stained with Flamingo fluorescent gel stain (Bio-Rad, Hercules, CA, United States) for minimum 5 h. After staining, gels were scanned at 50 μ m resolution on a Fuji FLA-5100 scanner. The digitalized images were analyzed using Delta 2D 3.4 (Decodon, Braunschweig, Germany). For protein visualization, 2-DE gels were additionally stained with colloidal Coomassie blue, Roti-Blue (Roth, Karlsruhe, Germany) overnight.

2D-DIGE

Protein extraction and methanol-chloroform-precipitation

To avoid the dye-specific protein labeling, every pair of protein samples from two independent cell extract preparations were processed in duplicate while swapping the dyes. Thereby four replicate gels were obtained, allowing to monitor regulation factors down to twofold changes^[35]. An internal standard consisting of a mixture of the samples under investigation was labeled with Cy2 and included on all gels to facilitate gel matching, thereby eliminating artifacts from experimental variation. The three differentially labeled fractions were pooled. Rehydration buffer (8 mol/L urea, 1% CHAPS, 13 mmol/L DTT and 1% ampholytes 3-10) was added to make up the volume to 185 μ L prior to IEF. The 2-DE was performed as described above. The CyDye-labeled gels were scanned at 50 μ m resolution on a Fuji FLA5100 scanner (Fuji Photo, Kanagawa, Japan) with laser excitation light at 473 nm and long pass emission filter 510LP (Cy2), 532 nm and long pass emission filter 575LP (Cy3), and 635 nm and long pass emission filter 665LP (Cy5). Fluorescent images were acquired in 16-bit TIFF files format. Spot matching across gels and normalization based on the internal standard was performed with Delta2D software (Decodon, Greifswald, Germany). To analyze the significance of protein regulation, a Student's *t*-test was performed, and statistical significance was assumed for *p* values less than 0.01. For protein visualization, the 2-DE gels were post-stained with colloidal Coomassie blue (Roti-Blue) overnight. Differentially regulated proteins were excised and processed for identification by mass spectrometry.

Manually excised gel plugs were subjected to an automated platform for the identification of gel-separated proteins^[36] as described in the framework of recent DIGE-based^[37] and large-scale proteome studies^[38]. An Ultraflex MALDI-TOF-TOF mass spectrometer (Bruker Daltonik) was used to acquire both PMF and fragment ion spectra, resulting in confident protein identifications based on peptide mass and sequence information. Database searches in the Swiss-Prot primary sequence database restricted to the taxonomy *mus musculus* were performed using the MASCOT Software 2.2 (Matrix Science). Carboxamidomethylation of Cys residues was specified as fixed and oxidation of Met as variable modifications. One trypsin missed cleavage was allowed. Mass tolerances were set to 100 ppm for PMF searches and to 100 ppm (precursor ions) and 0.7 Da (fragment ions) for MS/MS ion searches. The minimal requirement for accepting a protein as identified was at least one peptide sequence match above identity threshold in addition to at



least 20 % sequence coverage in the PMF.

least 20 % sequence coverage in the PMF.

The classification of the identified proteins according to their main known/postulated functions was carried out using DAVID bioinformatics^[39,40]. This classification together with the official gene symbol was used to investigate and categorize the gene ontology (GO)-annotations (biological processes and molecular functions).

Comparative analysis of differentially expressed proteins in RA and CPX treated SCs by 2-DE and ontogenic classification

The identified proteins were classified using DAVID bioinformatics^[39,40] focusing on its information considering the GO (Gene Ontology) annotations. The terms corresponding to the molecular function and biological process were regarded (Figures 5-7).

Examination of all of the proteins, which expression was altered either by CPX or RA treatment, was performed regarding their involvement in biological processes. We found that seven proteins are involved in regulation of

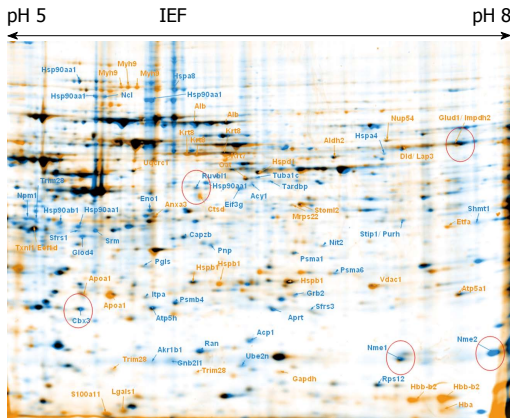


Figure 2 Multipotent adult germline stem cells control vs cyclopirox olamine treated cells. Overlay of 2-DE gels of samples from cyclopirox olamine treated multipotent adult germline stem cells (maGSCs) (orange) compared to control maGSCs (blue). The identified proteins are indicated with the gene names.

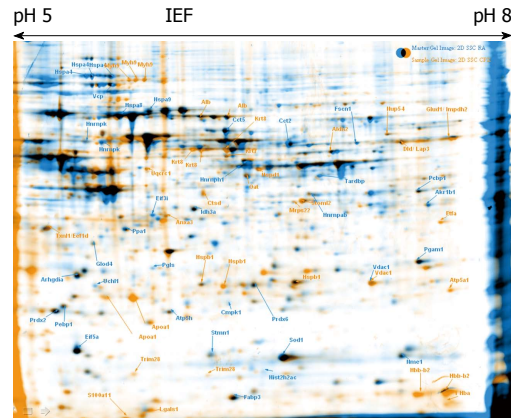


Figure 4 Multipotent adult germline stem cell control vs cyclopirox olamine treated cells. Overlay of 2-DE gels of samples from cyclopirox olamine treated Multipotent adult germline stem cells (maGSCs) (orange) compared to retinoic acid treated maGSCs (blue). The identified proteins are indicated with the gene names.

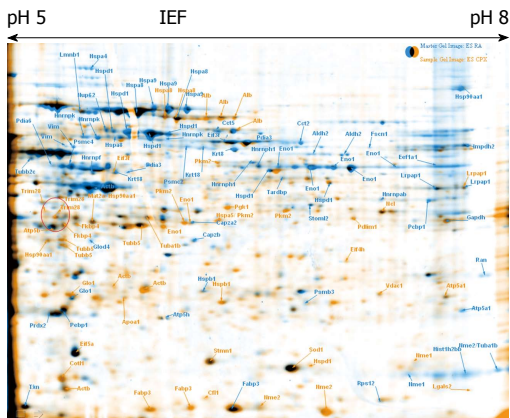


Figure 3 Embryonic stem cell control vs cyclopirox olamine treated cells. Overlay of 2-DE gels of samples from cyclopirox olamine treated embryonic stem cells (ESCs) (orange) compared to retinoic acid treated ESCs (blue). The identified proteins are indicated with the gene names.

transcription. Among these proteins Ube2n, Tardbp, Cbx3 and Hnrnpab were downregulated in CPX treated cells compared to control, whereas Nup62 was upregulated in CPX treated cells compared to control (Table 2). Two other proteins Trim28 and Ruvbl1 were downregulated in RA treated cells compared to control. Detailed information is given in Table 2 and the protein expression regulation folds are given in Tables 3-6.

When we looked at the molecular function of the regulated proteins, we observed that a major part of the proteins are involved in nucleotide binding (Table 7). Approximately half of these proteins were downregulated and the other half was upregulated upon CPX treatment compared to RA treated cells. About 13 proteins are involved in metal ion binding, of these five proteins (Acy1, Uqcrc1, Sfrs1, Trim28, Glo1) are involved in transition metal ion binding, like Fe^{3+} , which is known to be important in case of CPX, as CPX works as an inhibitor of the iron-dependent enzymes due to its role as a chelator of

intracellular iron. Three of the proteins involved in transition metal ion binding (Sfrs1, Trim28 and Glo1), were up-regulated upon CPX treatment compared to RA-treated cells.

Overall, it could be observed that most of the proteins of interest were downregulated in either CPX or RA treated cells compared to control.

Treated cells compared to control

About 56 of the 125 identified proteins showed different expression as a reaction to CPX treatment compared to control. Of these, 14 proteins were upregulated as a reaction to CPX treatment (Table 3), whereas 44 proteins were downregulated (Table 4). The expression of 52 proteins was found to be altered in both cell types, ESCs and maGSCs, under RA treatment compared to control (Tables 5 and 6). Of these proteins, 11 were upregulated and 41 were downregulated as a reaction to RA treatment.

In both experiments the majority of the regulated proteins were downregulated as a reaction to one of the treatments. Although mainly different proteins were regulated, bioinformatics analysis revealed that the downregulated proteins in both experiments are primarily involved in the same molecular functions (Figure 5). The downregulated proteins upon CPX treatment are mainly involved in nucleotide binding, GTP binding, peptidase activity and metal ion binding, particularly magnesium ion binding. The proteins which were downregulated upon RA treatment are involved in transition metal ion binding instead of magnesium ion binding, and furthermore involved in enzyme binding. Proteins, which were upregulated upon CPX treatment, are mainly involved in nucleotide binding, whereas proteins which were upregulated upon RA treatment are involved in nucleotide and metal ion binding.

When we look at the involvement of the regulated proteins in biological processes, more differences were observed (Figure 6). Both treatments showed downregu-

Table 1 Non-redundant proteins

Protein name	Gene name	Swiss-prot	Nominal mass	CPI	PMF-score	PMF sequence coverage	MS/MS-score	MS/MS-sequence coverage
Low molecular weight phosphotyrosine protein phosphatase	Acp1	PPAC_MOUSE	18 636	6.4	96	65	80	24
Actin, cytoplasmic 1	Actb	ACTB_MOUSE	42 052	5.2	170	70	312	15
Aminoacylase-1	Acy1	ACY1_MOUSE	45 980	5.9	167	56	44	5
Aldose reductase	Akr1b1	ALDR_MOUSE	36 052	6.9	128	43	136	10
Aldehyde dehydrogenase, mitochondrial	Aldh2	ALDH2_MOUSE	57 015	8.6	221	54	131	7
Annexin A3	Anxa3	ANXA3_MOUSE	36 520	5.2	84	47	111	14
Adenine phosphoribosyltransferase	Aprt	APT_MOUSE	19 883	6.4	88	67	216	27
Rho GDP-dissociation inhibitor 1	Arhgdia	GDIR1_MOUSE	23 450	5	123	54	66	11
ATP synthase subunit α , mitochondrial	Atp5a1	ATPA_MOUSE	59 830	9.7	100	28	53	4
ATP synthase subunit β , mitochondrial	Atp5b	ATPB_MOUSE	56 265	5.1	90	30	167	10
ATP synthase subunit d, mitochondrial	Atp5h	ATP5H_MOUSE	18 795	5.4	122	70	169	36
F-actin-capping protein subunit α -2	Capza2	CAZA2_MOUSE	33 118	5.5	148	69	19	9
F-actin-capping protein subunit β	Capzb	CAPZB_MOUSE	31 611	5.4	117	61	129	8
Chromobox protein homolog 3	Cbx3	CBX3_MOUSE	21 013	5	38	36	67	6
T-complex protein 1 subunit β	Cct2	TCPB_MOUSE	57 783	6	248	61	75	9
T-complex protein 1 subunit epsilon	Cct5	TCPE_MOUSE	60 042	5.7	186	60	138	6
Cofilin-1	Cfl1	COF1_MOUSE	18 776	9.1	95	45	87	13
UMP-CMP kinase	Cmpk1	KCY_MOUSE	22 379	5.6	74	52	29	10
Coactosin-like protein	Cotl1	COTL1_MOUSE	16 048	5.1	86	60	116	14
Cathepsin D	Ctsd	CATD_MOUSE	45 381	6.9	160	41	95	4
Dihydrolipoyl dehydrogenase, mitochondrial	Dld	DLDH_MOUSE	54 751	9	112	48	81	2
Elongation factor 1- α 1	Eef1a1	EF1A1_MOUSE	50 424	9.7	68	34	115	8
Elongation factor 1- δ	Eef1d	EF1D_MOUSE	31 388	4.8	86	54	79	9
Elongation factor 2	Eef2	EF2_MOUSE	96 222	6.4	52	26	29	1
Eukaryotic translation initiation factor 3 subunit F	Eif3f	EIF3F_MOUSE	38 090	5.2	109	45	106	14
Eukaryotic translation initiation factor 3 subunit G	Eif3g	EIF3G_MOUSE	35 901	5.6	54	35	23	7
Eukaryotic translation initiation factor 3 subunit I	Eif3i	EIF3I_MOUSE	36 837	5.3	228	78	89	16
Eukaryotic translation initiation factor 4H	Eif4h	IF4H_MOUSE	27 381	7.5	83	51	65	8
Eukaryotic translation initiation factor 5A-1	Eif5a	IF5A1_MOUSE	17 049	4.9	115	58	170	22
α -enolase	Eno1	ENOA_MOUSE	47 453	6.4	183	64	170	13
Electron transfer flavoprotein subunit α , mitochondrial	EtfA	ETFA_MOUSE	35 330	9.5	138	59	100	9
Fatty acid-binding protein, heart	Fabp3	FABPH_MOUSE	14 810	6.1	86	77	212	39
Peptidyl-prolyl cis-trans isomerase FKBP4	Fkbp4	FKBP4_MOUSE	51 939	5.4	122	38	168	9
Fascin	Fscn1	FSCN1_MOUSE	55 215	6.5	129	45	26	6
Glyceraldehyde-3-phosphate dehydrogenase	Gapdh	G3P_MOUSE	36 072	9.2	62	38	40	8
Lactoylglutathione lyase	Glo1	LGUL_MOUSE	20 967	5.1	134	66	114	20
Glyoxalase domain-containing protein 4	Glod4	GLOD4_MOUSE	33 581	5.2	167	69	115	13
Glutamate dehydrogenase 1, mitochondrial	Glud1	DHEF3_MOUSE	61 640	8.8	70	37	60	5
Guanine nucleotide-binding protein subunit β -2-like 1	Gnb2l1	GBLP_MOUSE	35 511	8.9	116	55	20	5
Growth factor receptor-bound protein 2	Grb2	GRB2_MOUSE	25 336	5.9	73	54	36	17
Histone H2B type 1-B	Hist1h2bb	H2B1B_MOUSE	13 944	10.8	52	41	93	19
Histone H2A type 2-C	Hist2h2ac	H2A2C_MOUSE	13 980	11.4	50	55	67	12
Heterogeneous nuclear ribonucleoprotein A/B	Hnmpab	ROAA_MOUSE	30 926	8.7	83	30	107	5
Heterogeneous nuclear ribonucleoproteins C1/C2	Hnmpc	HNRPC_MOUSE	34 421	4.8	57	32	57	6
Heterogeneous nuclear ribonucleoprotein F	Hnmpf	HNRPF_MOUSE	46 043	5.2	163	56	207	12
Heterogeneous nuclear ribonucleoprotein H	HnmpH1	HNRH1_MOUSE	49 454	5.9	166	61	134	15
Heterogeneous nuclear ribonucleoprotein K	Hnmpk	HNRPK_MOUSE	51 230	5.3	144	46	251	11
Heat shock protein HSP 90- α	Hsp90aa1	HS90A_MOUSE	85 134	4.8	131	31	130	5
Heat shock protein HSP 90- β	Hsp90ab1	HS90B_MOUSE	83 615	4.8	62	25	78	6
Heat shock 70 kDa protein 4	Hspa4	HSP74_MOUSE	94 872	5	242	54	102	3
78 kDa glucose-regulated protein	Hspa5	GRP78_MOUSE	72 492	4.9	78	25	122	5
Heat shock cognate 71 kDa protein	Hspa8	HSP7C_MOUSE	71 055	5.2	234	58	154	4
Stress-70 protein, mitochondrial	Hspa9	GRP75_MOUSE	73 768	5.8	219	50	272	7
Heat shock protein β -1	Hspb1	HSPB1_MOUSE	23 057	6.1	144	55	344	24
60 kDa heat shock protein, mitochondrial	Hspd1	CH60_MOUSE	61 088	5.8	334	69	232	10
Isocitrate dehydrogenase (NAD) subunit α , mitochondrial	Idh3a	IDH3A_MOUSE	40 069	6.3	70	31	158	12
Inosine-5'-monophosphate dehydrogenase 2	Impdh2	IMDH2_MOUSE	56 179	7	173	50	107	7
Inosine triphosphate pyrophosphatase	Itpa	ITPA_MOUSE	22 225	5.5	84	72	128	15
Keratin, type I cytoskeletal 18	Krt18	K1C18_MOUSE	47 509	5.1	199	65	58	9
Keratin, type II cytoskeletal 7	Krt7	K2C7_MOUSE	50 678	5.6	137	52	55	4
Keratin, type II cytoskeletal 8	Krt8	K2C8_MOUSE	54 531	5.6	245	55	237	9
Cytosol aminopeptidase	Lap3	AMPL_MOUSE	56 505	8.7	126	47	58	5
L-lactate dehydrogenase B chain	Ldhb	LDHB_MOUSE	36 834	5.6	84	46	30	6

