Universidade Federal do Rio Grande do Sul

Biologia de células-tronco mesenquimais pós-natais

Lindolfo da Silva Meirelles

Tese submetida ao Programa de Pós-Graduação em Genética e Biologia Molecular da UFRGS como requisito parcial para a obtenção do grau de Doutor em Ciências

Orientadora: Profa. Dra. Nance Beyer Nardi

Orientador no exterior: Prof. Dr. Arnold I. Caplan

Porto Alegre

Abril de 2007

Este trabalho foi desenvolvido no Laboratório de Imunogenética do Departamento de Genética, Instituto de Biociências da Universidade Federal do Rio Grande do Sul, e no Skeletal Research Center da Case Western Reserve University (Cleveland, OH, EUA) com o auxílio financeiro do CNPq.

Dedicada a minha esposa, Adriana, e a meus pais, Sônia e Telmo.

Agradecimentos

Nance, obrigado por me abrir as portas de um laboratório onde pude exercer minhas atividades, e também por permitir que eu pudesse colocar em prática minha criatividade e meu entusiasmo no intuito de fazer Ciência. Obrigado também por permitir e incentivar minha busca por independência científica.

Manifesto aqui minha gratidão ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) por ter-me concedido uma bolsa de doutorado. Devo também ao CNPq a chance de permanecer por seis meses no exterior, adquirindo conhecimentos em um laboratório de excelência em minha área de pesquisa.

My thanks are also due to The Company of Biologists that, by granting me a Travelling Fellowship through Journal of Cell Science, helped me visit Dr. Arnold I. Caplan's laboratory in the US.

Arnold, thanks for accepting me as a visiting student, for the help with my installation and for helping Adriana find a place where she could do some shadowing while we were there. Most importantly, thanks for sharing your scientific experience with me.

Thanks also to everyone in the Caplan lab during my stay there, namely Andreas, Dave, Debbie, Don, Jean, Kitsie, Lisa, Margie, Marilyn, Michael and Paul, for helping me out with everything.

Andrés, Elmo, Luisa, Melissa, Pedro, Renata e Zéca: obrigado por todas as vezes em que me ajudaram, em todos os sentidos.

And last, but not least, I didn't forget you, Pam. Thanks for your help with everything not only for the period of our stay there in the US, but also during all this time.

Sumário

Lista de abreviaturas.......... 6
Resumo.......... 7
Abstract.......... 8
Capítulo 1 – Introdução.......... 9
- Células-tronco.......... 10
- A medula óssea e o estroma medular.......... 10
- A célula-tronco mesenquimal.......... 11
- Obtenção e caracterização da MSC.......... 12
- Fontes adicionais de MSCs.......... 13
- Aplicações da MSC.......... 14

Capítulo 2 – Objetivos.......... 16
Capítulo 3 – Mesenchymal stem cells reside in virtually all post-natal organs and tissues.......... 18
Capítulo 4 – Manuscrito em preparação: Tetraploidy in long-term cultured murine mesenchymal stem cells: a link between cultured mesenchymal stem cells and the perivascular niche?.......... 29
Capítulo 5 – Manuscrito em preparação: Towards a unifying concept of the identity and natural distribution of mesenchymal stem cells.......... 43
Capítulo 6 – Manuscrito em preparação: Human mesenchymal stem cells take up perivascular locations when implanted in ceramic cubes *in vivo*.......... 78
Capítulo 7 – Discussão.......... 96
Capítulo 8 – Referências bibliográficas.......... 102
Capítulo 9 – Anexos.......... 109
- Mesenchymal stem cells: isolation, in vitro expansion and characterization.......... 110
- Functional characterization of cell hybrids generated by induced fusion of primary porcine mesenchymal stem cells with an immortal murine cell line.......... 145
- Polyethylene glycol-mediated fusion between primary mouse mesenchymal stem cells and mouse fibroblasts generates hybrid cells with increased proliferation and altered differentiation.......... 161

Lista de abreviaturas

CFU-F - *colony forming unit-fibroblast*, unidade formadora de colônia de fibroblastos

DNA - *deoxyribonucleic acid*, ácido desoxiribonucléico

FGF-2 - *fibroblast growth factor 2*, fator de crescimento de fibroblasto 2

G-CSF - *granulocyte colony-stimulating factor*, fator estimulatório de colônia de granulócitos

GM-CSF – *granulocyte macrophage colony-stimulating factor*, fator estimulatório de colônia de granulócitos macrófagos

HSC - *hematopoietic stem cell*, célula-tronco hematopoiética

LIF - *leukemia inhibitory factor*, fator inibitório de leucemia

M-CSF – *monocyte colony-stimulating factor*, fator estimulatório de colônia de monócitos

mMSC - *murine mesenchymal stem cell*, célula-tronco mesenquimal murina

MSC - *mesenchymal stem cell*, célula-tronco mesenquimal

OI - *osteogenesis imperfecta*

SCF - *stem cell factor*, fator de célula-tronco

VLA-1 - *very late activation antigen 1*, antígeno de ativação tardia 1 (integrina composta pelas cadeias $\alpha 1$ e $\beta 1$)

VLA-2 - *very late activation antigen 1*, antígeno de ativação tardia 2 (integrina composta pelas cadeias $\alpha 2$ e $\beta 1$)

VLA-3 - *very late activation antigen 1*, antígeno de ativação tardia 3 (integrina composta pelas cadeias $\alpha 3$ e $\beta 1$)

VLA-4 - *very late activation antigen 1*, antígeno de ativação tardia 4 (integrina composta pelas cadeias $\alpha 4$ e $\beta 1$)

VLA-5 - *very late activation antigen 1*, antígeno de ativação tardia 5 (integrina composta pelas cadeias $\alpha 5$ e $\beta 1$)

VLA-6 - *very late activation antigen 1* antígeno de ativação tardia 6 (integrina composta pelas cadeias $\alpha 6$ e $\beta 1$)

Resumo

Células-tronco mesenquimais (MSCs) são um tipo de célula-tronco pós-natal que se mostram muito promissoras como ferramentas terapêuticas porque elas exibem grande plasticidade, e podem ser isoladas e manipuladas de modo reprodutível e com poucos ou nenhum problema ético. Elas foram inicialmente descritas há mais de 30 anos, sob a designação de unidades formadoras de colônia de fibroblasto, e a maior parte do nosso conhecimento sobre elas advém de estudos *in vitro*. Compreender o comportamento das MSCs *in vivo* é um fator chave para o desenvolvimento de terapias celulares eficientes e para engenharia tecidual. Atualmente, as localização e função reais de MSCs *in vivo* ainda são pouco compreendidas. Em uma tentativa de melhor compreender a biologia da MSC, células apresentando características de tronco mesenquimal foram isoladas de vários tecidos diferentes de camundongos adultos, e foram caracterizadas *in vitro*. Os resultados obtidos, conjuntamente com dados da literatura, indicaram que as populações celulares obtidas eram derivadas da vasculatura, mais especificamente da região perivascular. Conseqüentemente, um modelo em que células perivascular ao longo dos vasos sangüíneos constituem uma reserva de células tronco/progenitoras para os tecidos a que pertencem foi concebido. Constatou-se que o conteúdo de DNA das células cultivadas era, em geral, tetraplóide, e esse resultado foi tomado como mais uma evidência a favor da visão de MSCs como células perivasculares, uma vez que tetraploidização em células perivasculares *in vivo* foi relatada como sendo usual em roedores. Uma análise das evidências indicando ligações entre MSCs e pericitos também foi realizada. Finalmente, constatou-se que MSCs humanas inseridas em cubos de cerâmica e implantadas em camundongos imunocomprometidos assumem uma localização perivascular, além de gerar tecido ósseo, dando mais embasamento para a visão de que MSCs cultivadas *in vitro* descendem de células perivasculares. Tomados em conjunto, as informações obtidas indicam que o compartimento perivascular abriga células tronco/progenitoras ao longo de toda sua extensão, e que MSCs isoladas classicamente da medula óssea são provavelmente um subtipo de célula-tronco perivascular.

Abstract

Mesenchymal stem cells (MSCs) are a type of post-natal stem cell that holds great promise as therapeutic tools because they exhibit great plasticity, and can be isolated and manipulated in a reproducible fashion with little or no ethical issues. They were initially described more than 30 years ago, under the designation of colony-forming unit-fibroblasts, and most of our current knowledge on them comes from *in vitro* studies. Understanding the behavior of MSCs *in vivo* is a key factor for the development of efficient cell-based therapies and for tissue engineering. To date, the actual location and function of MSCs *in vivo* are still poorly understood. In an attempt to better understand MSC biology, cells bearing mesenchymal stem characteristics were isolated from several different tissues of adult mice and were characterized *in vitro*. The results obtained, along with data from the literature, indicated the cell populations obtained were derived from the vasculature, more specifically from the perivascular region. As a consequence, a theoretical model in which perivascular cells along the blood vessels constitute a reservoir of stem/progenitor cells for the tissues where they belong was drawn. The DNA content of the cultured cells was found to be generally tetraploid, and this finding was taken as one more evidence towards the view of MSCs as perivascular cells, since tetraploidization in perivascular cells *in vivo* has been reported as usual in rodents. An analysis of the evidences indicating links between MSCs and pericytes was also performed. Finally, human MSCs loaded in ceramic cubes and implanted into immunocompromised mice were found to take up perivascular locations in addition to generate osseous tissue, providing further support for the view that *in vitro* cultured MSCs descend from perivascular cells. Taken together, the informations obtained indicate that the perivascular compartment harbors stem/progenitor cells throughout its extent, and that MSCs classically isolated from bone marrow are probably one subtype of perivascular stem cell.