Galectin-1	Lgals1	LEG1_MOUSE	15 198	5.2	109	70	172	25
Galectin-2	Lgals2	LEG2_MOUSE	14 984	7.9	120	88	60	14
Lamin-B1	Lmnbl	LMNB1_MOUSE	66 973	5	265	60	134	5
α -2-macroglobulin receptor-associated protein	Lrpap1	AMRP_MOUSE	42 189	7.9	139	49	177	14
S-adenosylmethionine synthase isoform type-2	Mat2a	METK2_MOUSE	44 003	6	73	41	59	3
28S ribosomal protein S22, mitochondrial	Mrps22	RT22_MOUSE	41 281	9.2	112	45	97	9
Myosin-9	Myh9	MYH9_MOUSE	227 429	5.4	73	15	103	2
Nucleolin	Ncl	NUCL_MOUSE	76 734	4.5	113	26	179	7
Omega-amidase NIT2	Nit2	NIT2_MOUSE	30 825	6.5	112	59	75	9
Nucleoside diphosphate kinase A	Nme1	NDKA_MOUSE	17 311	7.7	125	72	223	30
Nucleoside diphosphate kinase B	Nme2	NDKB_MOUSE	17 466	7.8	160	84	287	30
Nucleophosmin	Npm1	NPM_MOUSE	32 711	4.5	53	33	136	10
Nuclear pore complex protein Nup54	Nup54	NUP54_MOUSE	55 812	6.6	55	21	23	3
Nuclear pore glycoprotein p62	Nup62	NUP62_MOUSE	53 336	5.1		13	43	5
Ornithine aminotransferase, mitochondrial	Oat	OAT_MOUSE	48 723	6.2	174	64	125	9
Poly(rC)-binding protein 1	Pcbp1	PCBP1_MOUSE	37 987	6.8	175	69	115	12
Protein disulfide-isomerase A3	Pdia3	PDIA3_MOUSE	57 099	5.8	254	55	90	5
Protein disulfide-isomerase A6	Pdia6	PDIA6_MOUSE	48 469	4.9	75	40	47	2
PDZ and LIM domain protein 1	Pdlim1	PDL1_MOUSE	36 208	6.4	200	73	56	7
Phosphatidylethanolamine-binding protein 1	Pebp1	PEBP1_MOUSE	20 988	5.1	130	79	107	11
Phosphoglycerate mutase 1	Pgam1	PGAM1_MOUSE	28 928	6.8	157	66	192	21
Phosphoglycerate kinase 1	Pgk1	PGK1_MOUSE	44 921	9	136	52	128	7
6-phosphogluconolactonase	Pgl	6PGL_MOUSE	27 465	5.5	102	49	148	17
Pyruvate kinase isozymes M1/M2	Pkm2	KPYM_MOUSE	58 378	7.9	178	49	106	9
Purine nucleoside phosphorylase	Pnp	PNPH_MOUSE	32 541	5.8	119	67	138	13
Inorganic pyrophosphatase	Ppa1	IPYR_MOUSE	33 102	5.3	126	66	26	7
Peroxiredoxin-2	Prdx2	PRDX2_MOUSE	21 936	5.1	103	62	285	22
Peroxiredoxin-6	Prdx6	PRDX6_MOUSE	24 969	5.6	156	67	101	17
Proteasome subunit α type-1	Psma1	PSA1_MOUSE	29 813	6	71	52	140	17
Proteasome subunit α type-6	Psma6	PSA6_MOUSE	27 811	6.4	72	38	108	10
Proteasome subunit β type-3	Psmb3	PSB3_MOUSE	23 235	6.2	110	51	187	30
Proteasome subunit β type-4	Psmb4	PSB4_MOUSE	29 211	5.3	60	42	109	10
26S protease regulatory subunit 7	Psmc2	PRS7_MOUSE	49 016	5.6	166	60	72	8
26S protease regulatory subunit 6B	Psmc4	PRS6B_MOUSE	47 366	5	144	55	109	9
GTP-binding nuclear protein Ran	Ran	RAN_MOUSE	24 579	7.8	124	51	139	11
40S ribosomal protein S12	Rps12	RS12_MOUSE	14 858	7.7	77	62	95	11
RuvB-like 1	Ruvbl1	RUVB1_MOUSE	50 524	6	61	35	106	10
Protein S100-A11	S100a11	S10AB_MOUSE	11 247	5.1		36	147	27
Splicing factor, arginine/serine-rich 1	Sfrs1	SFRS1_MOUSE	27 842	10.8	80	43	156	18
Splicing factor, arginine/serine-rich 3	Sfrs3	SFRS3_MOUSE	19 546	12.3			87	14
Serine hydroxymethyltransferase, cytosolic	Shmt1	GLYC_MOUSE	53 065	6.5	98	43	19	2
Superoxide dismutase [Cu-Zn]	Sod1	SODC_MOUSE	16 104	6	83	45	126	31
Spermidine synthase	Srm	SPEE_MOUSE	34 543	5.2	141	73	129	15
Stress-induced-phosphoprotein 1	Stip1	STIP1_MOUSE	63 170	6.4	184	55	89	4
Stathmin	Stmn1	STMN1_MOUSE	17 264	5.7	28	24	69	8
Stomatin-like protein 2	Stoml2	STML2_MOUSE	38 475	9.5	144	61	165	15
TAR DNA-binding protein 43	Tardbp	TADBP_MOUSE	44 918	6.3	68	30	107	7
T-complex protein 1 subunit α	Tcp1	TCPA_MOUSE	60 867	5.8	61	27	28	4
Transcription intermediary factor 1- β	Trim28	TIF1B_MOUSE	90 558	5.4		10	139	4
Tubulin α -1B chain	Tuba1b	TBA1B_MOUSE	50 804	4.8	128	39	152	9
Tubulin α -1C chain	Tuba1c	TBA1C_MOUSE	50 562	4.8	53	24	52	6
Tubulin β -2A chain	Tubb2a	TBB2A_MOUSE	50 274	4.6	126	55	111	11
Tubulin β -2C chain	Tubb2c	TBB2C_MOUSE	50 255	4.6	150	56	49	8
Tubulin β -5 chain	Tubb5	TBB5_MOUSE	50 095	4.6	169	57	237	9
Thioredoxin	Txn	THIO_MOUSE	12 010	4.6	63	67	92	22
Thioredoxin-like protein 1	Txn1l	TXNL1_MOUSE	32 616	4.7	144	78	39	2
Ubiquitin-conjugating enzyme E2 N	Ube2n	UBE2N_MOUSE	17 184	6.2	119	71	20	6
Ubiquitin carboxyl-terminal hydrolase isozyme L1	Uchl1	UCHL1_MOUSE	25 165	5	77	64	16	8
Cytochrome b-c1 complex subunit 1, mitochondrial	Uqcrc1	QCR1_MOUSE	53 420	5.7	95	40	46	6
Transitional endoplasmic reticulum ATPase	Vcp	TERA_MOUSE	89 950	5	310	61	40	5
Voltage-dependent anion-selective channel protein 1	Vdac1	VDAC1_MOUSE	32 502	9.2	159	57	80	24
Vimentin	Vim	VIME_MOUSE	53 712	4.9	218	64	47	8

CPI: Calculated isoelectric point; PMF: Peptide mass fingerprint; MS/MS: Tandem mass spectrometry.

lation of proteins involved in protein complex biogenesis, nucleotide biosynthetic process, cell death and positive regulation of biosynthetic process. Additionally, proteins involved in proteolysis and positive regulation of protein

metabolic process were downregulated in SCs upon CPX treatment. Proteins which were downregulated in SCs upon RA treatment are, among others, involved in cell cycle, RNA processing, glycolysis and negative regulation

Table 2 Gene Ontology functional annotation of proteins which were regulated in this experiment according to their involvement in different biological processes

Biological process	Proteins	CPX > RA	CPX < RA	CPX > c	CPX < c	RA > c	RA < c
Monosaccharide metabolic/ catabolic processes	5	Pgls Gapdh Eno1 Pkm2	Eno1		Eno1 Pkm2		Eno1 Ldhd Pkm2
Nucleobase, nucleoside, nucleotide, and nucleic acid biosynthetic processes	7	Atp5a1	Aprt Nme2 Nme1	Atp5a1	Aprt Atp5a1 Impdh2 Nme2 Nme1 Pnp	Nme2 Nme1	Aprt Atp5a1 Atp5h Impdh2
RNA and mRNA processing	6	Sfrs1			Hnrnpk Tardbp Pcbp1		Hnrnpc Sfrs3
Regulation of transcription	7	Ruvbl1 Trim28		Nup62	Ube2n Tardbp Cbx3 Hnrnpab		Trim28 Ruvbl1
Embryonic development	5	Sfrs1 Eno1	Psmc4 Eno1	Atp5a1 Myh9	Eno1 Atp5a1	Myh9 Psmc4	Eno1
Gene expression	16	Trim28 Sfrs1 Ruvbl1	Rps12	Eif3f Eif3i	Eef1a1 Eif5a Eef1d Cbx3 Hnrnpk Hnrnpab Tardbp Pcbp1		Sfrs3 Eif3i Hnrnpc Ruvbl1 Eef1d Trim28
Cell cycle processes	6	Ruvbl1		Myh9		Krt7 Myh9	Npm1 Tubb5 Stmn1 Ruvbl1
Cell morphogenesis involved in differentiation	4	Trim28		Myh9	Uchl1 Hnrnpab	Myh9	Stmn1 Trim28
Regulation of cell proliferation	4			Nup62	Nme2 Pnp	Nme2	Npm1
Regulation of signal transduction	4			Nup62	Ube2n		Npm1 Hspa5

Ciclopirox olamine (CPX) > retinoic acid (RA): Proteins which were more than 2-fold higher expressed in CPX-treated cells compared to RA-treated cells; CPX < RA: Proteins which were more than 2-fold higher expressed in RA-treated cells compared to CPX-treated cells; CPX > c: Proteins which were more than 2-fold higher expressed in CPX-treated cells compared to control; CPX < c: Proteins which were more than 2-fold higher expressed in control compared to CPX-treated cells; RA > c: Proteins which were more than 2-fold higher expressed in RA-treated cells compared to control; RA < c: Proteins which were more than 2-fold higher expressed in control compared to RA-treated cells.

of protein metabolic process.

Proteins which were upregulated in SCs upon CPX treatment are involved in nucleotide binding, regulation of cell death and protein transport, whereas proteins which were upregulated upon RA treatment are involved in nucleotide binding, metal ion binding and proteolysis.

Proteins in CPX treated cells compared to RA treated cells

When the proteins in RA treated SCs were compared to CPX treated SCs, we observed that 54 proteins are differently regulated (Tables 8 and 9). Of these proteins, 31 were upregulated and 26 downregulated upon CPX treatment. In some cases, different forms of one protein, e.g., Actb, Eno1, and Hsp90aa1 were observed and showed different regulation.

The bioinformatics analysis of these proteins, focus-

ing on biological processes, showed involvement of the proteins in different categories (Figure 7). Proteins which were downregulated in CPX treated cells are involved in processes like protein complex biogenesis, nucleotide biosynthetic process, proteolysis, intracellular transport and regulation of cell death. Proteins which were downregulated as a reaction to RA treatment are involved in protein complex biogenesis, cell death, positive regulation of biosynthetic process, response to organic substance, glycolysis, anti-apoptosis and phosphorylation.

To get a better focus on proteins, which may play a key role in proliferation, we also focussed on proteins, which showed contrary regulation upon CPX treatment and RA treatment compared to control. This resulted in 15 proteins, of which eight were upregulated upon CPX treatment and concurrently downregulated upon RA treatment compared to control, and seven proteins,

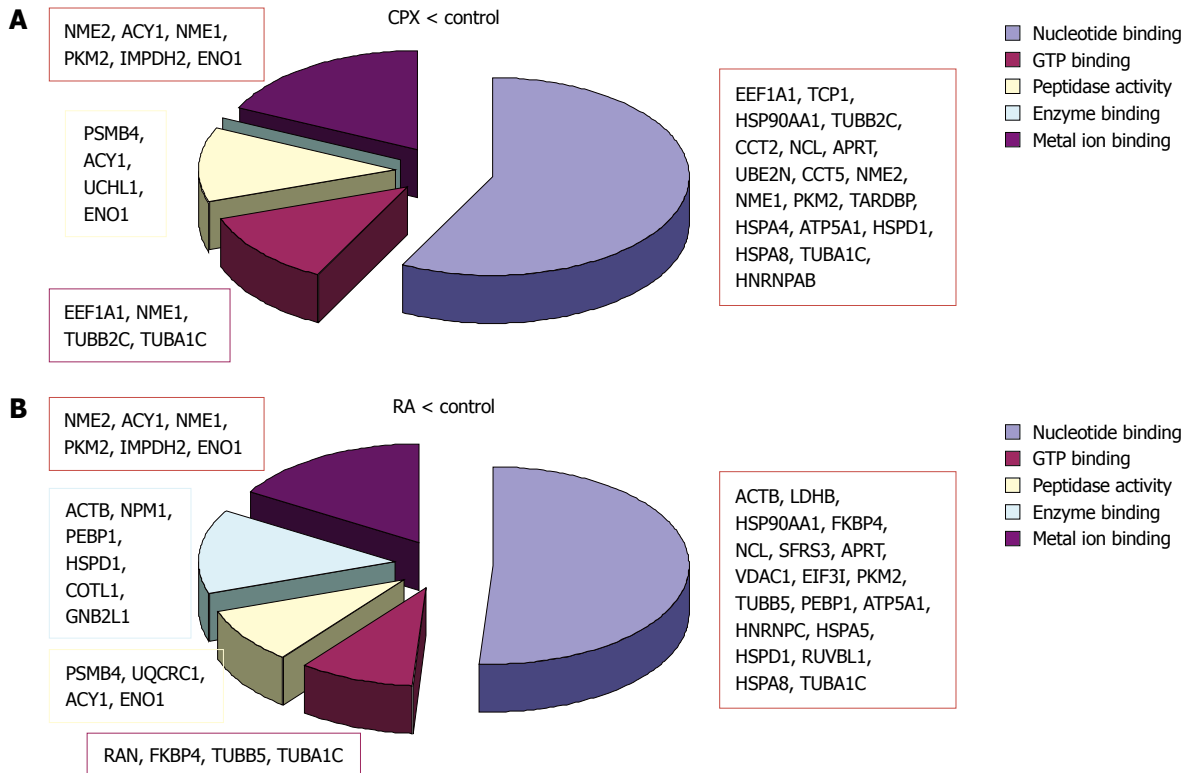


Figure 5 Molecular function. Classification of the downregulated proteins upon treatment with ciclopirox olamine (CPX) (A) or retinoic acid (RA) (B) according to their molecular functions.

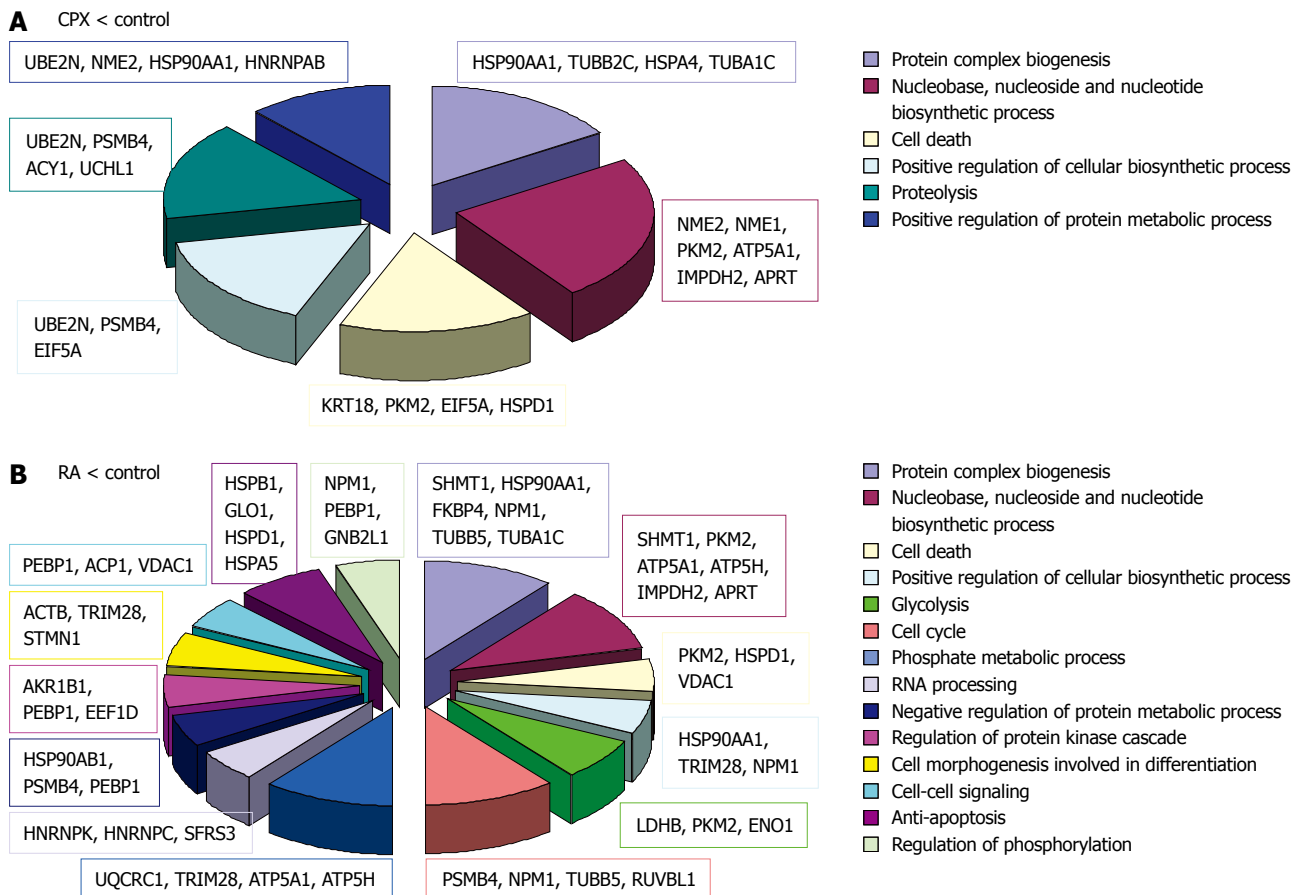


Figure 6 Biological process. Classification of the downregulated proteins upon treatment with ciclopirox olamine (CPX) (A) or retinoic acid (RA) (B) according to their biological processes.