Capítulo 1

Introdução

Células-tronco

Células-tronco podem ser definidas como aquelas capazes de auto-renovação ilimitada ou prolongada e que podem também dar origem a pelo menos um tipo celular em estágio de diferenciação mais avançado (revisado por Morrison et al., 1997; Watt e Hogan, 2000). A célula-tronco embrionária é totipotente e dá origem a todas as células do organismo (revisado por Odorico et al., 2001; van der Kooy e Weiss, 2000). Indivíduos adultos também possuem células-tronco, mas estas não são totipotentes como a embrionária. Em humanos adultos, a primeira célula-tronco relatada foi a hematopoiética (hematopoietic stem cell, HSC) (revisado por Weissman, 2000). Esta célula localiza-se na medula óssea, e já foi extensivamente caracterizada, demonstrando ser uma célula multipotente que dá origem às diferentes células do sangue (revisado por Nardi e Alfonso, 1999).

Além da HSC, outras células-tronco pós-natais já foram descritas. As células-tronco epitelial, neural e mesenquimal podem ser apontadas como exemplos. A primeira é encontrada no intestino e na epiderme, e dá origem a células em camadas epiteliais (revisado por Slack, 2000). A célula-tronco neural situa-se no cérebro, e origina neurônios, astrócitos, oligodendrócitos e, surpreendentemente, células sangüíneas (McKay, 1997; Bjornson et al., 1999; Gage, 2000). A célula-tronco mesenquimal (mesenchymal stem cell, MSC), é encontrada na medula óssea e gera ossos, tendão, cartilagem, tecidos adiposo e muscular, estroma medular e até mesmo células com características neurais (Caplan, 1991; Prockop, 1997; Pittenger et al., 1999; Kopen et al., 1999).

A medula óssea e o estroma medular

O compartimento medular é muitas vezes descrito como composto basicamente por três sistemas celulares: hematopoiético, endotelial e estromal. O termo estroma refere-se ao conjunto composto pelo sistema celular estromal – que inclui fibroblastos, células endoteliais, células reticulares, adipócitos e osteoblastos – conjuntamente com a matriz extracelular a ele associada, bem como outros tipos celulares, tais como macrófagos (revisado por Deans e Moseley, 2000). Estes, embora sejam de origem hematopoiética, são considerados componentes estromais funcionais (Dexter, 1982). O referido sistema celular

estromal foi proposto por Owen em 1985 e baseia-se em uma analogia com o sistema hematopoiético. Neste modelo, células-tronco estromais residem na medula óssea, mantêm um determinado grau de auto-renovação, e dão origem a células que podem diferenciar-se em várias linhagens de tecido conjuntivo e em tecidos estromais. Além dos sistemas celulares mencionados acima, é importante ressaltar que o compartimento medular abriga também um sistema nervoso, que desempenha funções importantes na integração da hematopoiese com o sistema nervoso central (Elenkov et al.,, 2000).

As células do estroma medular garantem suporte mecânico às células tronco/precursoras hematopoiéticas, além de produzirem matriz extracelular e fatores solúveis que regulam a hematopoiese (Dexter, 1982). Proteínas da matriz, tais como fibronectina, colágeno, vitronectina e tenascina atuam em conjunto com fatores solúveis como o fator de célula-tronco (stem cell factor, SCF), citocinas como o fator estimulatório de colônia de granulócitos-macrófagos (granulocyte-macrophage colony-stimulating factor, GM-CSF) e o fator estimulatório de colônia de granulócitos (granulocyte colony-stimulating factor, G-CSF), e moléculas de adesão tais como as da superfamília das integrinas (VLA-1, VLA-2, VLA-3, VLA-4, VLA-5, VLA-6) na constituição do nicho da célula-tronco hematopoiética (revisado por Whetton e Grahan, 1999).

O contato físico direto entre as células do estroma também é relevante para a regulação desse microambiente. Embora já tenha sido demonstrado que esse contato não é fundamental para que a hematopoiese ocorra (Verfaillie, 1992), existem evidências de que ele esteja relacionado com a qualidade das células hematopoiéticas produzidas (Breems et al., 1998).

A célula-tronco mesenquimal

A primeira evidência direta de que a medula óssea contém células precursoras de tecidos mesenquimais não hematopoiéticos advém do trabalho de Friedenstein e colaboradores, iniciado em Moscou nos anos 60 - 70 (revisado por Phinney, 2002). Em seus experimentos, Friedenstein dispensava amostras de medula óssea suspensas em meio de cultura em frascos de cultura de tecidos, e a fração aderente era cultivada. Por volta do terceiro ao quinto dia, focos discretos de dois a quatro fibroblastos surgiam nas culturas, entre histiócitos e células mononucleares (Friedenstein et al., 1976). Em uma revisão sobre

o assunto, Prockop (1997) acrescenta que a característica mais marcante dessas células era sua capacidade de se diferenciarem em colônias que lembravam pequenos depósitos de osso ou cartilagem. Nos anos 80, diversos estudos estabeleceram que células isoladas pelo método de Friedenstein eram multipotentes e capazes de se diferenciarem em osteoblastos, condroblastos, adipócitos, e até mioblastos (revisado por Prockop, 1997). Em 1991, Caplan, tomando como base modelo do sistema estromal proposto por Owen em 1985, criou o termo célula-tronco mesenquimal para designar a célula que dá origem aos diferentes tipos de tecidos mesenquimais da medula óssea.

Obtenção e caracterização da MSC

MSCs são encontradas na medula óssea de indivíduos adultos, onde estão imersas no estroma (Pittenger et al., 1999). Sua freqüência neste tecido é baixa. Wexler et al. (2003) estimaram que há 1 MSC em cada 34.000 células nucleadas da medula óssea humana por meio de ensaios de unidade formadora de colônia de fibroblastos (CFU-F). Em um estudo preliminar, a freqüencia dessas células na medula óssea murina foi estimada ser 1 em uma faixa de 11.300 – 27.000 células nucleadas (Meirelles e Nardi, 2003).

Os protocolos utilizados para a obtenção da MSC envolvem basicamente o cultivo das células aderentes da medula óssea em placas plásticas (Haynesworth et al., 1992; MacKay et al., 1998; Pittenger et al., 1999, Muraglia et al., 2000; Makino et al., 1999; Wakitani et al., 1995), embora protocolos envolvendo imunodepleção de contaminantes hematopoiéticos antes do cultivo primário já tenham sido utilizados (Kopen et al., 1999; Badoo et al., 2003).

A expansão *in vitro* prolongada das células humanas obtidas pelas técnicas convencionais tem sido difícil de ser obtida (Sekiya et al., 2002), e por isso ainda existem poucos dados abrangentes retratando o quão capazes de proliferação e diferenciação hMSCs são. Há, no entanto, trabalhos que demonstram que a proliferação de MSCs humanas *in vitro* pode ser aumentada por transdução retroviral de construções contendo o cDNA codificante da subunidade catalítica da telomerase humana (Okamoto et al., 2002; Mihara et al., 2003), ou por adição de fator de crescimento de fibroblasto 2 (fibroblast growth factor 2, FGF-2) (Bianchi et al., 2003). MSCs de camundongo, por sua vez, podem ser expandidas *in vitro* por período de tempo ainda indeterminado, sem manipulação

genética ou adição de fatores de crescimento além daqueles presentes no soro fetal bovino (Meirelles e Nardi, 2003).

Vários marcadores moleculares já foram descritos para progenitores mesenquimais comprometidos e para os estágios fenotípicos finais de células originadas da MSC (Seshi et al., 2000; Long, 2001; Bruder et al., 1994). Marcadores moleculares candidatos para a definição de MSCs humanas estão incluídos numa longa lista publicada em uma revisão feita por Deans e Moseley (2000). Dentre estes, podem-se destacar as moléculas de superfície CD44, CD29, CD90, e os fatores secretados LIF (*leukemia inhibitory factor*, fator inibitório de leucemia), M-CSF (*monocyte-colony-stimulating factor*, fator estimulador de colônia de monócitos), e SCF. Até pouco tempo, a verificação dos marcadores específicos da MSC murina (mMSC) ainda não era possível devido à dificuldade de obtenção de uma população pura através das técnicas convencionais (Phinney et al., 1999). Trabalhos recentes têm colaborado para reverter esta situação (Wieczorek et al., 2003; Badoo et al., 2003, Meirelles e Nardi, 2003).

A caracterização dos marcadores da MSC observados durante seu cultivo *in vitro*, conforme descrito no parágrafo anterior, não teria validade se a população de células em estudo não tivessem características funcionais de tronco e mesenquimal confirmadas, ou seja: células em cultura podem ser operacionalmente consideradas MSCs se forem capazes de auto-renovação e também de dar origem, em condições adequadas de cultivo, a diferentes tipos celulares mesenquimais, tais como adipócitos, condrócitos e osteócitos. Diferenciação neuronal (Woodbury et al., 2000; Zuk et al., 2002) e suporte à hematopoiese (Majumdar et al., 1998) são características também atribuídas a MSCs, e também devem ser levadas em consideração. Finalmente, para que o termo "operacional" possa ser deixado de lado é necessária, ainda, a demonstração de que tais células contribuem para a formação de tecidos mesenquimais quando infundidas in vivo (revisado por Verfaillie, 2002).

Fontes adicionais de MSCs

Além de estar presente na medula óssea de indivíduos adultos, a MSC também pode ser encontrada em outros tecidos e fases do desenvolvimento (Tabela 1).

Tabela 1. Ocorrência de células com características de tronco mesenquimal em diferentes órgãos/tecidos, durante o período pós-embrionário.

Local	Espécie	Período de vida	Referência (s)
Tecido adiposo	*Homo sapiens*	pós-natal	Zuk et al., 2001; Zuk et al., 2002
Tecido adiposo	*Mus musculus*	pós-natal	Safford et al., 2002
Pâncreas	*Homo sapiens*	fetal	Hu et al.,2003
Medula óssea	*Homo sapiens*	fetal	Campagnoli et al., 2001
Fígado	*Homo sapiens*	fetal	Campagnoli et al., 2001
Sangue	*Homo sapiens*	fetal	Campagnoli et al., 2001
Tendão	*Mus musculus*	pós-natal	Salingcarnboriboon et al., 2003
Membrana sinovial	*Mus musculus*	pós-natal	de Bari et al., 2003
Líquido amniótico	*Homo sapiens*	fetal	in ‘t Anker et al., 2003
Sangue periférico	*Homo sapiens*	pós-natal	Zvaifler et al., 2000; Kuwana et al., 2003
Sangue de cordão umbilical	*Homo sapiens*	fetal/pós-natal	Alfonso et al., 2000

A distribuição de células com características de MSC no organismo pós-natal foi estudada em maior detalhe no primeiro artigo desta tese (Capítulo 3).

Aplicações da MSC

MSCs já vêm sendo exploradas há algum tempo com o propósito de se obter regeneração esquelética (Bruder et al., 1994). Seu potencial de diferenciação em múltiplas linhagens, sua sensibilidade elevada a moléculas sinalizadoras específicas e uma relativa facilidade de manuseio *in vitro* (Caplan e Bruder, 2001; Beyer Nardi e da Silva Meirelles, 2006) as torna ferramentas importantes para a engenharia de tecidos, e para terapias celulares.

O potencial de utilização de MSCs para engenharia tecidual é reforçado ainda por dois fatores: a) elas podem diferenciar-se em tipos celulares não-mesenquimais *in vitro* (neurônios – Woodbury et al., 2000) e *in vivo* (células neurais – Kopen et al., 1999;

hepatócitos – Sato et al., 2005); e b) elas exibem a tendência de adquirir características tecido-específicas quando co-cultivadas com tipos celulares especializados ou quando expostas a extratos teciduais *in vitro* (células de câncer gástrico – Houghton et al., 2004; células secretoras de insulina – Choi et al., 2005; hepatócitos – Lange et al., 2005).

A MSC também apresenta potencial para o tratamento de doenças caracterizadas pela deficiência do produto de algum gene, de duas formas: a) utilizando-se a MSC *per se*, quando a deficiência genética puder ser suprida por seu patrimônio genético, ou b) modificando-se a MSC para que ela expresse altos níveis do produto gênico em questão, quando a primeira estratégia não é eficaz. Insere-se no primeiro caso a tentativa bem sucedida de correção da *osteogenesis imperfecta* (OI) – doença genética causada pela deficiência da produção de colágeno tipo I - em crianças utilizando-se transplante de medula óssea de doadores compatíveis (Horwitz et al., 1999; Horwitz et al., 2001). Esse trabalho baseou-se no fato de que a medula óssea contém MSCs capazes de inserir-se em vários pontos do organismo quando injetadas na circulação, de diferenciar-se em células envolvidas na produção de ossos e cartilagem e músculo, e que podem auxiliar a regeneração desses tecidos por suprirem a deficiência de colágeno tipo I nos indivíduos receptores.

Outras características que fazem da MSC uma boa candidata a agente terapêutico são seus efeitos imunomodulatórios, demonstrados quando usadas no tratamento de um paciente com doença do enxerto contra o hospedeiro (Le Blanc et al, 2004), e também seus efeitos tróficos (Caplan e Dennis, 2006), que podem ser úteis para o tratamento de acidentes vasculares cerebrais.

Capítulo 2

Objetivos

Em vista das características da MSC aqui apresentadas, este trabalho teve os seguintes objetivos:

Conhecer melhor a biologia da MSC, através da caracterização de MSCs obtidas de órgãos/tecidos de camundongos adultos que não a medula óssea, e da comparação das mesmas entre si, utilizando os critérios já estabelecidos para a mMSC derivada da medula óssea (Meirelles e Nardi, 2003);

Checar mMSCs obtidas quanto a alterações genéticas grosseiras, tais como aneuploidias, através da observação de seu conteúdo de DNA;

Compreender a relação da MSC com o nicho perivascular *in vivo*.

Capítulo 3

Mesenchymal stem cells reside in virtually all post-natal organs and tissues

Lindolfo da Silva Meirelles, Pedro Pedro Cesar Chagastelles & Nance Beyer Nardi

J Cell Sci. 2006 Jun 1;119(Pt 11):2204-13.

Mesenchymal stem cells reside in virtually all post-natal organs and tissues

Lindolfo da Silva Meirelles, Pedro Cesar Chagastelles and Nance Beyer Nardi*

Departamento de Genética, Universidade Federal do Rio Grande do Sul, Av Bento Gonçalves 9500, 91540-970 Porto Alegre, RS, Brazil

*Author for correspondence (e-mail: nardi@ufrgs.br)

Accepted 13 February 2006
Journal of Cell Science 119, 2204-2213 Published by The Company of Biologists 2006
doi:10.1242/jcs.02932

Summary

Mesenchymal stem cells (MSCs) are multipotent cells which can give rise to mesenchymal and non-mesenchymal tissues in vitro and in vivo. Whereas in vitro properties such as (trans)differentiation capabilities are well known, there is little information regarding natural distribution and biology in the living organism. To investigate the subject further, we generated long-term cultures of cells with mesenchymal stem cell characteristics from different organs and tissues from adult mice. These populations have morphology, immunophenotype and growth properties similar to bone marrow-derived MSCs. The differentiation potential was related to the tissue of origin. The results indicate that (1) cells with mesenchymal stem characteristics can be derived and propagated in vitro from different organs and tissues (brain, spleen, liver, kidney, lung, bone marrow, muscle, thymus, pancreas); (2) MSC long-term cultures can be generated from large blood vessels such as the aorta artery and the vena cava, as well as from small vessels such as those from kidney glomeruli; (3) MSCs are not detected in peripheral blood. Taken together, these results suggest that the distribution of MSCs throughout the post-natal organism is related to their existence in a perivascular niche. These findings have implications for understanding MSC biology, and for clinical and pharmacological purposes.

Key words: Mesenchymal stem cell, Pericyte, CFU-F, Mouse, In vitro cultivation

Introduction

Stem cells are defined as having the capacity for extensive self-renewal and for originating at least one type of highly differentiated descendant (Watt and Hogan, 2000). Post-natal tissues have reservoirs of specific stem cells which contribute to maintenance and regeneration. Examples include epithelial stem cells in epidermis and intestinal crypts (Slack, 2000), neural stem cells in the central nervous system (McKay, 1997) and satellite cells in muscle (Charge and Rudnicki, 2004). The adult bone marrow shelters different types of stem cells, including hematopoietic (Weissman, 2000) and mesenchymal (Prockop, 1997; Nardi and da Silva Meirelles, 2006) stem cells.

Experiments using bone marrow cells have raised the issue of phenotypic plasticity (Herzog et al., 2003; Wagers and Weissman, 2004), because they have shown the consequent generation of specialized cells derived from bone marrow in the central nervous system (Eglitis and Mezey, 1997; Mezey et al., 2000), skeletal muscle (Ferrari et al., 1998), liver (Petersen et al., 1999) and heart (Orlic et al., 2001). The specific cell type(s) involved in these phenomena is not clear. However, reports describing mesenchymal stem cell (MSC) differentiation capabilities suggest that they may contribute to the results observed: they can differentiate into specific cell types in vitro and in vivo (Woodbury et al., 2000; Kopen et al., 1999; Sato et al., 2005), and have a tendency to acquire tissue-specific characteristics when co-cultured with specialized cell types or exposed to tissue extracts in vitro (Houghton et al., 2004; Choi et al., 2005; Lange et al., 2005). In addition, the capacity to differentiate into mesodermal (Pittenger et al., 1999), ectodermal (Kopen et al., 1999) and endodermal (Sato et al., 2005) cell lineages characterizes MSCs as pluripotent cells, suggesting that the term ‘mesenchymal’ stem cell might be inappropriate to describe this particular stem cell. Since different methodologies are used to cultivate and characterize MSC-related cell types, there is still a lack of consensus on the hierarchy intrinsic to the MSC compartment (Nardi and da Silva Meirelles, 2006), reflected by the existence of similar cells such as multipotent adult progenitor cells (MAPCs) (Reyes et al., 2001), marrow-isolated adult multilineage inducible (MIAMI) cells (D’Ippolito et al., 2004), and recycling stem cells (RS-1, RS-2) (Colter et al., 2000).

The exact nature and localization of MSCs in vivo remain poorly understood; growing evidence indicates a relationship with pericytes (Doherty et al., 1998; Farrington-Rock et al., 2004). Approaches so far include the use of selected markers known to be expressed by MSCs in vitro to seek positive cells in vivo (Bianco et al., 2001; Shi and Gronthos, 2003) and the infusion of marked cultured cells in vivo to analyze their tissue distribution (Anjos-Afonso et al., 2004). The first type of approach, though sensitive, may be nonspecific as the majority of cell markers are specific only in a given context. The second strategy may be less accurate to study MSC natural distribution in vivo because the cells might engraft nonspecifically in different locations. The systematic isolation of MSCs from different organs and tissues and the evaluation of their characteristics could represent an alternative approach. Studies describing the isolation of post-natal mesenchymal stem cells from different sources – e.g. adipose tissue (Zuk et al., 2001),

Journal of Cell Science

tendon (Salingcarnboriboon et al., 2003), periodontal ligament (Seo et al., 2004), synovial membrane (De Bari et al., 2001) and lungs (Sabatini et al., 2005) – can be found in the literature; however, these isolated studies do not allow the consistent visualization of the distribution of MSCs in the post-natal organism.