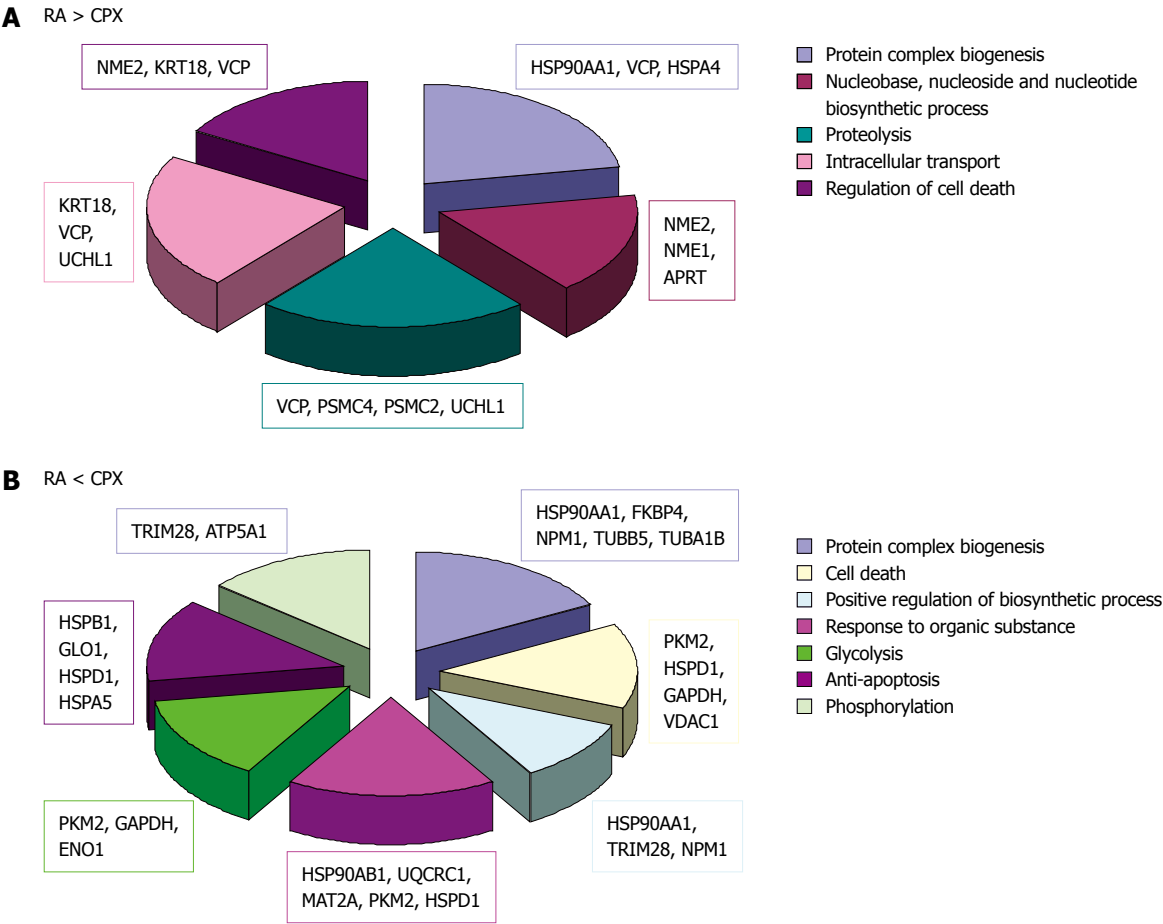


Figure 7 Biological process. Classification of the differently regulated proteins upon treatment with ciclopirox olamine (CPX) (A) or retinoic acid (RA) (B) according to their biological processes.

Table 3 Proteins which are upregulated upon ciclopirox olamine treatment compared to control		
	k/CPX	
	ESC	maGSC
Actb	0.13	0.19
Atp5a1 ¹	0.41	0.54
Ctsd	0.97	0.09
Eif3f ¹	0.94	0.43
Eif3i ¹	0.49	0.95
Etfp	0.63	0.39
Hspa9	0.92	0.31
Hspb1	0.63	0.05
Hspd1	0.19	0.21
Hspd1	0.36	0.69
Myh9 ¹	0.63	0.09
Nup62 ¹	0.30	0.60
S100a11		0.21
Tubb2a	1.00	0.21
Vdac1	0.58	0.18

¹The proteins are referred to in the text and following tables. CPX: Ciclopirox olamine; ESC: Embryonic stem cell; maGSC: Multipotent adult germline stem cell.

which were downregulated upon CPX treatment and concurrently upregulated upon RA treatment compared to control (proteins marked by asterisk in Tables 8 and 9).

Bioinformatics analysis of the proteins, which were downregulated upon CPX treatment along with upregulated upon RA treatment were primarily involved in metabolic processes (Nme2, Hsp90aa1, Psmc4, Rps12, Cct2 and Eno1) like protein folding (Hsp90aa1, Cct2), whereas proteins, which were upregulated upon CPX-treatment and concurrently downregulated upon RA-treatment were additionally involved in developmental processes (Psmc4, Eno1) and transport/localization (Vdac1, Hspa9).

Analysis of the molecular function of the differently regulated proteins upon CPX and RA treatment showed their important role in nucleotide binding (Nme2, Hsp90aa1, Psmc4, Hspa4, Cct2, Actb, Pkm2, Hspa5, Vdac1 and Hspa9) and metal ion binding (Pkm2, S100a11, Eno1).

DISCUSSION

CPX is a synthetic antifungal drug, which is currently used for the treatment of superficial mycoses^[41]. Since two decades CPX has also been used as an antitumor agent^[42]. It has been shown that CPX can be used to treat solid tumors due to its strong antiangiogenic activity^[43,23]. CPX might inhibit the cell proliferation and work as an antitumor agent due to its iron chelating function, as iron is essential for cell proliferation and function^[24]. In

Table 4 Proteins which are downregulated upon ciclopirox olamine treatment compared to control

	k/CPX	
	ESC	maGSC
Acp1	1.29	6.01
Acy1 ¹	1.38	2.81
Akr1b1	2.07	13.44
Aprt ¹	4.80	3.60
Atp5a1 ¹	3.50	1.21
Capzb	3.04	2.35
Cbx3 ¹	1.72	2.12
Cct2 ¹	12.00	1.28
Cct5 ¹	1.06	2.02
Eef1a1 ¹	2.47	1.74
Eef1d ¹	1.46	2.03
Eif5a ¹	1.31	2.07
Eno1 ¹	3.56	1.60
Fscn1	3.31	1.49
Glod4	3.35	1.60
Gnb2l1	2.61	12.92
Hist1h2bb	2.31	2.10
Hist2h2ac	17.33	67.90
Hnrnpab ¹	2.41	3.36
Hnrnpk ¹	2.00	1.17
Hsp90aa1 ¹	1.19	6.79
Hsp90aa1 ¹	1.84	3.02
Hspa4 ¹	3.28	1.51
Hspa4	1.13	3.14
Hspa8	1.74	7.17
Hspd1	1.67	3.07
Impdh2 ¹	2.59	2.13
Impdh2 ¹	> 100	27.94
Krt18 ¹	2.31	1.44
Lgals2	2.82	8.45
Ncl ¹	1.33	2.61
Nit2	1.24	2.13
Nme1 ¹	6.25	1.56
Nme2 ¹	4.77	4.51
Pcbp1 ¹	2.21	1.64
Pkm2 ¹	3.75	3.27
Pnp ¹	1.20	2.62
Psmb4 ¹	1.01	2.32
Ruvbl1 ¹	1.02	2.14
Srm	1.64	3.63
Shmt1 ¹	> 100	> 100
Tardbp ¹	1.38	3.52
Tcp1	1.47	3.76
Tuba1c ¹	1.87	3.11
Tubb2c ¹	1.38	3.41
Ube2n ¹	1.31	6.45
Uchl1 ¹	2.66	1.66

¹The proteins are referred to in the text and following tables. CPX: Ciclopirox olamine; ESC: Embryonic stem cell; maGSC: Multipotent adult germline stem cell.

a recent study, we investigated the effect of CPX on the cellular viability and proliferation of SCs. The study demonstrated that in contrast to RA, CPX treatment resulted in a reversible antiproliferative effect^[8]. The present study was conducted to understand the anti-proliferative effect of CPX on stem cells in terms of proteins and molecular processes which are involved in its mode of action.

With proteomic analysis of ESCs and maGSCs treated with CPX and RA, we could identify more than 90 single proteins which were differently expressed in both cell

Table 5 Proteins which are downregulated upon retinoic acid treatment compared to control

Label	RA/k	
	ESC	maGSC
Acp1	0.61	0.50
Actb ¹	0.53	0.13
Acy1 ¹	0.13	0.70
Akr1b1	0.43	0.11
Aprt ¹	0.46	0.39
Atp5a1 ¹	0.76	0.38
Atp5h ¹	0.69	0.40
Cbx3	1.01	0.47
Cotl1 ¹	0.50	0.44
Eef1d ¹	0.70	0.15
Eif3i ¹	0.09	0.92
Eno1 ¹	0.24	0.04
Eno1 ¹	0.55	0.22
Fabp3		0.45
Fkbp4 ¹	0.90	0.40
Glo1 ¹	0.74	0.41
Glod4	0.82	0.30
Impdh2 ¹	0.76	0.35
Impdh2 ¹	0.54	0.20
Gnb2l1 ¹	0.66	0.15
Hnrnpc ¹	0.76	0.43
Hsp90aa1	0.75	0.08
Hsp90aa1	0.49	0.06
Hsp90aa1	0.76	0.12
Hspa5 ¹	0.32	0.22
Hspa8 ¹	0.69	0.50
Hspb1 ¹	0.36	0.47
Hspb1 ¹	0.46	0.88
Hspb1 ¹	0.90	0.41
Hspd1 ¹	0.16	0.67
Hspd1 ¹	0.34	0.95
Itpa	0.57	0.07
Ldhab ¹	0.42	0.43
Lgals2	0.29	0.03
Ncl ¹	0.26	0.71
Npm1 ¹	0.46	0.04
Pebp1 ¹	0.89	0.42
Pkm2 ¹	0.38	0.15
Pkm2 ¹	0.32	0.65
Pkm2 ¹	0.42	0.76
Pkm2 ¹	0.21	0.43
Psmb4 ¹	0.62	0.43
Ruvbl1 ¹	0.63	0.22
Sfrs3 ¹	0.41	0.46
Shmt1 ¹	0.01	0.00
Srm	0.68	0.24
Trim28 ¹	0.23	0.11
Trim28 ¹	0.40	0.37
Tuba1c ¹	0.27	0.71
Tubb5 ¹	0.70	0.25
Uqcr1 ¹	0.24	0.22
Vdac1 ¹	0.30	0.52

¹The proteins are referred to in the text and following tables. RA: Retinoic acid; ESC: Embryonic stem cell; maGSC: Multipotent adult germline stem cell.

lines. Bioinformatics analysis of the regulated proteins demonstrated their involvement in various biological processes. To our interest, a number of proteins have potential roles in the regulation of cell proliferation either directly or indirectly.

One of the possible mechanisms of CPX action on

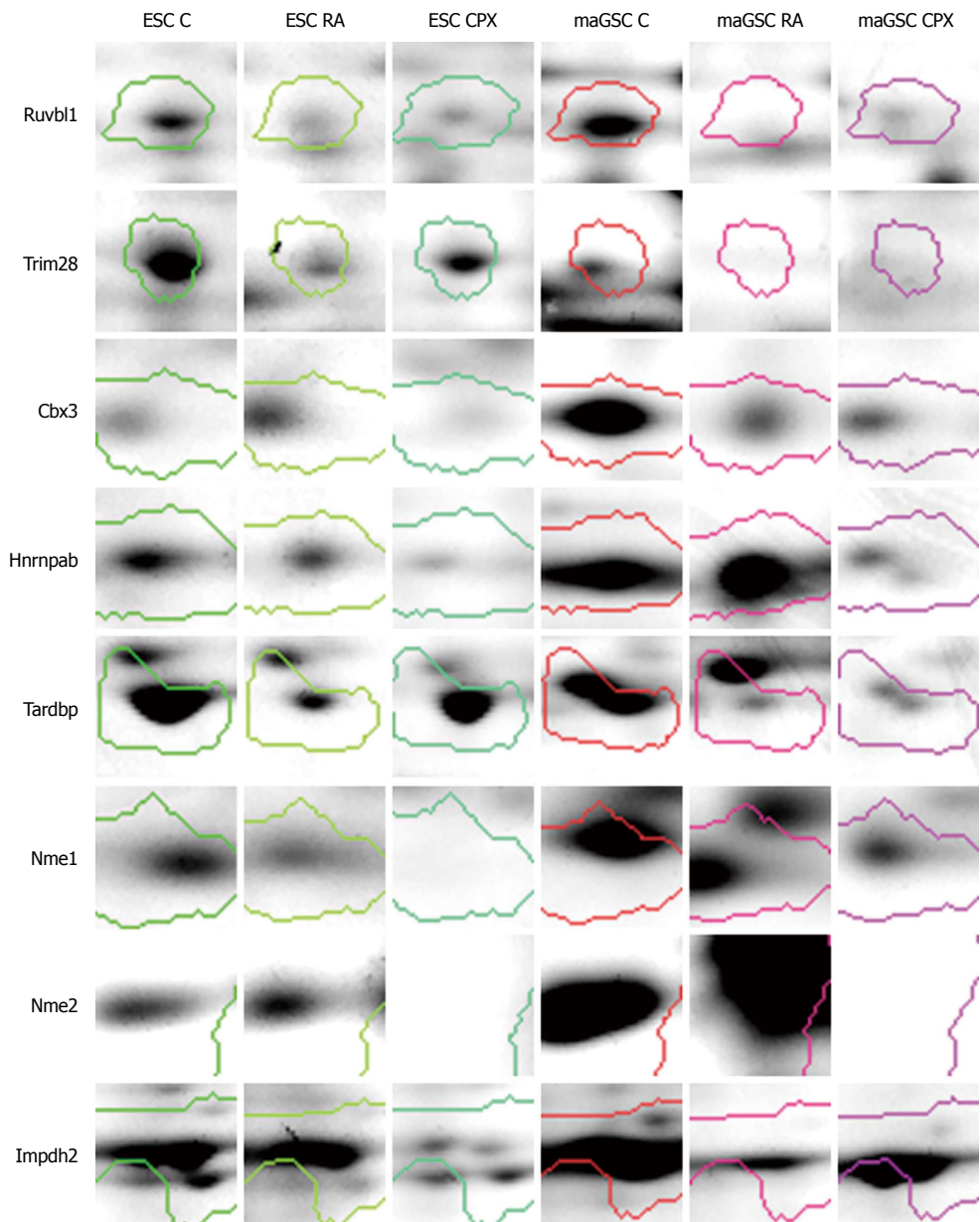


Figure 8 Enlargement of the gel spots of some proteins of interest. ESC: Embryonic stem cell; maGSC: Multipotent adult germline stem cell; CPX: Ciclopirox olamine; RA: Retinoic acid.

	RA/k	
	ESC	maGSC
Cct2	1.14	2.16
Hspa4	3.96	2.47
Krt7 ¹	1.01	38.11
Krt8	1.97	1.78
Myh9 ¹	3.06	3.24
Nme1 ¹	2.52	3.81
Nme2 ¹	1.20	2.48
Pdia6	1.72	20.48
Psmc4 ¹	2.17	2.17
Vcp	8.30	4.13
Vim ¹	3.85	1.16

¹The proteins are referred to in the text and following tables. RA: Retinoic acid; ESC: Embryonic stem cell; maGSC: Multipotent adult germline stem cell.

cell proliferation is through controlling the progression of the cell cycle^[44]. We identified a number of proteins which are involved in cell cycle processes. Ruvbl1 is one of the differentially regulated proteins which is involved in cell cycle processes, gene expression and transcription regulation. It was found to be downregulated in CPX and RA treated cells compared to control (Figure 8). Ruvbl1 is an evolutionarily highly conserved eukaryotic protein belonging to the AAA+ family of ATPase^[45]. It plays an important role in various cell cycle processes such as chromatin remodeling^[46], gene activation^[47], transcriptional regulation, DNA repair and transcription factor c-Myc^[48]. It also controls Wnt signaling pathway through transcription-associated protein β -catenin^[49,50]. Another protein, which was higher expressed in CPX treated cells compared to RA treated cells, is Trim28. Trim28 is involved in regulation of transcription and silencing gene

Table 7 Gene Ontology functional annotation of proteins which were regulated in this experiment according to their involvement in different molecular function

Molecular function	Proteins	CPX > RA	CPX < RA	CPX > c	CPX < c	RA > c	RA < c
Nucleotide binding	41	Hsp90ab1 Fkbp4 Tubb5 Hspa5 Tubb1b Gapdh Etfa Hspa9 Actb Hsp90aa1 Sfrs1 Vdac1 Pkm2 Atp5a1 Ruvbl1 Hspd1	Atp5b Cct2 Tardbp Hspa4 Actb Hsp90aa1 Ncl Aprt Nme2 Vcp Psmc4 Nme1 Psmc2	Tubb2a Etfa Hspa9 Actb Myh9 Vdac1 Atp5a1 Hspd1	Cct2 Tardbp Hspa4 Tuba1c Hspa8 Hnrnpab Eef1a1 Tcp1 Hsp90aa1 Ncl Aprt Ube2n Nme2 Cct5 Nme1 Pkm2 Atp5a1 Ruvbl1 Hspd1	Cct2 Hspa4 Myh9 Nme2 Vcp Psmc4 Nme1	Ldhb Fkbp4 Tubb5 Hnrnpc Hspa5 Tuba1c Hspa8 Actb Hsp90aa1 Ncl Sfrs3 Aprt Vdac1 Pkm2 Pebp1 Atp5a1 Ruvbl1 Hspd1
GTP binding	8	Fkbp4 Tubb5 Tuba1b	Nme1	Tubb2a	Eef1a1 Nme1 Tuba1c	Nme1	Fkbp4 Tubb5 Tuba1c
ATPase activity	8	Atp5a1	Vcp Psmc4 Atp5b Psmc2	Atp5a1 Myh9	Atp5a1 Hspa8	Vcp Psmc4 Myh9	Atp5a1 Atp5h Hspa8
Enzyme binding	8	Actb Npm1 Hspd1 Cotl1 Hspa9	Actb Gnb2l1	Actb Hspd1 Hspa9	Gnb2l1	Vim	Actb Pebp1 Hspd1 Cotl1
Cofactor binding	5	Gapdh Etfa		Etfa	Shmt1		Ldhb Shmt1
Peptidase activity	6	Ctsd Eno1	Uchl1 Eno1	Ctsd	Psmb4 Acy1 Uchl1 Eno1		Psmb4 Uqcrc1 Acy1 Eno1
Metal ion binding	13	Trim28 Sfrs1 Pkm2 Glo1 Eno1	Atp5b Pdia6 Nme2 Nme1 Eno1		Acy1 Nme2 Nme1 Pkm2 Impdh2 Eno1	Pdia6 Nme2 Nme1	Acy1 Uqcrc1 Trim28 Pkm2 Glo1 Impdh2 Eno1

Ciclopirox olamine (CPX) > Retinoic acid (RA): Proteins which were more than 2-fold higher expressed in CPX-treated cells compared to RA-treated cells; CPX < RA: Proteins which were more than 2-fold higher expressed in RA-treated cells compared to CPX-treated cells; CPX > c: Proteins which were more than 2-fold higher expressed in CPX-treated cells compared to control; CPX < c: Proteins which were more than 2-fold higher expressed in control compared to CPX-treated cells; RA > c: Proteins which were more than 2-fold higher expressed in RA-treated cells compared to control; RA < c: Proteins which were more than 2-fold higher expressed in control compared to RA-treated cells.

expression through its ability to bind to DNA through interaction with a KRAB-ZFP protein. Other proteins, like Cbx3, Tardbp, and Hnrnpab, which are important in gene expression and regulation of transcription, were down-regulated due to treatment with CPX. Tardbp is a DNA and RNA-binding protein, which regulates transcription and splicing. It is also involved in the regulation of CFTR (Cystic fibrosis transmembrane conductance regulator), microRNA biogenesis, apoptosis and cell division. It can repress HIV-1 transcription by binding to the HIV-1 long terminal repeat. Cbx3 seems to be involved in transcriptional silencing in heterochromatin-like complexes. It

recognizes and binds histone H3 tails methylated at K9, which leads to epigenetic repression. It is suggested that these proteins, which are involved in cell cycle processes, transcription regulation and gene expression, might be potential candidates for cell proliferation regulation and their repression through down-regulation might result in cell cycle stop without impact on stem cell pluripotency.

Proteins, which are involved in nucleotide biosynthetic process and proteolysis, were downregulated in CPX treated cells compared to control, as well as in RA treated cells (Figures 6A and 7A). Nucleoside diphosphatase kinases A and B (Nme1 and Nme2) are some of the

Table 8 Proteins which are upregulated in stem cells upon ciclopirox olamine treatment compared to retinoic acid treatment

	RA/CPX	
	ESC	maGSC
Actb ^{*1}	0.12	0.10
Actb ¹	0.14	0.15
Atp5a1 ¹	0.43	0.48
Cotl1	0.19	0.67
Ctsd	0.95	0.16
Eif3i	0.04	0.87
Eno1 ^{*1}	0.13	0.03
Eno1 ¹	0.57	0.40
Etfa	0.68	0.16
Fkbp4 ¹	0.46	0.43
Gapdh ¹	0.35	0.59
Glo1	0.31	0.66
Glod4	0.85	0.36
Hsp90aa1	0.28	0.38
Hsp90aa1	0.43	0.13
Hsp90ab1	0.41	0.26
Hspa5 ^{*1}	0.17	0.13
Hspa9 ^{*1}	0.50	0.28
Hspb1 ^{*1}	0.29	0.04
Hspb1 ^{*1}	0.20	0.42
Hspb1 ¹	0.82	0.50
Hspd1 ¹	0.49	0.74
Hspd1 ¹	0.42	0.33
Itpa	0.21	0.08
Mat2a ¹	0.41	0.14
Npm1 ¹	0.28	0.18
Nup62	0.57	0.26
Pgls ¹	0.40	0.68
Pkm2 ¹	0.22	0.25
Pkm2 ¹	0.29	0.86
Prdx6	0.46	0.94
Ruvbl1 ¹	0.64	0.42
S100a11 ^{*1}	²	0.17
Sfrs1 ¹	0.70	0.30
Trim28 ¹	0.44	0.08
Trim28 ¹	0.42	0.44
Trim28 ¹	0.33	0.28
Tuba1b ^{*1}	0.42	0.65
Tubb5 ¹	0.42	0.54
Tubb5 ¹	0.43	0.58
Vdac1 ^{*1}	0.17	0.09

¹The proteins are referred to in the text and following tables; ²The protein was not identified in embryonic stem cells. Proteins, which are assigned an asterisk, were upregulated upon ciclopirox olamine (CPX) treatment compared to control and concurrently downregulated upon retinoic acid (RA) treatment compared to control. ESC: Embryonic stem cell; maGSC: Multipotent adult germline stem cell.

proteins which are involved in nucleotide biosynthetic process. These proteins are known to be involved in the synthesis of nucleoside triphosphatases^[51] as well as in cell proliferation^[52], differentiation^[53] and development^[54], signal transduction, G protein-coupled receptor endocytosis and gene expression. Nme1 was downregulated in CPX treated cells compared to control and RA treated cells (Figure 8). This may explain the slowdown of the proliferation of CPX treated SCs. Impdh2 is a rate limiting enzyme in the *de novo* synthesis of guanine nucleotides and is therefore involved in the regulation of cell growth

Table 9 Proteins which are downregulated upon ciclopirox olamine treatment compared to retinoic acid treated stem cells

	RA/CPX	
	ESC	maGSC
Actb ¹	2.17	1.10
Aldh2	2.61	2.17
Aldh2	2.43	1.21
Aprt ¹	2.20	1.42
Atp5b	1.15	2.17
Capzb	2.79	2.07
Cct2 ^{*1}	13.70	2.77
Eno1 ¹	2.48	2.02
Eno1 ¹	2.71	1.51
Fscn1	2.20	2.14
Gnb2l1	1.81	2.24
Hist1h2bb	7.53	1.62
Hist2h2ac	3.89	211.81
Hnrnpk	2.37	1.58
Hsp90aa1 ^{*1}	2.69	6.36
Hsp90aa1	6.36	4.33
Hspa4 ^{*1}	12.98	3.72
Hspa4	1.35	3.42
Krt7	> 100	1.14
Krt18 ^{*1}	4.04	1.76
Ncl	1.80	3.48
Nme1 ¹	2.63	1.57
Nme2 ^{*1}	5.72	11.15
Pdia6	1.74	6.33
Psmc2 ¹	2.87	1.20
Psmc4 ^{*1}	3.06	2.26
Rps12 ^{*1}	²	2.05
Tardbp	1.11	3.85
Uchl1 ¹	2.02	1.10
Vcp ¹	8.94	2.57

¹The proteins are referred to in the text and following tables; ²The protein was not identified in embryonic stem cells. Proteins, which are assigned an asterisk, were downregulated upon ciclopirox olamine (CPX) treatment compared to control and concurrently upregulated upon retinoic acid (RA) treatment compared to control. ESC: Embryonic stem cell; maGSC: Multipotent adult germline stem cell.

and differentiation^[55-58]. It may have a role in the development of malignancy and the growth progression of some tumors. Impdh2 was downregulated in CPX treated cells compared to control (Figure 8).

Proteins which were involved in cell death, positive regulation of biosynthetic process, response to organic substance, glycolysis, anti-apoptosis, and phosphorylation were downregulated in RA treated cells compared to control and CPX treated cells (Figures 6B and 7B).

Analysis of the molecular function of the differently expressed proteins demonstrated a potential involvement of some of these in metal ion binding, mainly iron binding. Cazzola *et al*^[59] in 1990 established that iron is essential for proliferation, DNA synthesis and repair and mitochondrial electron transport. Therefore, it is assumed that CPX can stop the cell proliferation by regulating the expression of iron binding proteins.

The present study could give some insights into the mode of action of CPX in terms of expression regula-

tion of various proteins. It not only shed light on the previously discussed roles of CPX, but could also provide some further insight into their mechanism. We could identify some potential candidates which can effect the cell proliferation directly or indirectly through other cellular processes. By understanding the mode of action of CPX, this study may provide new aspect that will help in the future strategy to improve therapeutic intervention in the treatment with CPX.

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COMMENTS

Background

Ciclopirox olamine (CPX), a synthetic antifungal agent used in the treatment of fungal and yeast infection of skin or mucosa. Apart from its antimycotic activity, CPX is also effective against both gram-positive and gram-negative bacteria. CPX might also serve as an alternative agent for therapeutic angiogenesis. CPX was also shown to have an antiproliferative effect on stem cells without affecting their pluripotency.

Research frontiers

Although CPX is used as therapeutic for different aspect the mechanism of action is still not clear. In this study, the authors investigated the impact of CPX on stem cell proteome and identified cellular mechanisms that may explain the way of action of CPX. The authors provided evidence that CPX is involved in expression regulation of nucleotide binding proteins resulting in cell cycle arrest.

Innovations and breakthroughs

It is postulated that the CPX works as an inhibitor of the iron-dependent enzymes due to its potential role as a chelator of intracellular iron. The present study could give some insights into the mode of action of CPX in terms of expression regulation of various proteins especially nucleotide-binding proteins. It not only shed light on the previously discussed roles of CPX, but could also provide some further insight into their mechanism. We could also identify some potential candidates, which can effect the cell proliferation directly or indirectly through other cellular processes.

Applications

By understanding the mode of action of CPX, this study may provide new aspects that will be helpful in the future strategy for therapeutic intervention in the treatment with CPX.

Terminology

Multipotent adult germline stem cells (maGSCs) are spermatogonial stem cells isolated from murine testis. CPX, the ethanolamine salt of 6-cyclohexyl-1-hydroxy-4-methylpyridin-2(1H)-one, is a synthetic antifungal agent and is a hypusination inhibitor that controls the second step of the modification, which is catalyzed by deoxyhypusine hydroxylase. The hypusine is the result of a post-translational modification catalyzed by two enzymes: deoxyhypusine synthase and deoxyhypusine hydroxylase.

Peer review

This is a descriptive study in which the authors analyzed the proteome changes of embryonic stem cells and maGSCs accompanying the treatment with CPX and subsequent inhibition of hypusination using classical proteomic techniques like 2-DE, differential in-gel electrophoresis and mass spectrometry. The results are interesting and we could highlight that a treatment with CPX resulted in an alteration of the expression of 56 proteins compared to non-treated cells, and 54 proteins compared to retinoic acid treated cells. The majority of these proteins are involved in nucleotide binding and nucleotide biosynthetic processes, metal binding, DNA binding, and other processes which have been linked to CPX.

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Fetal cardiac mesenchymal stem cells express embryonal markers and exhibit differentiation into cells of all three germ layers

Garikipati Venkata Naga Srikanth, Naresh Kumar Tripathy, Soniya Nityanand

Garikipati Venkata Naga Srikanth, Naresh Kumar Tripathy, Soniya Nityanand, Stem Cell Research Facility, Department of Hematology, Sanjay Gandhi Post-Graduate Institute of Medical Sciences, Lucknow 226014, India

Author contributions: Srikanth GVN designed and performed the experiments and analyzed the data; Tripathy NK and Nityanand S designed the study and analyzed the data; Nityanand S provided the reagents and analytical tools; Srikanth GVN, Tripathy NK and Nityanand S wrote the manuscript.

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Correspondence to: Soniya Nityanand, MD, PhD, FNAsc, FASc, Professor, Head, Stem Cell Research Facility, Department of Hematology, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Rae Bareilly Road, Lucknow 226014, UP, India. soniya@sgpgi.ac.in

Telephone: +91-522-2494291 Fax: +91-522-2668017

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Abstract

AIM: To study the expression of embryonal markers by fetal cardiac mesenchymal stem cells (fC-MSC) and their differentiation into cells of all the germ layers.

METHODS: Ten independent cultures of rat fC-MSC were set up from cells derived from individual or pooled fetal hearts and studies given below were carried out at passages 3, 6, 15 and 21. The phenotypic markers CD29, CD31, CD34, CD45, CD73, CD90, CD105, CD166 and HLA-DR were analyzed by flow cytometry. The expression of embryonal markers Oct-4, Nanog, Sox-2, SSEA-1, SSEA-3, SSEA-4, TRA-1-60 and TRA 1-81 were studied by immunocytochemistry. The fC-MSC treated with specific induction medium were evaluated for their differentiation into (1) adipocytes

and osteocytes (mesodermal cells) by Oil Red O and Alizarin Red staining, respectively, as well as by expression of lipoprotein lipase, *PPAR γ 2* genes in adipocytes and osteopontin and *RUNX2* genes in osteocytes by reverse-transcription polymerase chain reaction (RT-PCR); (2) neuronal (ectodermal) cells by expression of neuronal Filament-160 and Glial Fibrillar Acidic Protein by RT-PCR and immunocytochemistry; and (3) hepatocytic (endodermal) cells by expression of albumin by RT-PCR and immunocytochemistry, glycogen deposits by Periodic Acid Schiff staining and excretion of urea into the culture supernatant.

RESULTS: The fC-MSC expressed CD29, CD73, CD90, CD105, CD166 but lacked expression of CD31, CD34, CD45 and HLA-DR. They expressed embryonal markers, viz. Oct-4, Nanog, Sox-2, SSEA-1, SSEA-3, SSEA-4, TRA-1-81 but not TRA-1-60. On treatment with specific induction media, they differentiated into adipocytes and osteocytes, neuronal cells and hepatocytic cells.

CONCLUSION: Our results together suggest that fC-MSC are primitive stem cell types with a high degree of plasticity and, in addition to their suitability for cardiovascular regenerative therapy, they may have a wide spectrum of therapeutic applications in regenerative medicine.

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Key words: Fetal cardiac mesenchymal stem cells; Embryonal markers; Multipotent differentiation potential

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INTRODUCTION

The heart is composed of multiple cell types, including cardiomyocytes, endothelial cells, vascular smooth muscle cells, connective tissue and cells of the conduction system; hence, stem cells that have a multipotent differentiation potential with ability to give rise to cells of different germ layers would be the most suitable for efficient regeneration of heart^[1,2]. Although embryonic stem cells are pluripotent, their clinical applications are restricted by their teratogenic and ethical concerns. Hence, different pre- and post-natal tissues are being extensively explored as an alternative source of pluripotent/multipotent stem cells for cardiovascular regenerative therapy^[3-5].

Recently, we have identified a population of mesenchymal stem cells (MSC) from rat fetal heart and termed them as fetal cardiac MSC (fC-MSC)^[6]. These stem cells possessed morphological and phenotypical characteristics of typical bone marrow derived MSC. In addition, they expressed cardiovascular markers and differentiated into all major cells of cardiovascular lineage. These cells exhibited several primitive characteristics, including extended self renewal properties, and expression of OCT-4, Nanog and Sox-2 at gene level and SSEA-1, SSEA-3 and SSEA-4 at protein level^[6]. However, it remains to be determined whether the differentiation of fC-MSC is restricted to cardiovascular or mesodermal lineage or if they possess a multipotent differentiation potential with ability to differentiate into cells of other germ layers as well.

Therefore, the objective of the present study was to evaluate further the primitive characteristics of fC-MSC and we studied the expression of a wide array of embryonic/pluripotency markers by fC-MSC and their capacity to differentiate into mesodermal, ectodermal and endodermal lineages.

MATERIALS AND METHODS

Isolation, culture and characterization of rat fC-MSC

Heart tissues obtained from 16 d gestation age fetuses of female Sprague-Dawley rats were minced and digested with 1 mg/mL collagenase type-IV (Worthington Biochemical, United States) in serum free α -MEM medium for 30 min at 37 °C with intermittent stirring. After washing, the minced tissues were cultured under standard tissue culture conditions in 25 cm² tissue culture flasks (Becton, Dickinson; United States) using complete culture medium consisting of α -MEM medium, 2 mg/mL of Glutamax (Gibco-Invitrogen), 16.5% fetal bovine serum (Hyclone, United States) and bacteriostatic level of penicillin-streptomycin (Gibco-Invitrogen). The semi confluent cultures of cells obtained within 72 h were harvested by trypsinization (0.05% Trypsin-EDTA) (Gibco-Invitrogen) and the cells were expanded in larger flasks up to 30 passages. Ten independent fC-MSC cultures were set up from cells derived from individual hearts or pooled from 2-3 hearts and studies given below were carried out at passages 3, 6, 15 and 21. All the procedures were per-

formed as per guidelines of the Institutional Animal Ethics Committee of Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow, India.

Flow cytometry

The cells were directly stained with pre conjugated anti-rat monoclonal antibodies to CD44-fluorescein isothiocyanate (FITC), CD90-FITC, CD45-phycoerythrin (PE) and HLA DR-PE. Indirect staining was performed using un-conjugated primary antibodies viz. mouse anti-rabbit CD29 (Abcam, United Kingdom), rabbit anti-mouse CD73 (Becton Dickinson), rabbit anti-mouse CD105 (Santa Cruz, United States), rabbit anti-mouse CD166 (LsBio, United States), rabbit anti-mouse CD34 (Santa Cruz, United States) and rabbit anti-mouse CD31 (Serotec, United Kingdom). Thereafter, cells were stained with FITC/PE conjugated polyclonal rabbit anti-mouse or mouse anti-rabbit secondary antibodies (Abcam) or isotype matched control monoclonal antibodies (Becton Dickinson). Stained cells were analyzed using FCS Express 3.0 in Flow Cytometer (FACS Calibur, Becton, and Dickinson, United States).

Immunocytochemistry

Expression of Oct-4, Nanog, Sox-2, SSEA-1, SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81 was studied by immunocytochemistry. The fC-MSC was fixed with 4% para-formaldehyde (Sigma Aldrich) in phosphate buffered saline (PBS), pH 7.4, for 1 h at room temperature. The fixed cells were incubated overnight at 4 °C with the following primary antibodies: Oct-4, Nanog and Sox-2, SSEA-1, SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81 (ES cell characterization kit; Chemicon, United States), diluted 1:50. After washing with PBS, cells were incubated with 1:500 diluted IgG (Fab)₂ FITC as the secondary antibody (Abcam) and stained with Hoechst dye. The pictures were taken using a fluorescent microscope (Nikon 80i, Japan).