Here, we analyzed the in vivo distribution of murine post-natal MSCs through the establishment, long-term culture and functional characterization of MSC populations from different tissues and organs. The possibility that MSC cultures were partially or entirely derived from circulating blood was excluded by perfusing the animals intravascularly before organ collection. Moreover, no MSC long-term culture could be established from blood when a controlled protocol to minimize vessel rupture was used. Our data demonstrate that the MSC compartment is more widely distributed than previously thought and we present evidence that MSCs are resident in vessel walls. Variations in immunophenotype and osteogenic or adipogenic differentiation potential according to the site of origin suggest that functional roles are at least partially organ specific. These findings provide insight on the biology of MSCs in vivo, and add new information to be considered when developing clinical protocols involving the MSC compartment.

Results

Establishment and morphological characterization of long-term cultures

The establishment of long-term cultures was highly dependent on the conditions used to set up the primary culture. For instance, the use of DMEM with 10 mM HEPES instead of common buffered saline to dissolve collagenase, and a digestion time limited to up to 1 hour at 37°C proved to be essential for reproducibility (not shown). Other important factors were the elapsed time between euthanasia and tissue processing – the shorter the better, particularly for bone marrow and liver – and the temperature of the culture medium, which should not be lower than room temperature. The amount of medium used during the collagenase digestion step was also important for the establishment of brain-derived cultures, because this organ rapidly acidifies small volumes of medium. Furthermore, when establishing primary cultures from perfused animals, we observed that using DMEM containing 10 mM HEPES and HB-CMF-HBSS (1:1), rather than culture medium or saline alone, seemed to improve the quality of the MSC-like cells in primary culture.

The MSC long-term cultures generated during this study are described in Table 1. Not all cultures, in particular the earlier ones, were generated using the optimized conditions described above. To minimize eventual differences due to different starting conditions, the analyses were mainly focused on population sets established under similar conditions, even though gross differences, as judged by flow cytometric analyses, morphological and functional characteristics, were not observed among long-term cultures generated in any of the conditions (not shown).

Using the optimized conditions mentioned above, cells that morphologically resembled characterized MSCs could be seen as early as 24 hours post-plating (Fig. 1A). In the case of glomerular cell culture, adherent cell outgrowths could be observed from the third or fourth day onwards (Fig. 1B). Depending on the starting amount and on which tissue was used to establish the cultures, confluence could be reached within 5 days. Individual glomerular cultures did not reach confluence even when cultured for over a month despite robust initial cell growth (Fig. 1B,C), possibly because of the contact inhibition among the cells in each colony after some cell divisions; on the other hand, cultures containing 20 glomeruli or more per well could become confluent within three weeks or so, if the outgrowths were evenly distributed on the bottom of the dish. During the initial culture period (passages 1 to 5) there was some morphological heterogeneity in the adherent fraction, particularly in bone marrow and spleen cultures, possibly because of the presence of hematopoietic contaminants (Fig. 1D). As the cultures were passaged, morphological homogeneity was gradually achieved in that flat cells bearing a large nucleoli-rich nucleus predominated; this was independent of the origin of the culture (Fig. 1E,F). Glomeruli-derived explants, on the other hand, showed this morphology right from the start of culture (Fig. 1B). The expression of surface markers was analyzed by flow cytometry preferably at this time (see below).

During the establishment of primary cultures, special care was taken to avoid contamination by adjacent tissues. Muscle was thoroughly removed from femora and tibiae before bone

Table 1. MSC populations generated and donor animals

Animal	Strain	Age (weeks)	Gender	Perfused	MSC populations generated
001B	BALB/c	14	Male	No	001Bbm
002B	BALB/c	15	Male	No	002Bs
004B	BALB/c	8	Female	No	004Bbm, 004Bs, 004Bt, 004Bk
005B	BALB/c	9	Male	No	005Bs
006B	BALB/c	11	Male	No	006Bb
009B	BALB/c	16	Female	No	009Bl
010B	BALB/c	16	Female	No	010Bb
011B	BALB/c	10	Male	No	011Bt
012B	BALB/c	37	Male	No	012Ba
013B	BALB/c	37	Male	No	013Ba1, 013Ba2
014B	BALB/c	30	Male	No	014Ba1, 014Ba2
015B	BALB/c	31	Male	No	015Ba1, 015Ba2, 015Bs, 015Bl
016B	BALB/c	30	Male	Yes	016Ba1, 016Ba2, 016Bs, 016Bm, 016Bk
017B	BALB/c	43	Male	Yes	017Bk
018B	BALB/c	18	Female	Yes	018Ba1, 018Ba2, 018Bs, 018Bm, 018Bk, 018Bl
019B	BALB/c	20	Female	Yes	019Bs, 019Bbm, 019Blu, 019Bk
021B	BALB/c	27	Female	No	021Bkg
001C	C57Bl/6	39	Female	No	001Ck
002C	C57Bl/6	9	Male	No	002Cbm
008C	C57Bl/6	35	Female	No	008Clu, 008Cvc
009C	C57Bl/6	39	Female	Yes	009Ck
001R	ROSA26	20	Male	No	001Rlu
003R	ROSA26	13	Male	No	003Rbm, 003Rp
001G	GFP	22	Male	No	001Gvc

In the right column, lowercase letters indicate the tissue or organ from which the culture was derived: a, aorta; b, brain; bm, bone marrow; k, kidney; kg, kidney glomeruli; l, liver; lu, lungs; m, muscle; p, pancreas; s, spleen; t, thymus; vc, vena cava. In the case of aorta-derived cultures, the numbers after the ‘a’ indicate the first and second fraction-derived populations (see Materials and Methods for details).

marrow extraction, to avoid contamination with muscle-derived cells; adipose tissue was likewise separated from the aorta through serial enzymatic dissociation. In this particular case, the two fractions obtained could generate long-term cultures exhibiting the same morphological, immunophenotypic and kinetic characteristics (not shown), indicating that the enzymatic fractionation procedure is not necessary. On the other hand, contamination from surrounding tissue proved to be a serious issue for blood. The establishment of MSC-like long-term cultures from blood collected through cardiac puncture, or from the thoracic cavity, was possible but not easily reproducible (not shown). However, attempts to establish MSC cultures from blood collected from the portal vein were consistently unsuccessful. We consider this a very important observation, because of two facts: first, MSC long-term cultures can be generated from only one cell (see the Cloning section below); second, it is possible that a few MSCs detach from the walls of ruptured vessels and are collected with the blood. In this case, the results would suggest that blood can originate MSC long-term cultures. The insertion of an intravenous catheter cranially into the portal vein helps minimize this problem. As the catheter is introduced contrary to the blood flow, the few cells that eventually detach from the vessel wall during the needle insertion are likely to be lost in the circulation before blood collection starts.

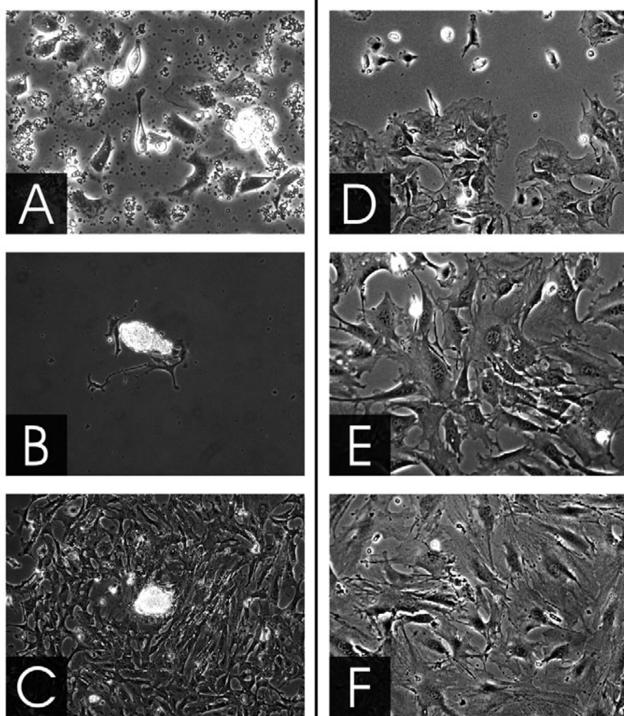

Fig. 1. Morphology of MSC cultures derived from different organs and tissues. (A) Phase-contrast micrographs of MSC-like cells in primary culture of aorta 24 hours after plating. Glomerulus outgrowth on the fourth (B) and sixth (C) day post-plating. (D) Heterogeneity among bone marrow-derived cells at passage 4, with MSC-like cells (lower portion), spindle-shaped and round cells. (E) Pancreas-derived MSCs at passage 30. (F) Vena-cava-derived MSCs at passage 22. Magnifications, ×100 (B-F); ×200 (A).

Immunophenotyping

The analysis of surface markers indicated that the MSC populations originating from multiple sources have a very similar immunophenotype. Examples of surface molecule profiles for selected markers are shown in Fig. 2. All the populations studied expressed CD29 (integrin β_1 chain) and CD44 (hyaluronan receptor). The expression of molecules such as CD34 (hematopoietic progenitor marker), Sca-1 (stem cell antigen-1) and CD49e (integrin α_5 chain) was variable among the different populations, and could also show variation during extended subculture (Fig. 3). CD90.2 expression, when present, was high; however, the proportion of positive cells was variable, ranging from ~30% to nearly 100%. High expression of CD117, the stem cell factor receptor, was infrequent; some populations however seemed to express it at very low levels, and a small percentage of positive cells could sometimes be detected. CD117 expression showed a tendency to decrease during extended serial passage, reaching control levels (Fig. 3), as reported previously for bone-marrow-derived murine MSCs (da Silva Meirelles and Nardi, 2003). The granulocyte marker Gr-1 was expressed in low levels by some populations (not shown). The hematopoietic markers CD45 and CD11b were not expressed by MSCs. They were only observed during the initial passages in cultures derived from bone marrow and spleen, presenting a small proportion of cells with morphological characteristics of macrophages, indicating hematopoietic contamination. MSC cell populations were also negative for the monocyte marker CD13, the leukocyte markers CD18 and CD19, the endothelial marker CD31 and surface Ig. The expression of CD49d (integrin α_4 chain) remains to be analyzed in more detail. When some populations were collected using either trypsin-EDTA or EDTA alone, CD49d was observed on cells collected with EDTA but not on those treated with trypsin, indicating its sensitivity to the enzyme (not shown).