Reverse-transcription polymerase chain reaction

Expression of lipo-protein lipase, PPAR γ 2, osteopontin, RUNX2, neuronal filament (NF)-160, glial fibrillar acidic protein (GFAP), albumin and GAPDH was determined by reverse-transcription polymerase chain reaction (RT-PCR). RNA of the cells was extracted using RNeasy Mini RNA isolation kit (Gibco-Invitrogen). One μ g of total RNA was reverse transcribed into cDNA using random hexamers (Gibco-Invitrogen). The gene expression was analyzed for the following genes using primers from MWG Biotech, Germany (Table 1). The amplicons were resolved on 2% agarose gel (Sigma-Aldrich, United States) and pictures acquired using a gel documentation system (Alpha Imager, United States).

Osteogenic differentiation

The cells were treated with osteogenic medium consisting of DMEM medium (Gibco-Invitrogen) containing 10% FBS (Hyclone), 1 mmol/L dexamethasone, 10 mg/mL glyceraldehyde 3-phosphate and 0.1 mmol/L ascorbic

Table 1 Primers used in the present study

Gene	Primer sequence	Product size (bp)	Accession number
Osteopontin	F: TCGGAGGAGAAGGCGCATTACAGC R: TCCTCATGGCTGTGAAATCGTGG	778	AB001382.1
RUNX2	F: TTCGTCAGCGTCCTATCAGTTC R: CTTCCATCAGCGTCAACACC	149	NM_053470.2
PPAR γ 2	F: TTGATTTCTCCAGCATTTTC R: GCTCTACTTTGATCGCACT	125	NM_001145366.1
Lipoprotein Lipase	F: GGGTCGCCTGGTCGAAGT R: AAAGTGCCTCCATTGGGATAAA	450	L03294.1
Neurofilament-160	F: CTCGACTTCAGCCAGTCTCTTCG R: TCTTTCGCTCTACGGTGATGTGC	550	NM_017029.1
GFAP	F: AGCTGAACCAGCTTCGAGCCAAGG R: GGAAGCAACGTCGTGAGGTCTGC	508	NM_017009.2
Albumin	F: TCGTGACAACCTACGGTGAATGGC R: TGTTCGTCTCAGCGAGACACTGG	640	NM_134326.2
GAPDH	R: CCTCTCTCTTGCTCTCAGIAT F: GTATCCGTTGIGGATCTGACA	284	NM_017008.3

GFAP: Glial fibrillar acidic protein; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

acid (Osteogenesis kit, Chemicon). Control cells were treated with complete medium alone. After 21 d, the experimental and control cells were fixed with 4% para-formaldehyde and stained with Alizarin Red Stain to visualize mineralization.

Adipogenic differentiation

The cells were treated with adipogenic medium consisting of DMEM medium (Gibco-Invitrogen) containing 10% FBS (Hyclone), 500 mmol/L isobutylmethylxanthine, 1 mmol/L dexamethasone, 10 mg/mL insulin and 100 mmol/L indomethacin (Adipogenesis kit, Chemicon). Control cells were treated with complete medium alone. Cells were fixed and stained with Oil Red O Stain to visualize fat droplets in the cells.

Neuronal differentiation

The cells were treated with neurogenic medium consisting of complete culture medium supplemented with 100 ng/mL of basic fibroblast growth factor, 100 ng/mL of noggin, 20 ng/mL of neurotrophin, 10 ng/mL of brain derived growth factor, 10 ng/mL of glial derived growth factor and 20 μ mol/L of retinoic acid (all from (R and D systems, United States) and 1X of B-27 (Gibco-Invitrogen) and 1X of 2-mercaptoethanol (Sigma Aldrich) (experimental cells) or complete culture medium alone (control cells). The cultures were terminated after 21 d and the neuronal nature of the cells was characterized by immunocytochemistry using primary antibodies against NF-160 (ready to use) and GFAP (ready to use) (Biovision Inc., United States). After washing with PBS, cells were incubated with 1:500 diluted IgG (Fab) γ FITC secondary antibody (Abcam) and stained with Hoechst dye (Sigma). The pictures were taken using a fluorescent microscope (Nikon).

Hepatocytic differentiation

The cells were treated with hepatogenic medium con-

sisting of complete culture medium supplemented with 50 ng/mL of hepatocyte growth factor, 50 ng/mL of fibroblast growth factor-4 (R and D systems) (experimental cells) or complete culture medium alone (control cells). Hepatocytic characterization was done by immunocytochemistry using 1:500 diluted primary antibody to albumin (Sigma-Aldrich). After washing with PBS, cells were incubated with 1:500 diluted IgG (Fab) γ FITC as the secondary antibody (Abcam) and stained with Hoechst dye (Sigma). The pictures were taken using a fluorescent microscope (Nikon).

The hepatocytic differentiation was further confirmed by urea assay and Periodic Acid-Schiff assay.

The urea assay was performed by estimating the concentration of urea in the supernatant of experimental and control cells at 11, 14, 17 and 21 d using urea assay kit (Merck diagnostics, India), following the manufacturer's instructions.

In the Periodic Acid-Schiff assay, experimental and control cells after fixation with 4% para-formaldehyde were oxidized in 10 g/L periodic acid for 10 min and rinsed thrice in dH $_2$ O. Thereafter, cells treated with Schiff's reagent for 10 min were rinsed in dH $_2$ O for 10 min and stained with hematoxylin for 2 min to visualize glycogen deposits.

Statistical analysis

Results are presented as mean \pm SE. Statistical significance was defined as $P < 0.05$ by analysis of variance using SPSS 16.0 software.

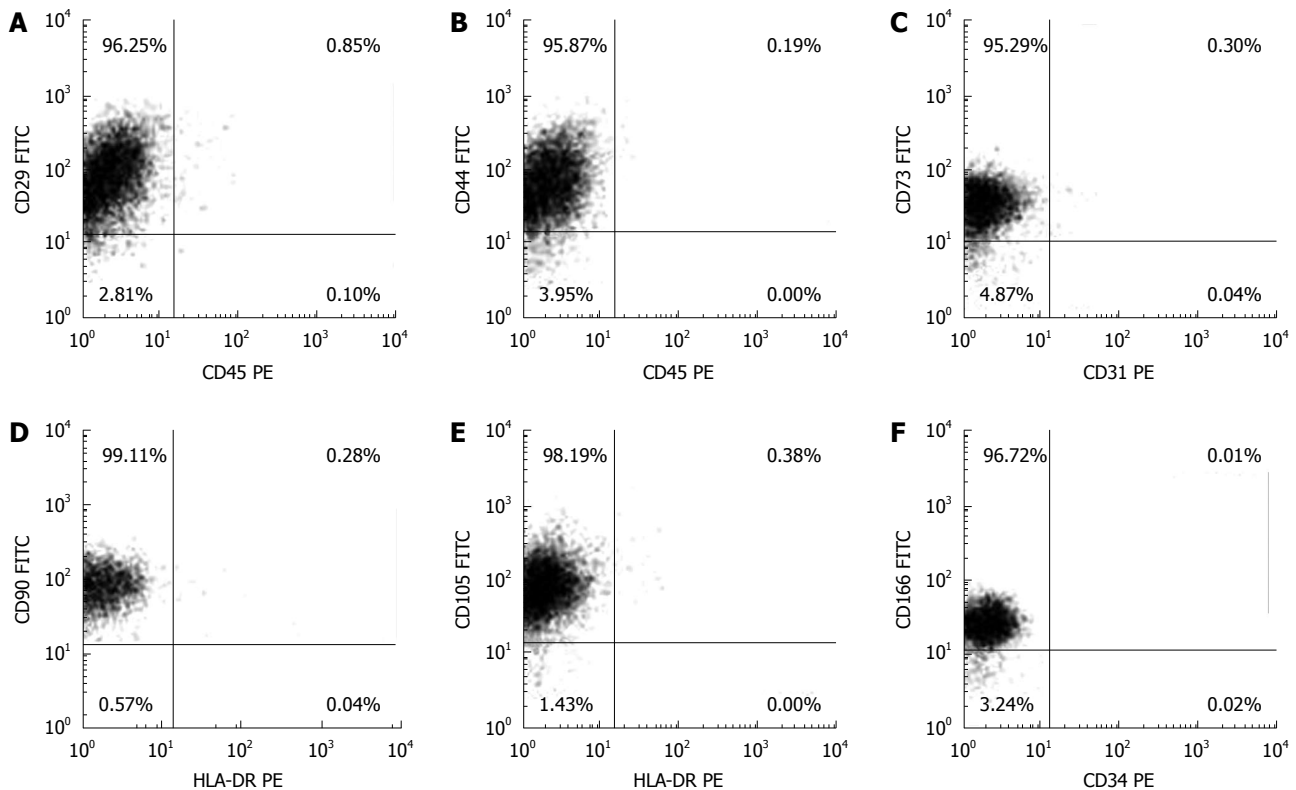
RESULTS

Immunophenotypic characteristics of fC-MSC

Flow cytometric analysis showed a typical mesenchymal phenotype of fC-MSC with expression of CD29, CD44, CD73, CD90, CD105 and CD166 markers and no expression of CD31, CD34, CD45 and MHC-II markers (Figure 1);

Table 2 Immunophenotype of rat fetal cardiac mesenchymal stem cells in primary culture and at passages 3, 6, 15 and 21 (mean \pm SE)

Markers	Primary culture	Passage 3	Passage 6	Passage 15	Passage 21
CD29	96.62 \pm 0.26	96.54 \pm 0.44	96.16 \pm 0.58	96.41 \pm 0.34	95.80 \pm 0.44
CD44	95.80 \pm 0.18	95.96 \pm 0.10	96.00 \pm 0.30	96.50 \pm 0.44	95.66 \pm 0.84
CD73	95.78 \pm 0.12	95.10 \pm 0.16	95.60 \pm 0.24	95.60 \pm 0.30	95.30 \pm 0.32
CD90	98.56 \pm 0.30	98.90 \pm 0.18	98.68 \pm 0.28	98.60 \pm 0.12	98.80 \pm 0.12
CD105	98.40 \pm 0.30	98.00 \pm 0.12	97.68 \pm 0.18	97.60 \pm 0.22	97.40 \pm 0.24
CD31	0.44 \pm 0.08	0.50 \pm 0.02	0.52 \pm 0.02	0.50 \pm 0.02	0.51 \pm 0.01
CD45	0.66 \pm 0.02	0.64 \pm 0.02	0.54 \pm 0.02	0.62 \pm 0.01	0.58 \pm 0.04
HLA-DR	0.38 \pm 0.02	0.30 \pm 0.08	0.28 \pm 0.04	0.34 \pm 0.04	0.32 \pm 0.02

**Figure 1** Representative flow cytometric dot-plots of rat fetal cardiac mesenchymal stem cells showing. A: CD29⁺/CD45⁺; B: CD44⁺/CD45⁺; C: CD73⁺/CD31⁺; D: CD90⁺/HLA-DR⁺; E: CD105⁺/HLA-DR⁺; F: CD166⁺/CD34⁺ phenotype.

this phenotype was maintained over the successive passages (Table 2).

Expression of embryonal markers by fC-MSC

The fC-MSC expressed embryonal markers Oct-4, Nanog, Sox-2, SSEA-1, SSEA-3, SSEA-4, TRA 1-81 but not TRA 1-60, as revealed by immunocytochemistry (Figure 2).

Differentiation of fC-MSC into cells of all three germ layers

Treatment of fC-MSC with adipogenic and osteogenic induction media resulted in their differentiation into adipocytes and osteocytes (mesoderm), as demonstrated by Oil Red O and Alizarin Red staining, as well as expression of lipoprotein lipase, *PPAR γ 2* and osteopontin and *RUNX2* genes by RT-PCR, respectively (Figure 3).

The neurogenic induction medium treated fC-MSC differentiated into neuronal cells (ectoderm), as revealed by expression of NF-160 and GFAP by RT-PCR and im-

munochemistry (Figure 4).

Similarly, on treatment with hepatogenic medium, fC-MSC exhibited differentiation into hepatocytic cells (endoderm), as demonstrated by expression of albumin by RT-PCR and immunocytochemistry, glycogen deposits by Periodic Schiffs staining and excretion of urea in the supernatant (Figure 5).

DISCUSSION

We have recently isolated a population of rat fC-MSC with typical MSC characteristics, including trigonal/spindle shaped morphology, expression of CD29, CD44, CD73, CD90 and CD105, but not of CD31, CD45 and HLA-DR, and potential to differentiate into adipogenic and osteogenic cells^[6]. The fC-MSC exhibited a cardiovascular commitment, as revealed by expression of cardiovascular genes *Isl-1*, *flk-1*, *Nkx2.5* and *GATA-4*, and differentiated

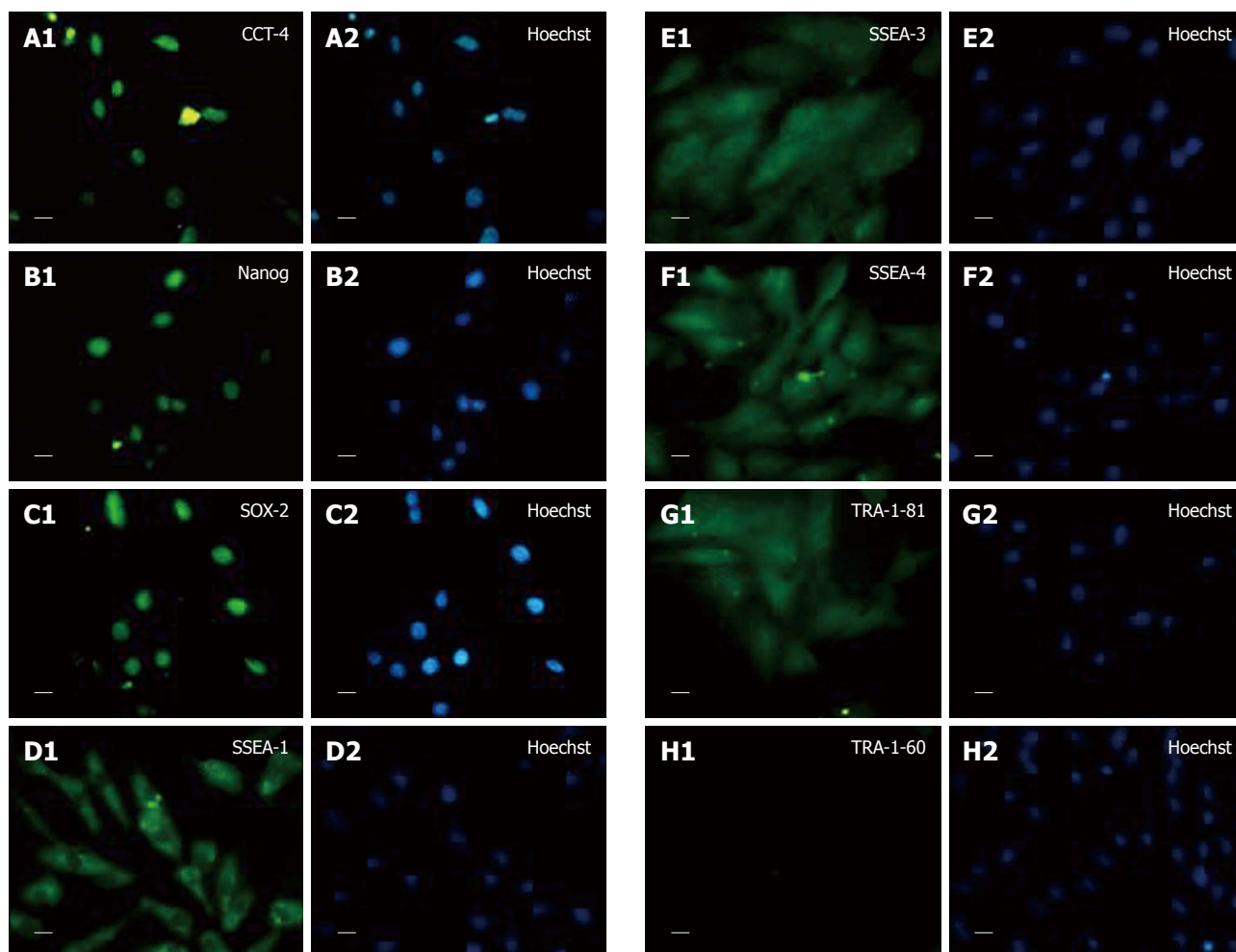


Figure 2 Representative immunocytochemistry photomicrographs (40 ×, 20 μm) of rat fetal cardiac mesenchymal stem cells showing expression. A: OCT-4 (A1: OCT-4 and A2: Hoechst dye); B: Nanog (B1: Nanog and B2: Hoechst dye); C: SOX-2 (C1: SOX-2 and C2: Hoechst dye); D: SSEA-1 (D1: SSEA-1 and D2: Hoechst dye); E: SSEA-3 (E1: SSEA-3 and E2: Hoechst dye); F: SSEA-4 (F1: SSEA-4 and F2: Hoechst dye); G: TRA 1-81 (G1: TRA 1-81 and G2: Hoechst dye); H: TRA1-60 (H1: TRA 1-60 and H2: Hoechst dye).

into all major cardiovascular lineage cells, including cardiomyocytes, endothelial cells and smooth muscle cells. In addition, these cells expressed some embryonal markers and had an extensive expansion potential with continuous expression of telomerase reverse transcriptase and maintenance of a normal karyotype throughout the monitoring period for up to the 21st passage^[6].

These primitive characteristics of fC-MSC prompted us to undertake the present study and we found that, similar to embryonic stem cells^[7], the fC-MSC expressed a wide range of embryonal markers, including Oct-4, Nanog, Sox-2, SSEA-1, SSEA-3, SSEA-4 and TRA-1-81, but not TRA-1-60. In addition, on treatment with lineage specific induction medium, they differentiated into adipocytes and osteocytes (mesoderm), neural cells (ectoderm) and hepatocytic cells (endoderm). To the best of our knowledge, this is the first report showing differentiation of fC-MSC into cells of all three germ layers.