The immunophenotyping of MSCs at early stages revealed the heterogeneity within cell populations before an eventual subpopulation selection owing to extensive cultivation. The expression of αSMA, a vascular smooth muscle cell marker, was analyzed in cultures that had been tested for their differentiation potential. All the MSC populations examined expressed αSMA as exemplified by representative results in Fig. 4, suggesting their relationship to perivascular cells.

Growth kinetics

Growth curves describing culture kinetics were generated as previously described (da Silva Meirelles and Nardi, 2003). Representative examples of growth curves for MSC cultures established from each of the organs or tissues are shown in Fig. 5. The culture kinetics varied depending on the origin of the cells and the culture stage. In general, the growth rate was low during the early passages, and increased with serial subculture (and time) until a stable value was reached. This behavior was previously reported for bone-marrow-derived murine MSCs alone (da Silva Meirelles and Nardi, 2003). However, differences in the kinetics of the cultures related to the tissue of origin of the MSCs could be observed. The growth rate of brain-derived MSC cultures, for instance, was slow for a longer period when compared with the other cultures. In addition, the stable growth ratios achieved by the different MSC populations, reflected in the inclination of their growth curves, differed.

Journal of Cell Science

Journal of Cell Science

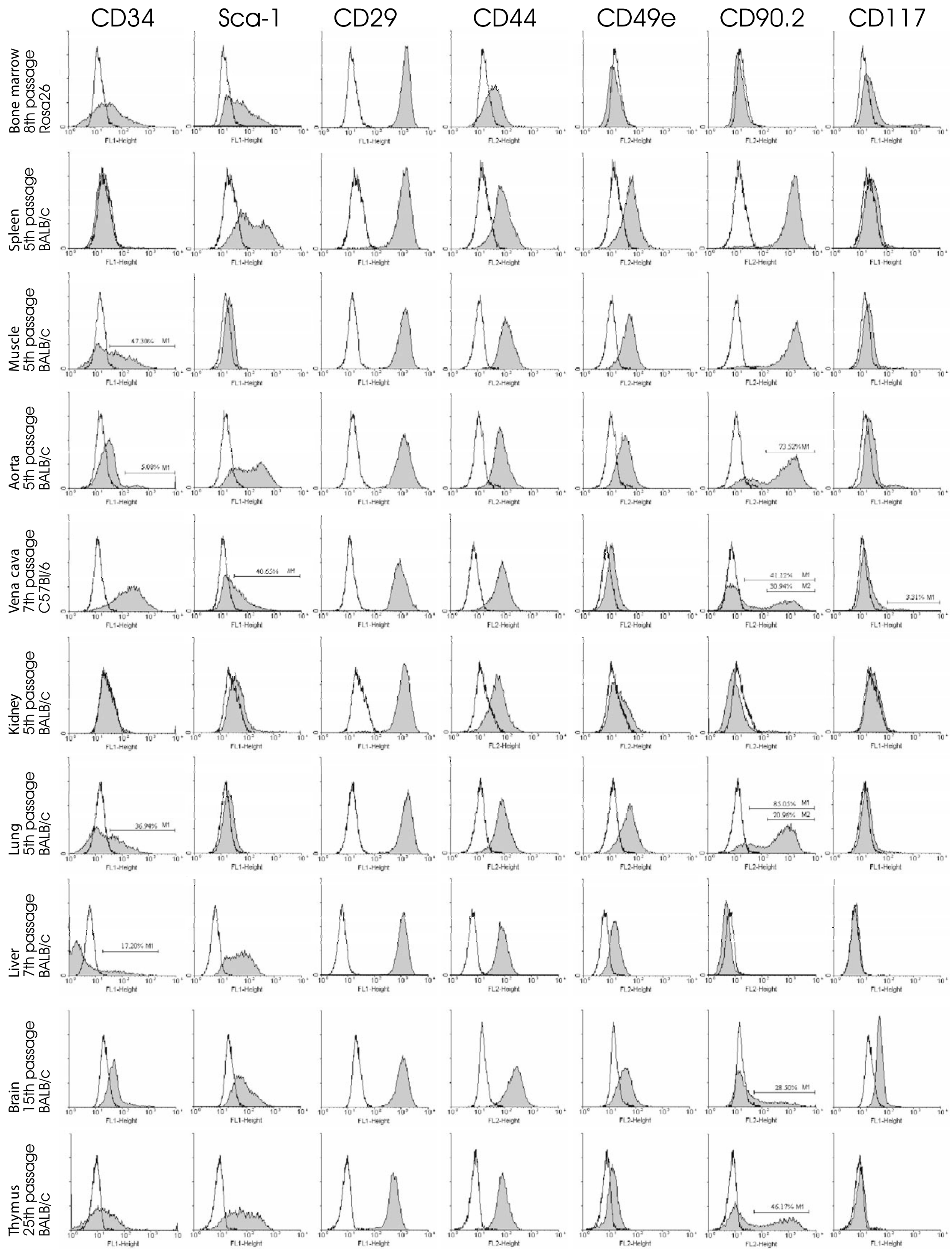

Fig. 2. Immunophenotypic profile of MSCs derived from different sources. Flow cytometry histograms show the expression (shaded) of selected molecules (CD34, Sca-1, CD29, CD44, CD49e, CD90.2 and CD117) by different MSC populations compared with controls (unshaded peaks). Kidney-derived MSCs and kidney glomerulus-derived MSCs share essentially the same surface profile.

Journal of Cell Science

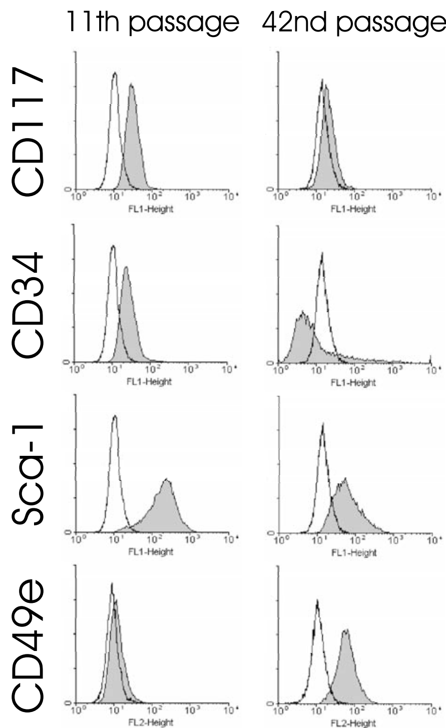

Fig. 3. Effect of long-term culture on the expression of CD117, CD34, Sca-1 and CD49e. Thymus-derived MSCs were analyzed by flow cytometry at passages 11 and 42. Whereas the level of expression of Sca-1 was around 2 log values (versus 1 log control), CD117 expression decreased to nearly control levels, CD34 expression was lost, and expression of CD49e increased 1 log value.

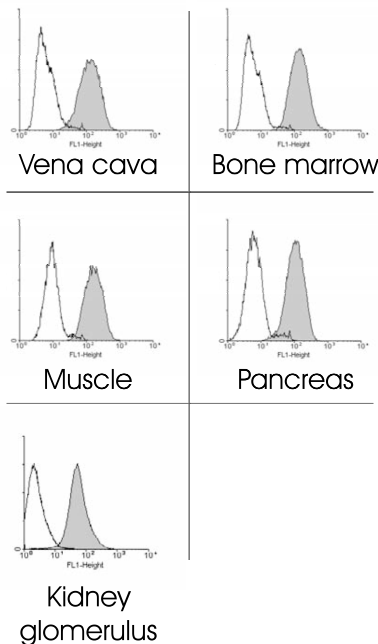

Fig. 4. αSMA expression by MSCs. The histograms represent levels of expression of αSMA (shaded) in MSCs from vena cava, bone marrow, muscle, pancreas and kidney glomeruli compared with controls (unshaded).

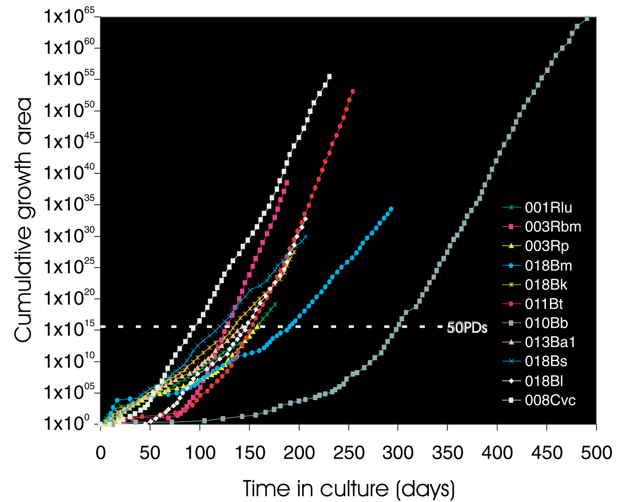

Fig. 5. Comparative growth kinetics of cultures originated from different organs and tissues. Growth curves of representative MSC populations from each source (as presented in Table 1) are plotted. The threshold for 50 population doublings (50 Pds) is shown as a dashed line. Lowercase letters indicate the tissue or organ from which the culture was derived: a, aorta; b, brain; bm, bone marrow; k, kidney; l, liver; lu, lungs; m, muscle; p, pancreas; s, spleen; t, thymus; vc, vena cava. Numbers and capital letters refer to the animal used. Growth area is represented as a multiple of the area occupied by a confluent primary culture, arbitrarily set to 1.