In two studies on stem cells from human fetal heart, the stem cells were demonstrated to express phenotypic markers of MSC, but they were not evaluated for expression of embryonal markers and tri-lineage differentiation

potential^[8,9]. Our observation on embryonic stem cell like characteristics of fC-MSC corroborates with results of a few previous studies on different fetal MSCs of rat and humans. MSC derived from rat and human amniotic membranes has been reported to exhibit expression of pluripotency markers and to differentiate into cells of ectodermal, mesodermal and endodermal lineages^[10,11]. Similarly, human MSC derived from fetal lung are reported to express various embryonal markers, including Oct-4, Nanog, Sox-2, TRA-1-60, TRA-1-81 and SSEA-4, as well as to differentiate into cells of three germ layers^[12]. In another human study, stem cells derived from different fetal organs have been shown to express Oct-4 and could be differentiated into cells of all three germ layers^[13].

In conclusion, our study has shown that fC-MSC are primitive stem cell types that have a high degree of plasticity, as demonstrated by their expression profile of embryonal markers and potential to differentiate into cells of all the three germ layers, suggesting that, in addition to their suitability for cardiovascular regenerative therapy, they may have a wide spectrum of therapeutic applications in regenerative medicine.

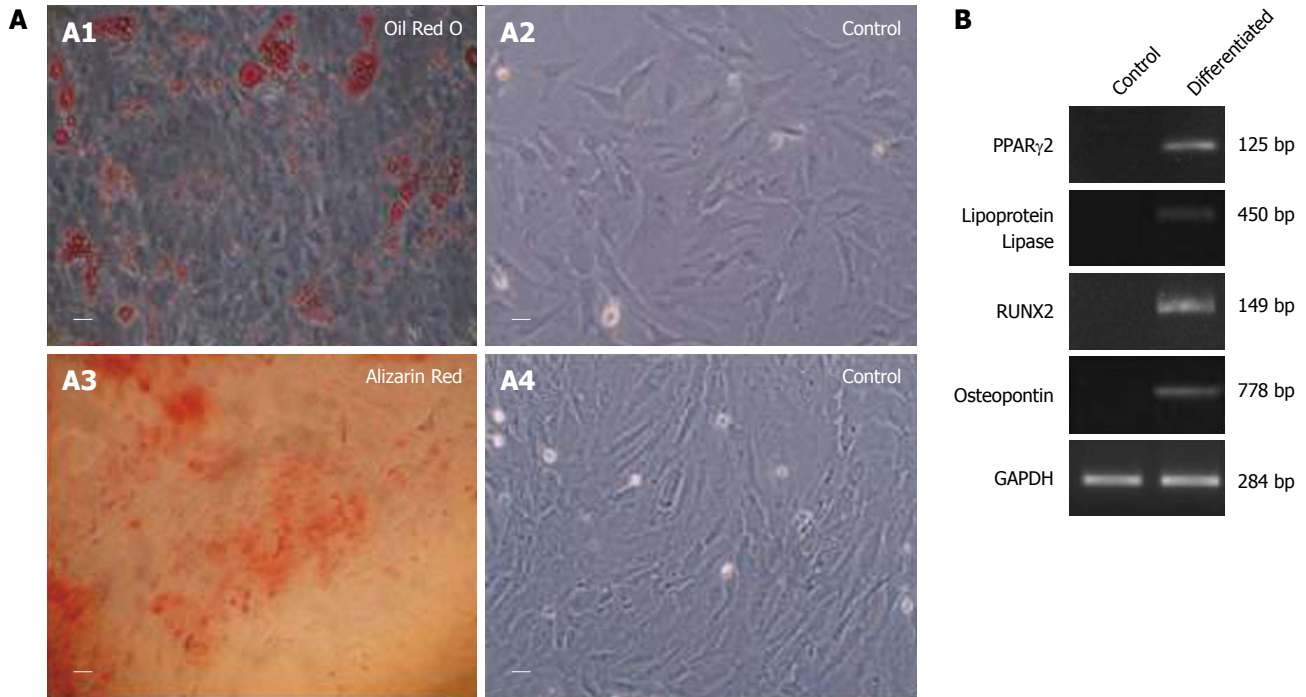


Figure 3 Representative photomicrographs (A) and representative reverse-transcription polymerase chain reaction gel photomicrographs (B). A: Representative photomicrographs (40 \times , 20 μ m) showing differentiation of rat fetal cardiac mesenchymal stem cells into osteocytes (A1: differentiated cells positive for Alizarin Red stain and A2: control cells negative for Alizarin Red stain) and adipocytes (A3: differentiated cells positive for Oil Red O stain and A4: control cells negative for Oil Red O stain); B: Representative reverse-transcription polymerase chain reaction gel photomicrographs showing expression of lipoprotein lipase and PPAR γ 2 by adipocytes and osteopontin and RUNX2 by osteocytes cells, induced from rat fetal cardiac mesenchymal stem cells. Control cells not treated with induction medium did not show expression of above markers.

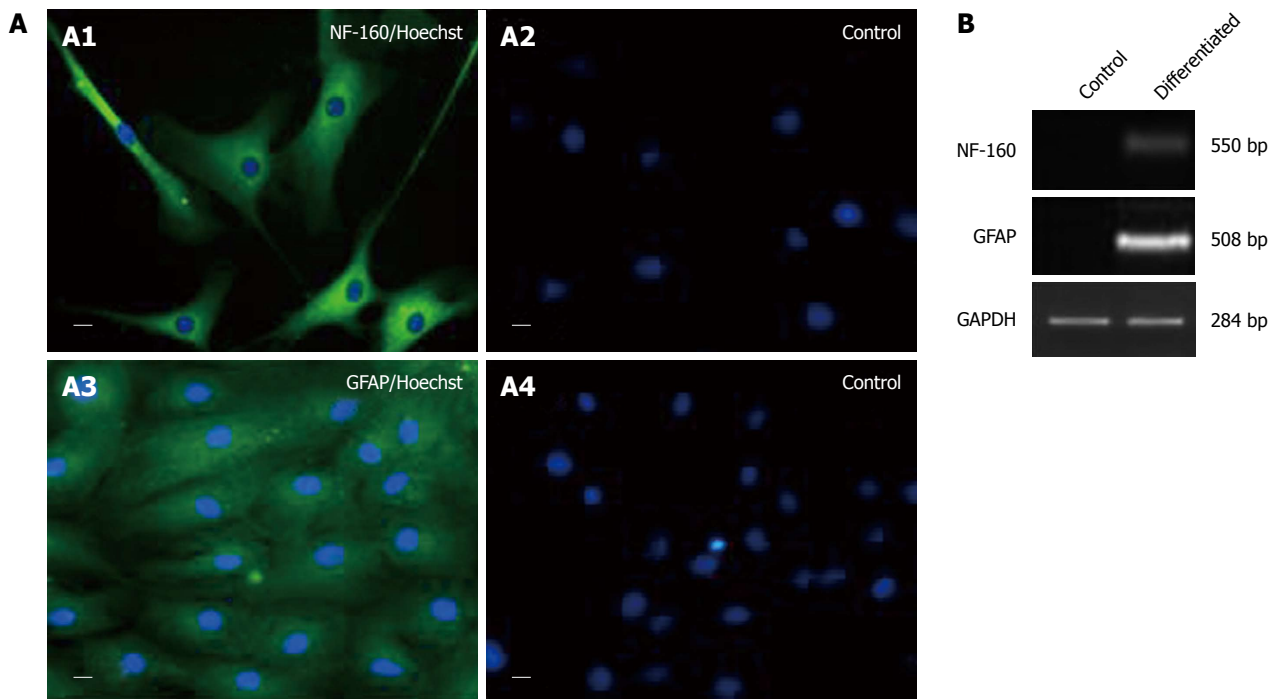


Figure 4 Representative immunocytochemistry photomicrographs (A) and representative reverse-transcription polymerase chain reaction gel photomicrographs (B). A: Representative immunocytochemistry photomicrographs (40 \times , 20 μ m) showing differentiation of rat fetal cardiac mesenchymal stem cells into neuronal cells [A1: Neuronal filament (NF)-160 and Hoechst and A2: no NF-160 only Hoechst dye in control cells]; [A3: Glial fibrillar acidic protein (GFAP) and Hoechst dye and A4: no GFAP only Hoechst dye in control cells]; B: Representative reverse-transcription polymerase chain reaction gel photomicrographs showing expression of NF-160 and GFAP by neuronal cells differentiated from rat fetal cardiac mesenchymal stem cells. Control cells without induction medium did not show expression of above markers.

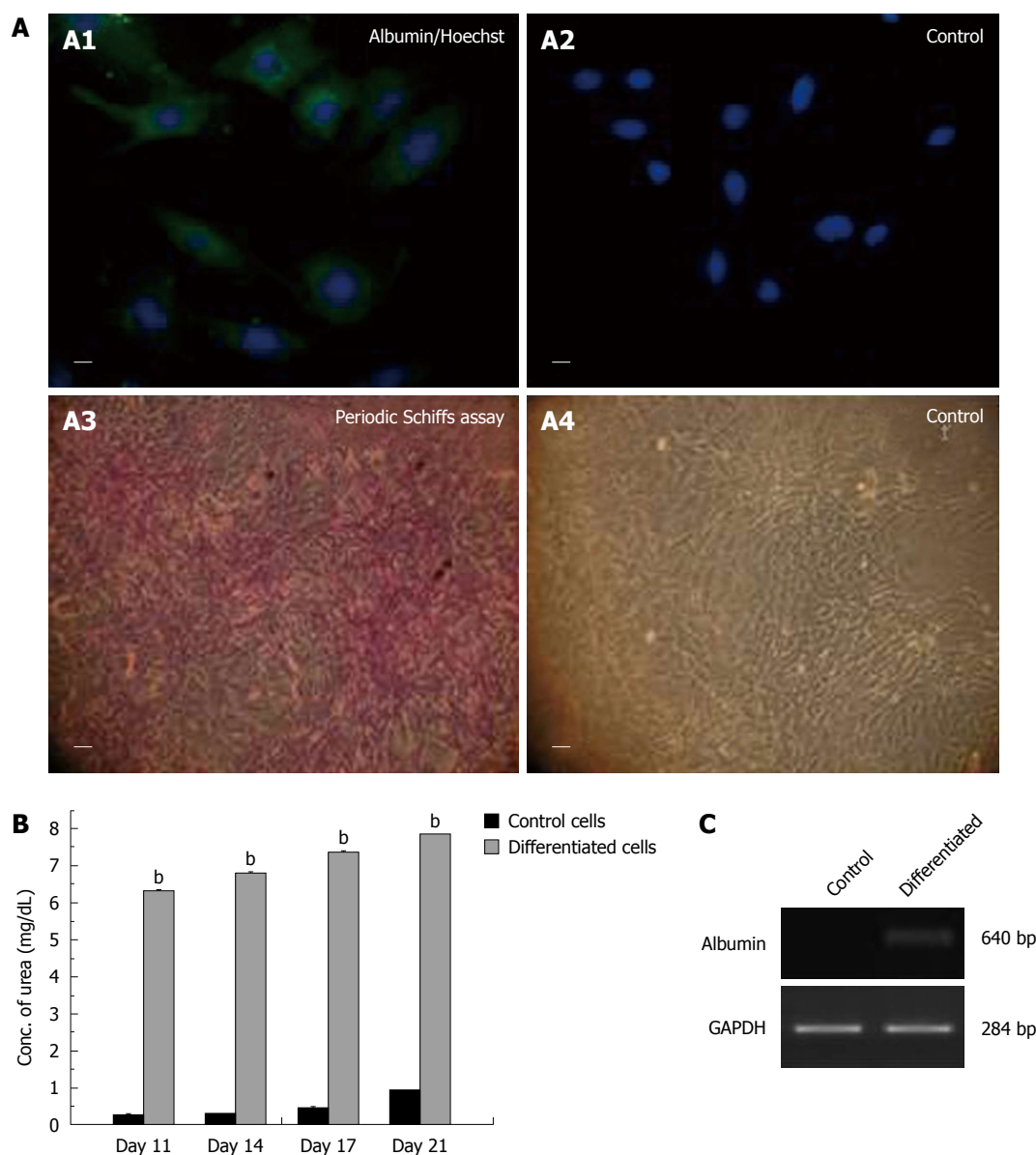


Figure 5 On treatment with hepatogenic medium, fetal cardiac mesenchymal stem cells exhibited differentiation into hepatocytic cells (endoderm), as demonstrated by expression of albumin by reverse-transcription polymerase chain reaction and immunocytochemistry, glycogen deposits by Periodic Schiff's staining and excretion of urea in the supernatant. **A:** Representative immunocytochemistry photomicrographs (40 ×, 20 μm) showing differentiation of rat fetal cardiac mesenchymal stem cells into hepatocytes (A1: albumin and Hoechst dye and A2: no albumin only Hoechst dye in control cells); (A3: Periodic Acid Schiff stain and A4: control cells negative for Periodic Acid Schiff stain); **B:** Urea levels in the supernatant of control and differentiated cells at days 11, 14, 17 and 21. Values are mean ± SE of three experiments; ^b*P* < 0.001 for control cells vs differentiated cells; **C:** Representative reverse-transcription polymerase chain reaction gel photomicrographs showing expression of albumin by hepatocytic cells induced from fetal cardiac mesenchymal stem cells. Control cells without induction medium did not show any expression of albumin.

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COMMENTS

Background

The authors' previous observation of fetal cardiac mesenchymal stem cells (fC-MSC) expressing primitive markers and differentiation ability to cardiovascular or mesodermal lineage led us to determine multipotent differentiation potential with ability to differentiate into cells of other germ layers as well.

Research frontiers

In this study, the authors have demonstrated that fC-MSC are primitive stem cells with capacity to differentiate into cells of all the three germ layers. Future studies on their basic biology and *in vivo* differentiation and function may be important for cell based therapy, generation of artificial tissues and organs and gene therapy.

Innovations and breakthroughs

To the best of our knowledge, this is the first report expressing a wide range of embryonal/pluripotent markers and showing differentiation of fC-MSC into cells of all three germ layers.

Applications

The fC-MSC with primitive stemness and multi-lineage differentiation potential might be novel stem cell types for regeneration/repair of tissues and organs derived from different primary germ layers and thus they may have a wide spectrum of therapeutic applications in regenerative medicine.

Terminology

The fC-MSC are non-hematopoietic stem cells derived from fetal heart that share morphological and phenotypical characteristics of typical bone marrow derived MSC but they are very primitive nature and express cardiovascular markers, pluripotent markers and have potential to differentiate into cells of all the three germ layers.

Peer review

This is an interesting study, wherein the authors have demonstrated that fC-MSC are primitive and highly multipotent stem cells and, in addition to their cardiovascular commitment, they could differentiate into cells of all the three germ layers; thus, they may have a wide spread application in regenerative therapy.

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Immunophenotype and differentiation capacity of bone marrow-derived mesenchymal stem cells from CBA/Ca, ICR and Balb/c mice

Yin Yin Ooi, Zul'atfi Rahmat, Shinsmon Jose, Rajesh Ramasamy, Sharmili Vidyadaran

Yin Yin Ooi, Zul'atfi Rahmat, Shinsmon Jose, Rajesh Ramasamy, Sharmili Vidyadaran, Immunology Laboratory, Department of Pathology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Malaysia

Yin Yin Ooi, Department of Anatomy, Yong Loo Lin School of Medicine, NUS, MD10, 4 Medical Drive, Singapore 117597, Singapore

Author contributions: Ooi YY performed the research and analysed the data; Rahmat Z assisted with the differentiation assays; Jose S assisted with the immunophenotyping; Ramasamy R provided technical advice on culture and characterisation of the mesenchymal stem cells; Vidyadaran S conceived the study, designed the experiments and wrote the manuscript.

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Correspondence to: Dr. Sharmili Vidyadaran, PhD, Immunology Laboratory, Department of Pathology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Malaysia. sharmili@medic.upm.edu.my

Telephone: +60-3-89472376

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marrow of all 3 strains, albeit differences in the temporal expression of certain surface antigens. Their differentiation into osteocytes and adipocytes were also observed. MSC from all 3 mouse strains demonstrated a shift from a haematopoietic phenotype (CD106⁺CD45⁺CD11b⁺Sca-1^{low}) to typical MSC phenotype (CD106⁺CD45⁺CD11b⁺Sca-1^{high}) with increasing passages.

CONCLUSION: Information garnered assists us in the decision of selecting a mouse strain to generate MSC from for downstream experimentation.

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Key words: Mesenchymal stem cells; Mouse bone marrow; CBA/Ca strain; ICR strain; Balb/c strain; Immunophenotyping; Differentiation

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Abstract

AIM: To assess the capacity to isolate and expand mesenchymal stem cells (MSC) from bone marrow of CBA/Ca, ICR and Balb/c mice.

METHODS: Bone marrow of tibia and femur were flushed, cultured and maintained in supplemented Dulbecco's modified Eagle's medium. MSC immunophenotype of cultures were tracked along increasing passages for positivity to CD106, Sca-1 and CD44 and negativity to CD45, CD11b and MHC class II. Differentiation capacity of MSC towards osteogenic and adipogenic lineages were also assessed.

RESULTS: MSC were successfully cultured from bone

INTRODUCTION

Mesenchymal stem cells (MSC) are a great therapeutic interest for tissue regeneration and immunomodulation. To elucidate the role of MSC in these two paradigms, experimentation is often performed with MSC generated from mouse bone marrow. For this, researchers have a choice to make in selecting the mouse strain to generate their MSC from. Here, we provide a report on the comparative description of mouse MSC derived from 3 different strains under the same experimental conditions. The mouse strains assessed were CBA/Ca, ICR and Balb/c.

MSC are stromal cells with self-renewal and differen-

tiation properties^[1]. MSC have been isolated from various tissues including bone marrow, adipose, skin, bone, synovial membrane, placenta and umbilical cord^[2-4] and proliferate *in vitro* as plastic-adherent cells with fibroblast-like morphology^[5]. The long term self-renewal and multilineage differentiation properties of MSC reflect their potential for tissue regeneration and cell/gene therapy-based treatment of diseases^[6] including acute graft-vs-host disease^[7], osteogenesis imperfecta^[8], cardiomyopathy^[9] and Crohn's disease^[10]. Recent findings that MSC have immunosuppressive properties^[11-14] have also increased the need for a suitable supply of MSC for experimental research. The expansion of undifferentiated MSC is a research tool for functional and genetic studies for subsequent development of preclinical protocols to treat a wide range of diseases. Murine sources of MSC are of great use for the extensive preclinical studies that are required within this research area.