The growth curves shown in Fig. 5 are based on the area occupied by the MSCs. To estimate the expansion in terms of population doublings (PDs), a simple correlation can be made in that 2^{50}, which represents 50 PDs, corresponds to ~1.13×10^{15} times the initial population. On the same basis, 2^{100} equals ~1.27×10^{30} times the initial population, and so on. The MSC populations depicted in Fig. 5 underwent over 50 PDs, some of them reaching 100-200 PDs. We have not observed replicative senescence in any murine MSC long-term cultures. The cultures were generated at different time points and some of them were cultured longer than others. Most of them were cryopreserved by the end of this study.

Differentiation

Functional assays to confirm the MSC identity of the populations studied were preferably performed later (see below), to evaluate the effect of prolonged cultivation on the capacity of the cells to differentiate. When subjected to osteogenic or adipogenic differentiation conditions, the MSC populations confirmed their mesenchymal stem characteristics by depositing a calcium-rich mineralized matrix as evidenced by Alizarin Red S staining, or by acquiring intracellular lipid droplets, evidenced by Oil Red O staining (Fig. 6).

Differences in the frequency of differentiated cells, as well as in the degree of differentiation, could be observed among the cultures originating from different tissues. Vena-cava-derived MSCs, for instance, were very efficient at depositing mineralized matrix, whereas muscle-derived MSCs showed little efficiency (Fig. 6C,G). On the other hand, muscle-derived MSCs were easily induced to differentiate into mature

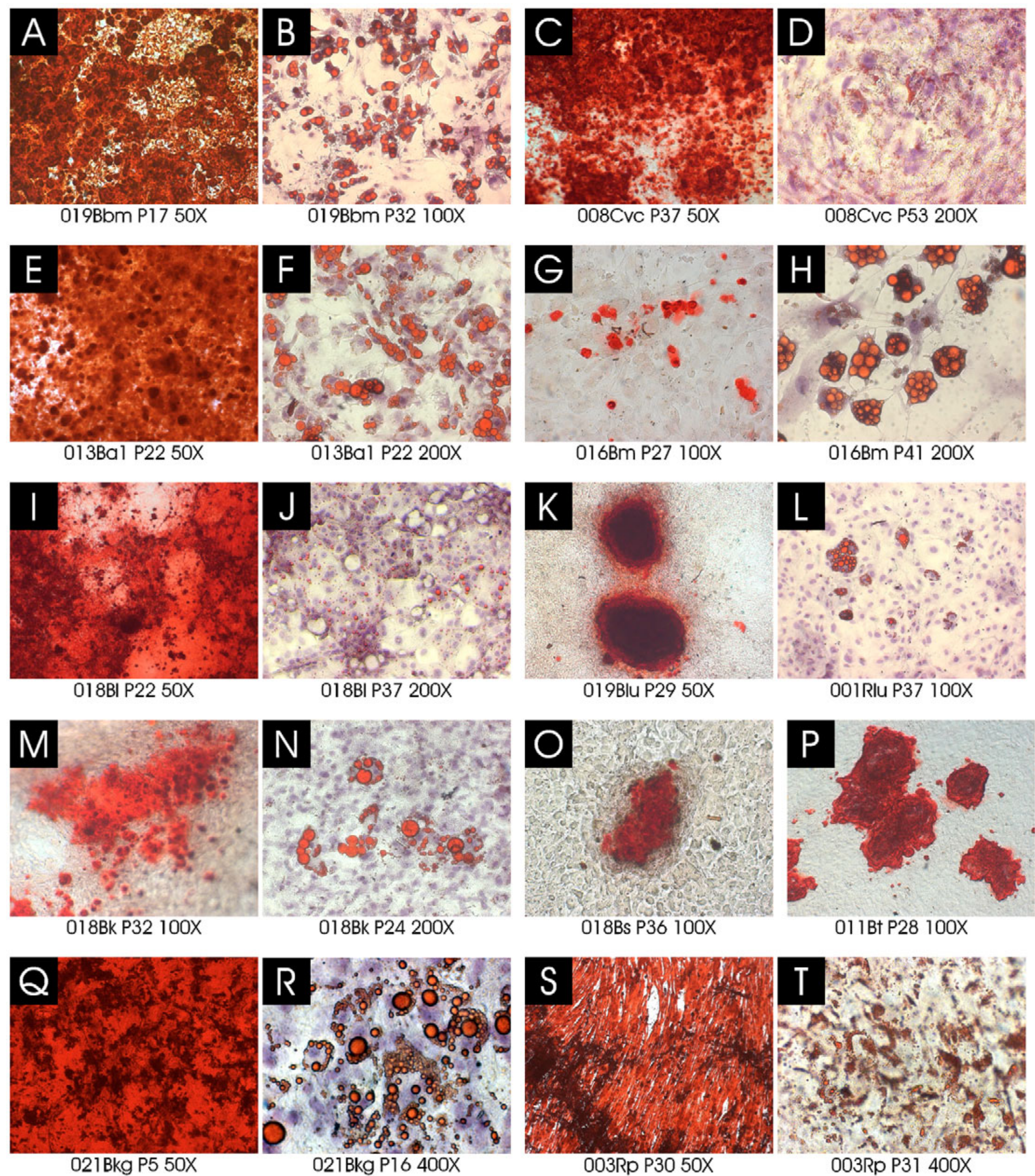

Fig. 6. Differentiation of MSCs derived from different sources, as presented in Table 1. MSCs were cultured in osteogenic or adipogenic medium for up to 2 months. Calcium deposited in the extracellular matrix is stained red by Alizarin Red S (A,C,E,G,I,K,M,O-Q,S). Lipid vacuoles are stained orange with Oil Red O (B,D,F,H,J,L,N,R,T). Magnifications and passage number (P) are indicated below each image. Cell lines are identified as described in the legend to Fig. 5.

adipocytes whereas the vena-cava-derived cultures presented small, poorly developed lipid vacuoles (Fig. 6H,D). Those differences were maintained even when the cultures were exposed to differentiation conditions for longer periods. The differentiation properties of aorta- and bone-marrow-derived MSCs were similar in terms of efficiency and quality (Fig. 6A,B,E,F). Spleen-, thymus-, lung- and kidney-derived MSCs exhibited mineralized nodules when subjected to osteogenic differentiation rather than mineralization of the whole monolayer (Fig. 6K,M,O,P), in contrast to bone-marrow- or aorta-derived MSCs (Fig. 6A,E). The adipogenic differentiation observed in lung-, brain- and kidney-derived MSCs seemed to be less efficient (Fig. 6L,N; brain-derived MSC adipogenic differentiation not shown), even though the degree of adipogenic differentiation presented by kidney- and lung-derived MSCs was comparable to that of bone-marrow-derived MSCs (Fig. 6B). Furthermore, these populations required a longer induction period to differentiate into adipocytes as compared with bone marrow-derived MSCs. Glomeruli-derived MSCs, tested at the fifth passage, differed from the whole-kidney-derived ones regarding osteogenesis: they deposited a rich mineralized matrix that could be detected by the first week of differentiation (Fig. 6Q), and were thus equivalent to bone-marrow-derived MSCs regarding their osteogenic potential. Glomeruli-derived MSCs could differentiate into adipocytes (Fig. 6R), similarly to the whole-kidney-derived cells. Pancreas-derived MSCs also exhibited osteogenic (Fig. 6S) and adipogenic (Fig. 6T) potential.

The protocols used in this study are quite simple, and the use of more complex differentiation strategies (e.g. special substrates and growth factors) might increase the efficiency of differentiation process, so that gross differences may disappear. The results obtained with the assays used in the present study, however, show that the propensity of MSC cultures to respond to different stimuli varies according to their in vivo location.

In addition to differentiation induced as described above, in

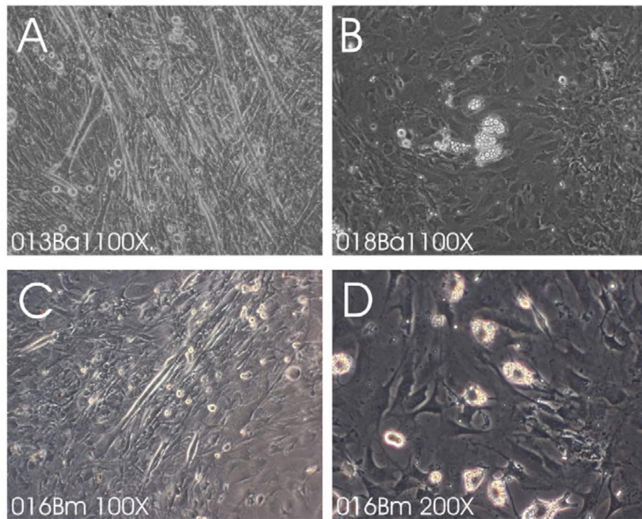

Fig. 7. Non-induced MSC differentiation in primary culture. Aorta primary cultures exhibit myogenic (A) and adipogenic (B) differentiation. The same happens in muscle primary cultures (C and D, respectively). Magnifications are indicated on each image.

some cases spontaneous differentiation was seen in primary cultures. In cultures derived from aorta and muscle, for instance, myotube-like cells and adipocytes were often observed (Fig. 7). This phenomenon could be caused by the short-term exposure to amphotericin-B present in antibiotic-antimycotic solution (Phinney et al., 1999), or by the presence of myogenic- and adipogenic-committed progenitors in the primary culture. Spontaneous differentiation was not observed in primary cultures originating from the other organs and tissues studied.

Cloning

Two cloning processes were performed, with long-term MSC cultures. In each of them, three 96-well plates were seeded with individual cells and analyzed 2 weeks later. The results were very similar, as presented in Table 2, and showed that around 50% of the cells were able to originate clones, with different potentials for expansion. The morphology of the clones was the same observed for the MSC cultures. Eight of the clones were selected for further expansion, and were able to establish cultures with normal growth kinetics, maintained for around 4 months. Two of these cultures were subcloned, and one of the subclones was submitted to a further subcloning process, with results similar to those of the initial cloning (Table 2). One clone from each cloning procedure was tested for the ability to differentiate along osteogenic or adipogenic pathways and exhibited differentiation capabilities similar to those of parental cultures in terms of both efficiency and quality.

Discussion

Mesenchymal stem cells have been conventionally isolated from bone marrow (Pittenger et al., 1999; Kopen et al., 1999) and, more recently, from some other tissues (Zuk et al., 2001; De Bari et al., 2001; Seo et al., 2004; Sabatini et al., 2005). This study was originally designed to investigate whether, using the same conditions established for the cultivation of murine bone-marrow-derived MSCs (da Silva Meirelles and Nardi, 2003), these cells could be found in other organs. Surprisingly, the results showed that long-term MSC cultures could be established from all the organs and tissues studied, irrespective or their embryonic origin. The cell populations thus obtained can be operationally defined as MSCs, because they exhibit the capacity of prolonged self-renewal and differentiate along mesenchymal cell lineages. The possibility that the long-term cultures might represent early progenitors exhibiting multipotent capabilities was tested with cloning experiments. The results showed that a percentage of cells plated at the single-cell level were able to regenerate long-term cell cultures indicating that, at a given time point, a proportion of the cells in each population is committed to self-renewal whereas the remainder are not. When clones were cultured under osteogenic or adipogenic conditions, they exhibited differentiation characteristics similar to those of parental cultures. This indicates that the long-term cultures represent populations containing stem cells, and suggests that population asymmetry is responsible for the maintenance of the stem cell pool (Watt and Hogan, 2000).

The MSC cell populations originating from brain, spleen, liver, kidney, kidney glomeruli, lung, bone marrow, muscle, thymus and pancreas presented similar morphology and, to a certain extent, surface marker profile. On the other hand, the differentiation assays showed some variation among the cultures in the frequency of cells which actually differentiated

Table 2. Results of cloning and subcloning of long-term MSC cultures

	Culture				
	Cloning 1	Cloning 2	Subcloning 1	Subcloning 2	Sub-subcloning
–	112 (38.8)	144 (50.0)	61 (31.8)	42 (21.9)	25 (26.0)
+	36 (12.5)	12 (4.2)	34 (17.7)	53 (27.6)	16 (16.7)
++	75 (26.0)	84 (29.2)	35 (18.2)	21 (10.9)	n.a.
+++	49 (17.0)	39 (13.5)	51 (26.6)	73 (38.0)	55 (57.3)
Aborted	12 (4.2)	6 (2.1)	9 (4.7)	3 (1.6)	–
Differentiated	4 (1.5)	3 (1.0)	2 (1.0)	–	–
Osteogenic differentiation	+ (Clone H4)	+ (Clone 3D10)	n.a.	n.a.	n.a.
Adipogenic differentiation	+ (Clone H4)	+ (Clone 3D10)	n.a.	n.a.	n.a.

MSC long-term cultures were cloned by micromanipulation. Individual cells were plated in 96-well plates (three plates for each cloning process) with MSC-conditioned medium and maintained for 2 weeks. Two well-developed clones were subcloned (two plates for each process), and one of the resulting cultures was further subcloned (one plate prepared). The plates were analyzed for the number (and percentage) of negative (–) and positive wells which were classified according to the size of the colonies (+, ++, +++). Aborted clones were those in which the cells proliferated and then spontaneously died. Differentiated clones developed mature morphologies, which were not further investigated. n.a., not applicable.

Journal of Cell Science

in the osteogenic or adipogenic phenotype, as well as on the degree of differentiation, related to their site of origin. This might be due to the influence of the local environment from which they originate, reflecting the importance of the niche in establishing the phenotype of the stem cells it interacts with (Fuchs et al., 2004).

Whereas most other studies have approached the question of the natural distribution of MSCs in the organism by infusing cultured cells into the animal models and analyzing their multi-site engraftment, this is, to our knowledge, the first one to apply the opposite approach. The simultaneous analysis of different organs and tissues for their MSC contents can provide more accurate information regarding their natural in vivo distribution. The wide distribution of MSCs observed raised the question of the relationship among these populations. Three hypotheses were then considered: first, MSCs are tissue-resident cells, and can be collected from individual tissues or organs; second, MSCs are resident in tissues, and circulate in blood; third, MSCs are derived from the circulating blood.

To test these hypotheses, the possibility that the MSC long-term cultures were derived from cells circulating in the blood was first analyzed by perfusing the animals before collection of the tissues or organs. Cultures could be normally established under these conditions. Since no long-term culture could be derived from circulating blood collected under controlled conditions, MSCs seem to be absent from the circulation under normal physiological conditions, in agreement with Wexler et al. (Wexler et al., 2003). The possibility that they circulate systemically or locally under other circumstances, e.g. during tissue injury, is not however excluded and was not tested in the present study. Endothelial progenitor cells, for instance, can be found at higher frequency in the peripheral blood of patients with acute myocardial infarction (Shintani et al., 2001).

The characteristics of MSC populations obtained from the different organs were however very similar, suggesting a closer relationship between them. Since literature reports have suggested that MSCs derive from perivascular cells (Doherty et al., 1998; Bianco et al., 2001; Shi and Gronthos, 2003; Farrington-Rock et al., 2004), it is possible that MSCs are actually derived from the vasculature. To test this hypothesis, the aorta and the vena cava were investigated and long-term MSC cultures could be established from both tissues. We next analyzed these and the MSC populations obtained from other sources and found them positive for the vascular smooth muscle cell marker αSMA (Owens, 1995). To demonstrate that perivascular cells at the capillary level have mesenchymal stem cell properties, we isolated a structure comprising capillaries only – the kidney glomerulus. Decapsulated glomeruli are composed of endothelial cells, podocytes and mesangial cells – which are considered specialized pericytes (Schlondorff, 1987). Based on the expression of αSMA, reported as a marker for activated mesangial cells (Johnson et al., 1991), lack of expression of CD31 by the resultant population, and morphology, the cultures can be regarded as originating from mesangial cells within the glomerulus. Their osteogenic and adipogenic differentiation capabilities, along with their self-renewal capacity, allow them to be operationally defined as MSCs. Taking these results as a whole, we conclude that the MSC compartment extends through the whole post-natal organism as a result of its perivascular location.

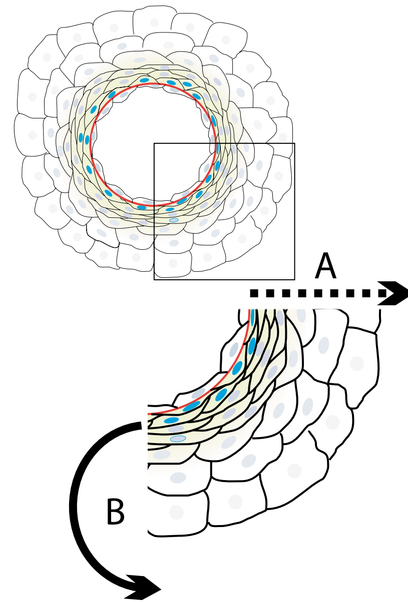

Fig. 8. A proposed model of MSC contribution to tissue maintenance. In this schematic representation of the transverse section of a simple vessel, MSCs lie in the basement membrane (red line), opposed to endothelial cells. Cues provided by the tissue-specific microenvironment coordinate a gradual transition (represented by green color gradient) from undifferentiated cells to progenitor and mature cell phenotypes. This process can occur naturally as represented by the dotted arrow (A). In case of tissue injury, undifferentiated MSCs can be mobilized directly into the tissue without the progenitor transition as represented by the curved arrow (B).

Early in vivo experiments have suggested that pericytes may act as a source of undifferentiated cells during adipose (Richardson et al., 1982) and osseous (Diaz-Flores et al., 1992) tissue repair. These data, along with reports describing the MSC differentiation capabilities (Pittenger et al., 1999; Kopen et al., 1999; Woodbury et al., 2000; Sato et al., 2005; Choi et al., 2005; Lange et al., 2005), and the functional differences between the populations studied here according to their origin, led us to propose the model depicted in Fig. 8. In this model, MSCs act as a reservoir of undifferentiated cells to supply the cellular (and non-cellular) demands of the tissue they belong to, acquiring local phenotypic characteristics. When necessary, and after signs from the microenvironment, they give rise to committed progenitors that gradually integrate into the tissue (Fig. 8A). Tissue injury can activate alternative processes (Fig. 8B). The model does not exclude the possible existence of other tissue-specific stem cells; however, it suggests that a portion of the apparent post-natal stem cell diversity may be attributed to local MSCs behaving as tissue-specific stem cells. Once again the term 'mesenchymal' stem cell seems inappropriate, and possibly the term 'perivascular stem cell' might best represent this particular cell type.