Murine MSC is commonly isolated from the bone marrow aspirates of the femur and tibia. Studies demonstrate that isolation of MSC from murine bone marrow using standard methods usually results in a heterogeneous cell population with a high degree of non-mesenchymal contaminants^[2,15-17]. Furthermore, there are reports of preferential growth of MSC from different mouse strains^[17]. MSC isolated from four different inbred mouse strains C57Bl/6J, Balb/c, FVB/N and DBA1 showed great variation in growth rate, differentiation capacity and immunophenotype^[17]. In this study, we compare phenotypic characteristics of MSC derived from bone marrow of 3 strains of mice (CBA/Ca, ICR and Balb/c) to determine a suitable source for these cells for downstream experimentation. As MSC that are cultured lack specific markers^[5], cells were immunophenotyped for a range of surface markers classically associated with MSC expression. The differentiation capacity of MSC confirmed the multipotency of these stem cells. Although there were differences in temporal pattern of surface marker expression and preferential differentiation, bone marrow cultures from all 3 strains of mice tested successfully yielded MSC.

MATERIALS AND METHODS

Isolation and culture of bone marrow-derived MSC

Bone marrow-derived MSC were generated from 3 different strains of mice, namely CBA/Ca, Balb/c and ICR. Mice used were male and between the ages of 6-10 wk. Animals were sacrificed by a CO₂ overdose and bone marrow cells were obtained by flushing femurs and tibias with Dulbecco's modified Eagle's medium (DMEM). Cells were centrifuged at 289 *g* for 10 min, resuspended and seeded into three 25 cm² flasks in DMEM with high glucose supplemented with 10 nmol/mL GlutaMAXTM-I Supplement, 100 U/mL penicillin and 100 µg/mL streptomycin, 1 mL/L gentamycin, 250 µg/mL Fungizone (all Invitrogen), 1.5 g/L sodium bicarbonate and 15% Mesenchymal Stem Cell Stimulatory Supplement (STEM-

CELL Technologies, Canada). Cultures were maintained at 37 °C in 95% humidified air and 5% CO₂. After 48 and 72 h, cells were washed gently using 1X PBS and replaced with fresh culture media. Adherent cells were further cultured with a medium change every 3-4 d. At approximately 80% confluency (occasionally localised), cells were harvested by treating with 0.25% trypsin containing 1 mmol/L EDTA for 5 min at 37 °C. Trypsin was neutralised with culture medium and detached cells were replated in a new 25 cm² culture flask. Cell cultures were routinely assessed using an inverted phase contrast microscope and cell viability was determined *via* trypan blue staining.

Flow cytometry analysis of cell surface markers

The following antibodies were obtained from Becton Dickinson: fluorescein isothiocyanate-conjugated anti-mouse CD106 (vascular adhesion molecule-1), CD11b, and MHC I ; phycoerythrin-conjugated anti-mouse CD45, Sca-1, and MHC II and allophycocyanin-conjugated anti-mouse CD44. Cells were trypsinised, washed with 0.1% BSA/PBS and incubated with antibody (1 µL per 10⁶ cells) for 30 min at 4 °C. Cells were then resuspended in 0.1% BSA/PBS and analysed by a FACS Calibur cytometer (BD Biosciences, San Jose, CA) using CellQuest Pro software. Ten thousand gated events were recorded. For each antibody, gating was determined based on appropriate isotype-stained controls.

MSC differentiation

Mouse bone marrow cultures of passages 10-16 were plated at a density of 6×10^4 cells/well in a 24-well plate and incubated at 37 °C in 95% humidified air, 5% CO₂ till confluency was reached. For adipocytic differentiation, the Mesenchymal Stem Cell Adipogenesis Kit (Chemicon; Cat. No. SCR020) was used. Briefly, cells were stimulated with induction medium (DMEM with high glucose supplemented with 10% FBS and 1X penicillin and streptomycin, 1 µmol/L dexamethasone, 0.5 mmol/L IBMX, 10 µg/mL insulin and 50 µmol/L indomethacin) and maintained with maintenance medium (DMEM with high glucose supplemented with 10% FBS and 10 µg/mL insulin) according to kit instructions. For analysis, cells were fixed with 4% paraformaldehyde and stained with 0.36% Oil Red O solution for 50 min. For osteogenic differentiation, the Mesenchymal Stem Cell Osteogenesis Kit (Chemicon; Cat. No. SCR028) was used. Briefly, cover slips were coated with 12 µg/mL vitronectin and 12 µg/mL collagen prior to cell seeding. Cells were then stimulated with induction medium (DMEM with high glucose supplemented with 10% FBS and 1X penicillin and streptomycin, 0.1 µmol/L dexamethasone, 0.2 mmol/L ascorbic acid 2-phosphate, 10 mmol/L glycerol 2-phosphate and 1X glutamine). Cells were replaced with fresh induction medium every 2 d. For analysis, osteocytes were fixed with 70% ethanol and stained with Alizarin Red S. For both differentiation assays, controls were MSC cultures without adipocytic/osteogenic induc-

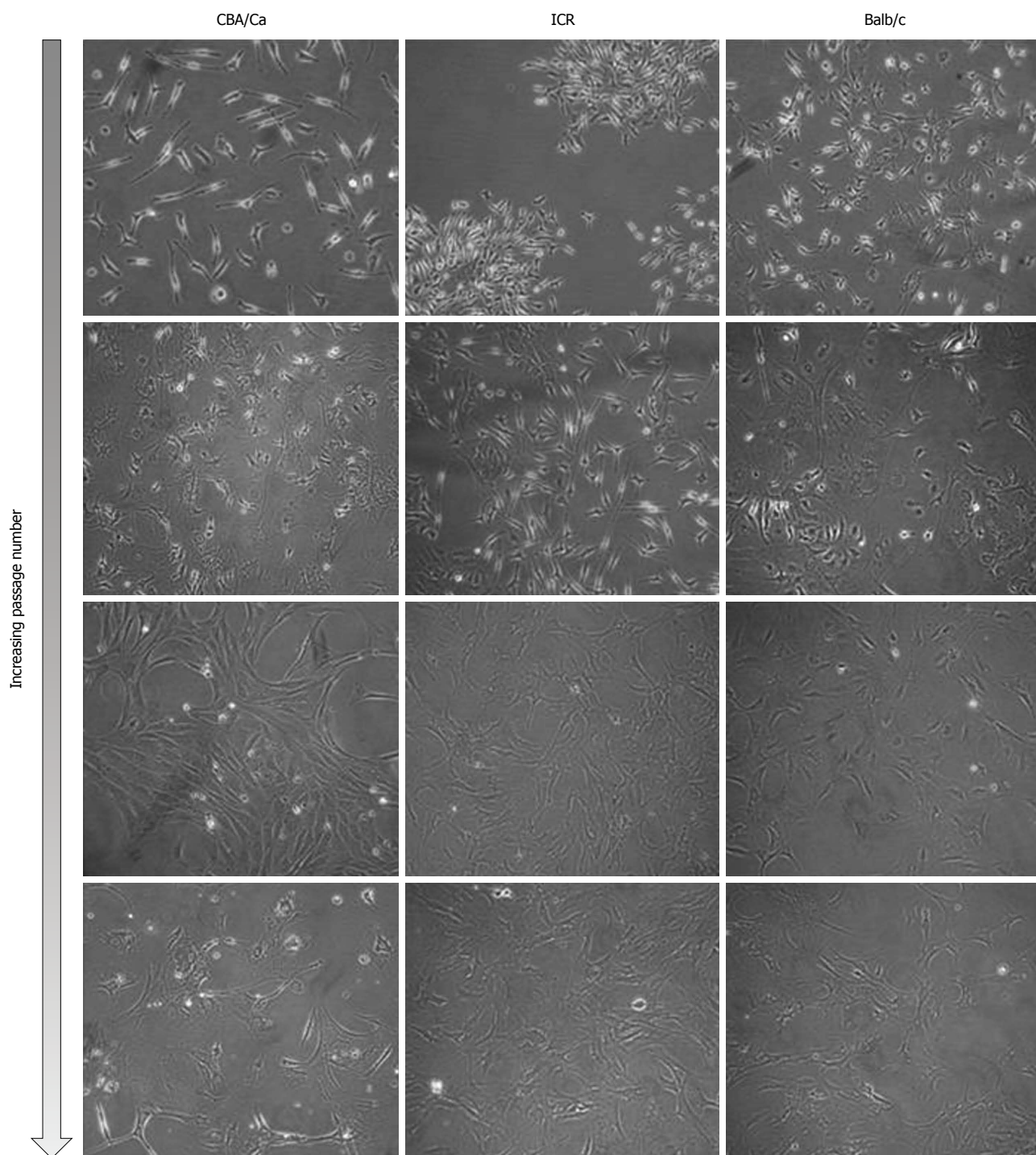


Figure 1 Morphology of mouse bone marrow cultures. Cells shown here represent passages 0-19 of bone marrow cells from CBA/Ca, ICR and Balb/c mice. Original magnification: $\times 200$.

tion medium.

RESULTS

Morphology of bone marrow cell cultures

Bone marrow cells were isolated from the femurs and tibias of a total of 11 mice (4 ICR, 2 CBA/Ca and 5 Balb/c mice) and plated onto culture flasks. Non-adherent cells were removed by replacing media and cultures were ob-

served *via* phase-contrast microscopy for their morphology. Initially, cells of all 3 strains of mice were small and exhibited spindle-shaped morphology (Figure 1, first and second row). Cells also tended to be locally confluent, growing in distinct colonies (as seen for ICR mice, first row; Balb/c mice, second row). A small number of round and flattened cells were observed within the colonies. As cells approached confluency within culture flasks, they were trypsinised and replated into new flasks. Bone mar-

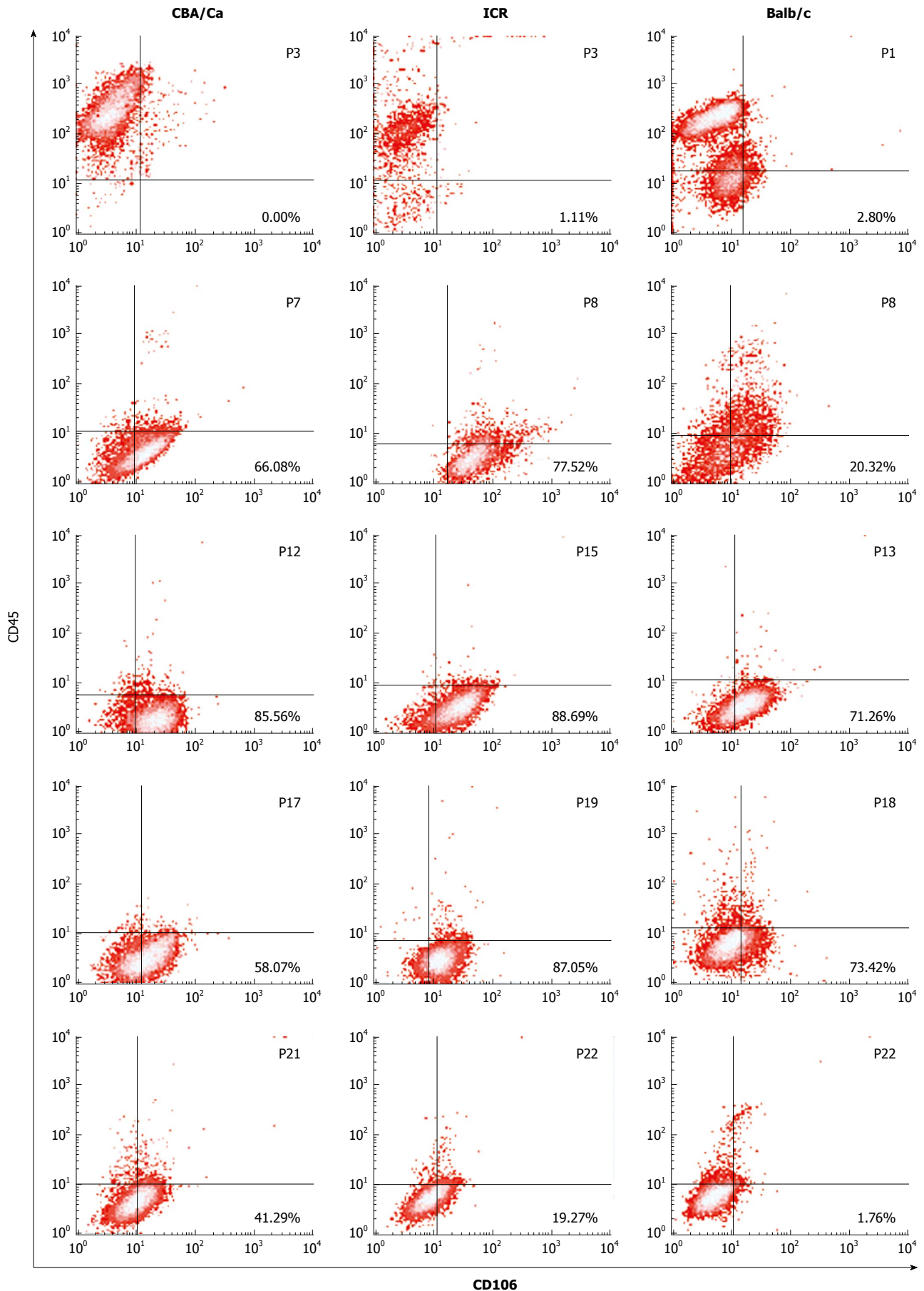


Figure 2 Bone marrow cultures acquire CD106⁺CD45⁻ immunophenotype at later passages. Bottom right quadrants show percentage of CD106⁺CD45⁻ cells for mesenchymal stem cells of CBA/Ca, ICR and Balb/c mice. Numbers in the upper right region indicate passage number.

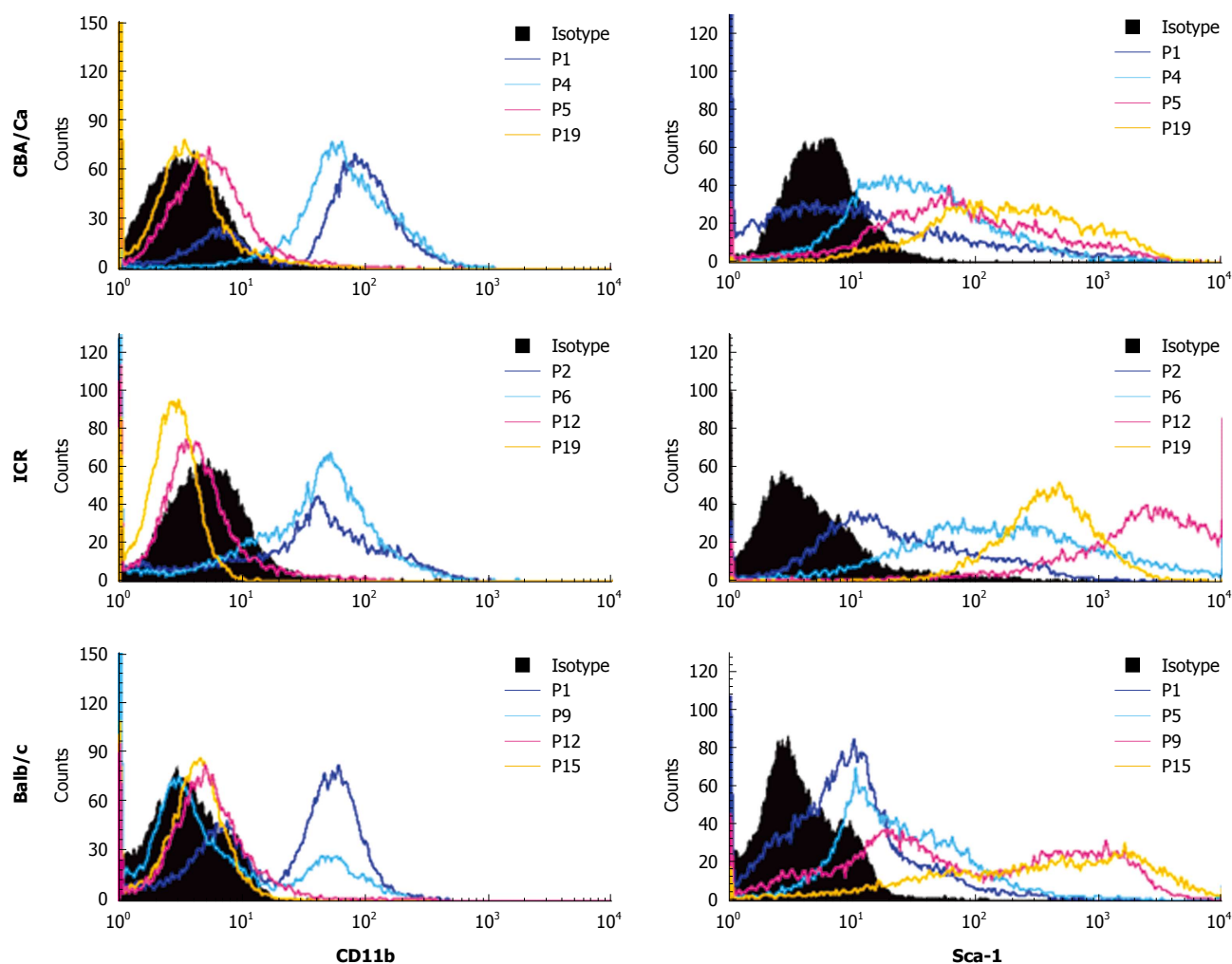


Figure 3 Bone marrow cultures show decreased CD11b and increased Sca-1 expression at later passages. Histograms show the shift of CD11b⁺ at earlier passages to CD11b⁻ at later passages for each strain of mice. Expression of Sca-1 is increased with mesenchymal stem cells passages.

row cultures became increasingly homogeneous, with cells displaying a fibroblast-shape (Figure 1, third and last row). At this point within the culture protocol, cells from all 3 mouse strains took 3-4 d to reach confluency within a 25 cm² flask. Bone marrow cells continued to proliferate in culture beyond passage 35.

Immunophenotypic profile of bone marrow cultures

Immunophenotyping of bone marrow cultures derived from the 3 mouse strains were performed at various passages throughout the culture period. Presence of MSC were confirmed by positivity to CD106, CD44, Sca-1 and MHC I markers and negativity to CD45, CD11b, and MHC II by flow cytometry.

Flow cytometric analyses revealed that MSC demonstrated a shift from a haematopoietic phenotype to typical MSC phenotype with increasing passages. MSC of all 3 strains of mice showed this shift in phenotype from CD106⁺CD45⁺ cells at earlier passages to CD106⁺CD45⁻ at later passages (Figure 2). ICR mice maintained their CD106 positivity for longer, remaining > 77% from passage 8 to 19. For CBA/Ca and Balb/c mice, CD106 ex-

pression began reverting after passage 17 and passage 18 respectively. Expression of CD45 (a common leukocyte antigen) was strong (70%-99%) at early passages for all 3 strains but decreased with increasing passages to < 9% positivity. Cultures derived from Balb/c mice took longer to lose their CD45 positivity compared to CBA/Ca and ICR strains (Figure 2).

Similar to CD45, expression of haematopoietic marker CD11b also revealed a shift from CD11b⁺ to CD11b⁻ with increasing passages (Figure 3). MSC cultures from all 3 strains of mice showed high positivity to CD11b (69%-99%) at early passages. Positivity for CD11b decreased with later passages. Cultures from CBA mice achieved CD11b⁻ phenotype earlier than the other strains, beginning from passage 5. For the MSC marker Sca-1, cultures showed lower positivity to Sca-1 at early passages with only 24%-50% cells positive (Figure 3). Sca-1 expression increased to a higher percentage after passage 5 for CBA/Ca mice, passage 6 for ICR mice and passage 9 for Balb/c mice. At late passages, MSC from all 3 mouse strains showed strong positivity for Sca-1 at 84%-100%. ICR mice demonstrated a distinctly positive (96%-100%)

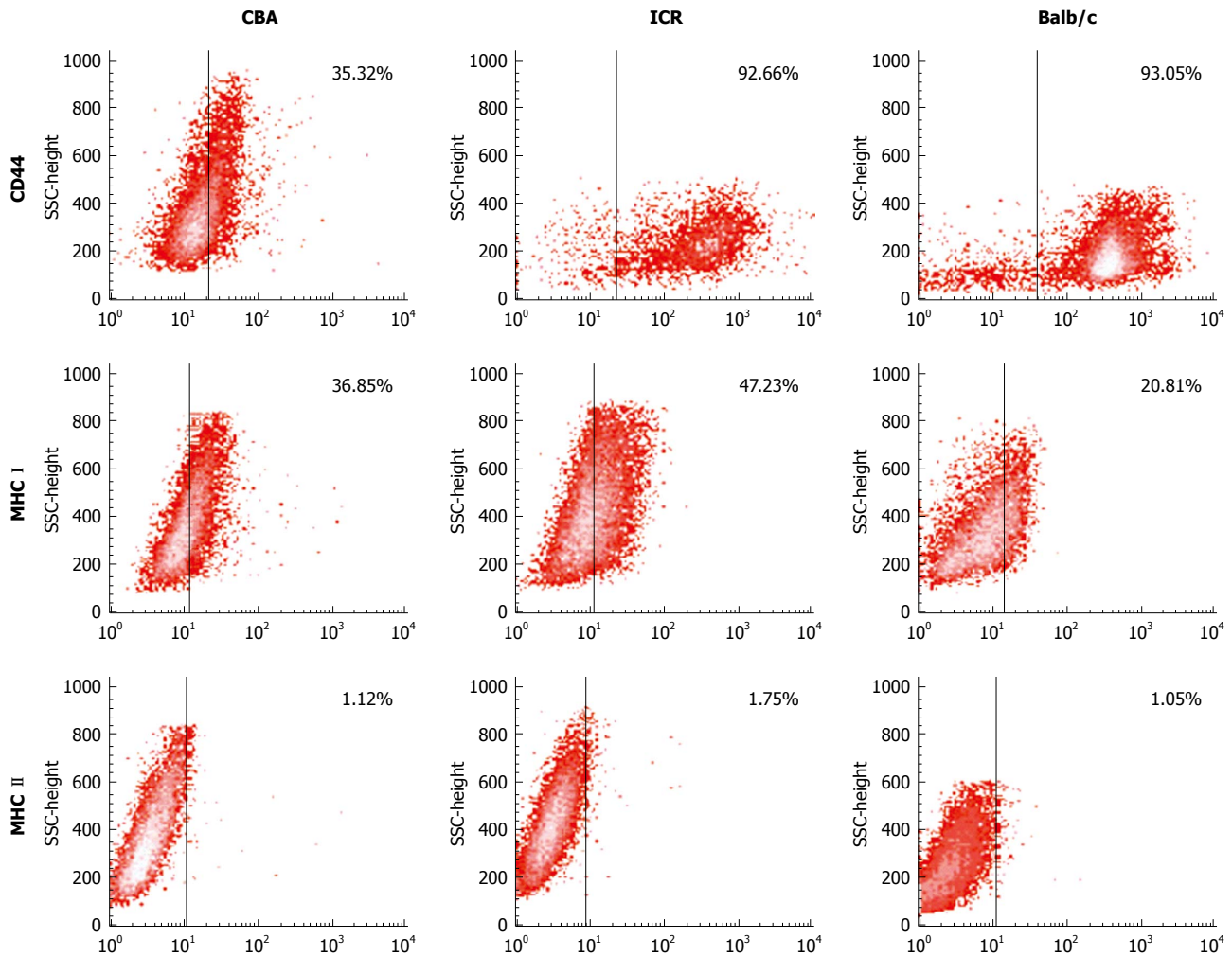


Figure 4 Mesenchymal stem cells cultures from all 3 strains of mice show positivity to CD44 and MHC I but negativity to MHC II. Numbers within plots indicate the percentage of positivity to each marker.

population for Sca-1 after passage 6, while expression in CBA/Ca and Balb/c mice were 91%-97% after passage 15 and 84%-99% after passage 12 respectively.

Next, CD44, MHC I and MHC II expression was examined in bone marrow cultures with CD106⁺CD45⁻ phenotype. For CD44 expression, cells from ICR and Balb/c mice had the highest expression (77%-99% and 79%-99% accordingly), compared to CBA/Ca mice that had varying CD44 expression ranging from 13%-90% (Figure 4). All 3 strains of mice consistently demonstrated positivity to MHC I but not MHC II (< 2%) (Figure 4).

Results from MSC immunophenotyping shown above suggest that bone marrow cultures from all 3 strains of mice acquire an MSC phenotype (CD106⁺CD44⁺Sca-1⁺MHC I⁺CD45⁻CD11b⁻MHC II⁻) after multiple rounds of passaging. Also, amongst the 3 mouse strains tested, bone marrow cells for ICR and Balb/c maintained MSC phenotype for longer compared to CBA/Ca.

Differentiation assay

Bone marrow cultures from all 3 strains of mice differentiated into adipocytes and osteocytes when stimulated

with adipocytic and osteogenic induction medium respectively (Figure 5). Three weeks of exposure to an adipocytic induction medium resulted in formation of lipid vacuoles in cultured MSC that could be observed with phase-contrast microscope. When cultured MSC were exposed to osteogenic induction medium, they aggregated and formed calcium deposits after 2 wk. Both of these cell differentiations were confirmed with cell-specific stains - Oil Red O for adipocytes and Alizarin Red S for osteocytes. Oil Red O-stained lipid vacuoles appeared bright red whereas Alizarin Red S stained precipitated calcium deposits dark red (Figure 5).

DISCUSSION

Isolation of mouse MSC is generally more difficult than human and rat MSC due to a high number of contaminating haematopoietic lineage cells and slow cell growth^[2,15,17]. Studies have also demonstrated mouse strain-dependent variability of MSC in terms of growth kinetics, surface markers and potential for differentiation^[16-18]. With the protocol described here for bone

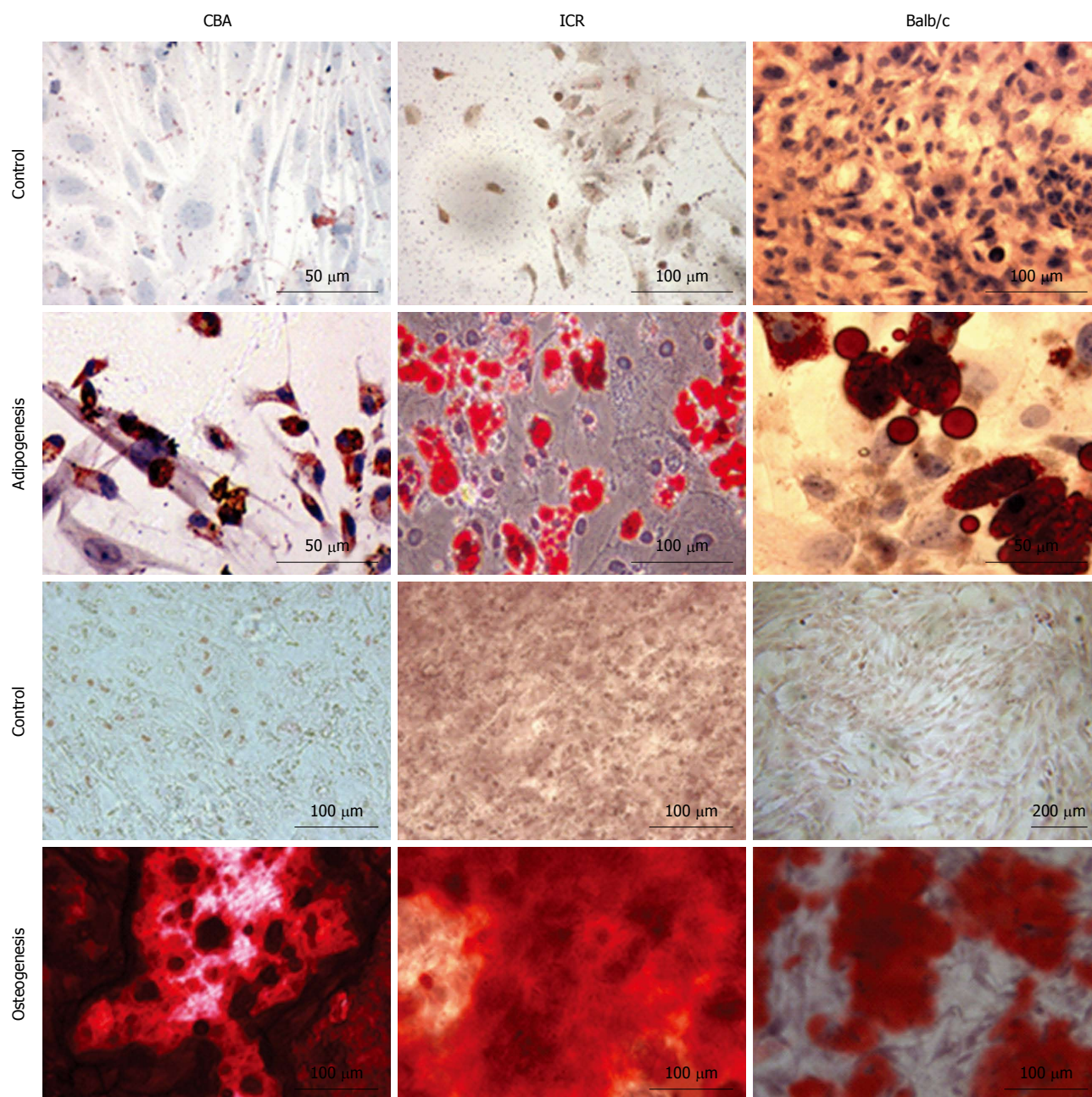


Figure 5 Mesenchymal stem cells from all 3 strains of mice differentiate into adipocytes and osteocytes. First and second row: images of non-induced mesenchymal stem cells (MSC) control and MSC differentiated into adipocytes, stained with Oil Red O. Third and fourth row: images of non-induced MSC control and MSC differentiated into osteocytes, stained with Alizarin Red S. Differences in quality of images are because they were taken using different camera/microscope systems.

marrow-derived MSC expansion, we provide descriptive data that MSC can be successfully generated from 3 different mouse strains, namely CBA/Ca, ICR and Balb/c. Bone marrow cells were cultured and each culture was characterised for their morphology, immunophenotype and differentiation potential. MSC from all strains tested showed expression of relevant surface markers and differentiation potential into osteogenic and adipocytic cells, albeit some differences in terms of time to achieve MSC immunophenotype.

Isolation of MSC from mouse bone marrow usually results in a highly heterogenous cell population with a high degree of haematopoietic contaminants such as

macrophages and fibroblasts^[2,16]. Immunophenotyping analysis revealed early passages of our bone marrow cultures to be positive for haematopoietic markers CD45 and CD11b (a macrophage-specific marker). Continuous change of cell culture medium removes non-adherent haematopoietic populations from the cultures^[3]. Passaging bone marrow cultures also helps eliminate haematopoietic contaminants and yield purer MSC cultures^[17]. With these approaches, we accordingly observed the MSC cultures become increasingly homogenous at later passages with minimal CD45 expression profile by P7-P8, although it took a little longer for cells from Balb/c mice. Better quality MSC cultures were also obtained by dis-

carding cells that remained attached to flasks following trypsinisation. These firmly adherent cells are probably contaminating fibroblasts and have been reported by other laboratories^[17,19]. Expression of stem cell marker Sca-1 also increased at later passages. Sca-1 is a marker associated with MSC and also haematopoietic and endothelial progenitors^[20]. MSC also expressed positivity to CD44 and CD106 and negativity to MHC II. To further encourage MSC growth and colony formation, we used 15% of a mouse mesenchymal supplement (MesenCult®, STEMCELL Technologies) instead of the routine 10% FBS to obtain enriched MSC cultures.

In addition to morphology and phenotype, the characterisation of MSC is complete with demonstration of their capacity for multilineage mesenchymal differentiation^[15,18,19,21]. All 3 mouse strains exhibited multilineage potential *in vitro*, differentiating into adipocytes and osteocyte when stimulated with appropriate induction media. MSC from Balb/c mice more readily differentiate into adipocytes than the other strains, also shown by Peister and colleagues who found Balb/c more readily differentiating into adipose cells than C57Bl/6J and DBA 1 mice^[17]. Conversely, MSC from Balb/c took longer to differentiate into osteocytes compared to the other 2 strains tested in our study. In view of the duration that MSC cultures remained in suitable phenotype and their ease to differentiation, our group has primarily utilised ICR mice for downstream experimentation where we show these stem cells to have immunomodulatory properties^[13].

MSC from all 3 different strains of mice tested were suitable sources for bone marrow-derived MSC as they showed typical morphology, immunophenotype and differentiation capacities of MSC. However, bone marrow cultures from Balb/c mice took longer than the other strains to achieve MSC immunophenotype.

COMMENTS

Background

Experimental research concerning mesenchymal stem cells (MSC) relies heavily on cells isolated from animals. In this paper, we sought to determine whether MSC could be successfully generated from three different mouse strains. The ICR, CBA/Ca and Balb/c mice strains were available to us in the laboratory, and are strains commonly used in research. However, there is no literature stating the feasibility of each of these strains to yield bone marrow-derived MSC. It is important to determine the strain to use, as strain-dependent variability has already been reported by Peister and colleagues.

Research frontiers

The therapeutic relevance for MSC is not limited to their regenerative properties, but also their capacity for modulation of immune responses. Beyond the efficacy of MSC to ameliorate disease, it is essential to test the immunogenicity and tumorigenicity of these cells. The use of murine-derived MSC for preclinical research is indisputable and their applicability in experimental settings should be determined for researchers to be able to accurately assess their use.

Innovations and breakthroughs

In this article, the authors show that bone marrow MSC can be derived from ICR, CBA/Ca and Balb/c mice strains with the protocol described. Similar information for these three mouse strains was previously unavailable. With passaging, bone marrow cultures achieved MSC immunophenotype and displayed multipotency by differentiating into osteocytes and adipocytes.

Applications

This study demonstrates that ICR, CBA/Ca and Balb/c mice strains can yield

bone marrow-derived MSC for downstream experimentation. Based on the protocol described for culture of MSC, the authors describe the passage range of bone marrow cultures for each mouse strain that yields MSC (based on immunophenotyping data). This information provides data that can assist researchers faced with a choice of setting up mouse bone marrow-derived MSC cultures.

Peer review

This article assessed bone marrow-derived MSC cultivated among three different mouse strains. The authors found some differences between strains with regards to the expression of certain surface antigens and differentiation capacity. These results will be useful for the selection of mouse for preclinical studies concerning bone marrow MSC.

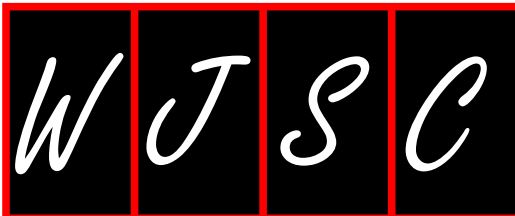
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Oscar Kuang-Sheng Lee, MD, PhD, Professor, Medical Research and Education of Veterans General Hospital-Taipei, No. 322, Sec. 2, Shih-pai Road, Shih-pai, Taipei 11217, Taiwan

Editorial Office

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Instructions to authors

No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-85381891
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Telephone: +86-10-85381892
Fax: +86-10-85381893

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A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

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- 2 Lin GZ, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarhoea. *Shijie Huaren Xiaobua Zazhi* 1999; **7**: 285-287

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- 5 Vallancien G, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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- 10 Sherlock S, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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- 12 Breedlove GK, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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- 15 Morse SS. Factors in the emergence of infectious diseases. *Emerg Infect Dis serial online*, 1995-01-03, cited 1996-06-05;

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- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Write as mean \pm SD or mean \pm SE.

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