We believe that, in addition to providing insight into MSC biology, our findings and hypotheses can be useful for designing therapeutic strategies for a range of diseases. Irradiation, or drugs able to transiently destabilize the vessel wall integrity, might facilitate cell engraftment in cell or cell-

mediated therapies; also, drugs and genetic therapy vectors could be directed to the perivascular compartment to achieve tissue-specific activity, as perivascular-derived cells gradually assume a tissue-specific phenotype. These approaches validate the circulatory system and more specifically its stem cell compartment as a vehicle for reaching the whole organism.

Materials and Methods

Reagents, culture media and solutions

Complete culture medium (CCM) was composed of Dulbecco's modified Eagle's medium (DMEM) with HEPES (free acid, 2.5–3.7 g/l) and 10% fetal bovine serum (Cultilab, Sao Paulo, Brazil). Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution containing 10 mM sodium HEPES (HB-CMF-HBSS) combined with DMEM (1:1) was used as perfusion medium. All reagents used in this study were from Sigma Chemical Co. (St Louis, MO), unless otherwise stated. Plasticware was from TPP (Trasadingen, Switzerland).

Animals

Adult mice (8-43 weeks old) from the C57Bl/6 and BALB/c strains were used in this study. ROSA26 (The Jackson Laboratory, Bar Harbor, ME) and eGFP mice (green mouse FM131, kindly provided by M. Okabe, Osaka University, Japan), derived from the C57Bl/6 strain, were also used. The animals were kept under standard conditions (12 hours light/12 hours dark, water and food ad libitum) in our animal house. All the experimental procedures were performed according to institutional guidelines.

Perfusion

The animals were anesthetized with a combination of ketamine and xilazine (1.16 g and 2.3 g per kg body weight, respectively) delivered intraperitoneally. The abdominal cavity was opened, the diaphragm was ruptured, and 100 units of heparin in 200 μl HB-CMF-HBSS were injected into the beating heart. The ascending aorta was catheterized with a 27G intravenous catheter inserted through the left ventricle. The caudal vena cava was cut, and around 50 ml of perfusion medium were pumped in. For lung perfusion, the pulmonary artery was catheterized instead.

MSC isolation and long-term culture

MSCs from bone marrow were isolated and cultured as previously described (da Silva Meirelles and Nardi, 2003). MSCs from liver, spleen, pancreas, lung, kidney, aorta, vena cava, brain and muscle were obtained as follows. Organs and tissues were collected from perfused or non-perfused animals, rinsed in HB-CMF-HBSS, transferred to a Petri dish and cut into small pieces. When dissecting organs, care was taken to discard the portions containing visible vessels (e.g. the portal vein and the vena cava in the liver). The dissected pieces (around 0.2-0.8 cm^3) were washed with HB-CMF-HBSS, cut into smaller fragments, and subsequently digested with collagenase type I (0.5 mg/ml in DMEM/10 mM HEPES) for 30 minutes to 3 hours at 37°C. To separate the adipose layer surrounding the aorta, the vessel was digested for around 30 minutes and subjected to vigorous agitation, yielding a first cell fraction. The remnant of the vessel was then washed in 20 ml HB-CMF-HBSS, and transferred to a new tube where digestion proceeded yielding a second cell fraction. Both cell fractions were used to establish separate primary cultures.

Whenever gross remnants persisted after collagenase digestion, they were allowed to settle for 1 to 3 minutes, and the supernatant was transferred to a new tube which was then completed with CCM. In some experiments, the cells were further cleared from debris by centrifugation on Ficoll-Hypaque (Amersham Pharmacia, Piscataway, NJ), followed by an additional washing step. After centrifugation at 400 ***g*** for 10 minutes at room temperature (RT), the pellets were resuspended in 3.5 ml CCM containing 1% antibiotic-antimycotic solution (GIBCO BRL, Gaithersburg, MD), seeded in six-well dishes (3.5 ml/well) and incubated at 37°C in a humidified atmosphere containing 5% CO_2. Three days later, if the cultures were not confluent, the whole volume of CCM (with no antibiotics or antimycotics) was replaced, and the adherent layer was refed every 3 or 4 days.

For subculture, the adherent layer was washed once and incubated with 0.25% trypsin and 0.01% EDTA in HB-CMF-HBSS. The cultures were split whenever they reached confluence, at ratios empirically determined for two subcultures a week at most. Initial split ratios were 1:2 or 1:3, and as the culture kinetics accelerated the ratios were set to values ranging from 1:6 to 1:25, until they stabilized at different ratios as described below.

To evaluate the presence of MSCs in blood, animals were anesthetized, the abdominal cavity was opened, and 100 units of heparin in 200 μl HB-CMF-HBSS were injected into the beating heart. Either a 27G intravenous catheter was introduced cranially into the portal vein and 500-750 μl blood were collected, or the vessels arising from the heart were cut and 500-750 μl blood were collected from the thoracic cavity. Blood was also collected directly from the exposed heart in some cases. The collected blood was either added to a 25 cm^2 flask containing 7 ml CCM incubated at 37°C, or fractionated on Ficoll-Hypaque. In this case, mononuclear cells were collected, washed once in complete medium, resuspended in 3.5 ml fresh complete medium, transferred to a well of a six-well dish and incubated at 37°C. In either case, after 3 days, non-adherent cells were removed along with the culture medium and fresh complete medium was added. The adherent cells were then re-fed every 3 or 4 days.

To establish glomeruli-derived MSC cultures, kidneys were placed into a 15 ml centrifuge tube containing 5 ml CCM, and mechanically disrupted by several rounds of aspiration/expulsion using a 10 ml pipette. Single glomeruli devoid of the Bowman's capsule were isolated from the cell suspension by micromanipulation, and transferred either individually or collectively to 12-well dishes containing CCM and 1% antibiotic-antimycotic solution. Subsequent passages were performed as described above.

Cell cloning

To clone MSCs to the single-cell level, cultures were trypsinized, resuspended in MSC-conditioned medium which had been previously filtered through a 0.22 μm membrane, and individually transferred to 96-well dishes using a micromanipulator. The number, morphology and kinetics of resulting clones were analyzed, and some of them were selected for subcloning and differentiation assays.

Morphological analysis and photographs

MSC cultures were routinely observed with an inverted phase-contrast microscope (Axiovert 25; Zeiss, Hallbergmoos, Germany). For detailed observation, cells were rinsed with phosphate-buffered saline (PBS), fixed with ethanol for 5 minutes at RT in some cases, and stained for 2.5 minutes with Giemsa. Photomicrographs were taken with a digital camera (AxioCam MRc, Zeiss), using AxioVision 3.1 software (Zeiss).

Flow cytometry

For detection of surface antigens the cells were trypsinized, centrifuged, and incubated for 30 minutes at 4°C with phycoerythrin (PE)- or fluorescein isothiocyanate (FITC)-conjugated antibodies against murine Sca-1, Gr-1, CD11b, CD13, CD18, CD19, CD29, CD31, CD44, CD45, CD49d, CD49e, CD90.2, CD117 and IgG (Pharmingen BD, San Diego, CA). Excess antibody was removed by washing.

For the detection of α-smooth muscle actin (αSMA), the cells were collected, washed once in HB-CMF-HBSS and fixed with 4% paraformaldehyde in PBS for 1 hour at RT. After centrifugation, the cells were kept for 15 minutes at RT in 5 ml PBS containing 0.2% Triton X-100. Cells were collected, washed once in PBS, and incubated with or without primary antibody against αSMA (Chemicon, Temecula, CA) overnight at 4°C. The cells were then incubated with FITC-conjugated anti-mouse IgG secondary antibody for 1 hour at 4°C.

The cells were analyzed using a FACScalibur cytometer equipped with 488 nm argon laser (Becton Dickinson, San Diego, CA) with the CellQuest software. At least 10,000 events were collected. The WinMDI 2.8 software was used for building histograms.

MSC differentiation

Osteogenic differentiation was induced by culturing MSCs for up to 8 weeks in CCM supplemented with 10^{-8} M dexamethasone, 5 μg/ml ascorbic acid 2-phosphate and 10 mM β-glycerophosphate (Phinney et al., 1999). To observe calcium deposition, cultures were washed once with PBS, fixed with 4% paraformaldehyde in PBS for 15-30 minutes at RT, and stained for 5 minutes at RT with Alizarin Red S stain at pH 4.2. Excess stain was removed by several washes with distilled water.

To induce adipogenic differentiation, MSCs were cultured for up to 8 weeks in CCM supplemented with 10^{-8} M dexamethasone, 2.5 μg/ml insulin, 100 μM indomethacin and, in some experiments, 3.5 μM rosiglitazone or 5 μM 15-deoxy-$D^{12,14}$-prostaglandin J_2. Later in this study, DMEM with 10 mM HEPES, heparin and 20% platelet-free human plasma (Krawisz and Scott, 1982) was used to induce kidney glomerulus-derived MSC adipogenic differentiation. Adipocytes were easily discerned from the undifferentiated cells by phase-contrast microscopy. To further confirm their identity, cells were fixed with 4% paraformaldehyde in PBS for 1 hour at RT, and stained with either Oil Red O solution (three volumes of 3.75% Oil Red O in isopropanol plus two volumes of distilled water) or Sudan Black B solution (three volumes of 2% Sudan Black B in isopropanol plus two volumes of distilled water) for 5 minutes at RT. When stained with Oil Red O, the cultures were counterstained with Harry's hematoxylin (1 minute at RT).

We thank Ricardo Ribeiro dos Santos (FIOCRUZ, Salvador, Brazil) and Antonio Carlos Campos de Carvalho (Universidade Federal do Rio de Janeiro, Brazil) for their assistance with the establishment of the colonies of transgenic mice. This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS).

Journal of Cell Science

Journal of Cell Science

References

Anjos-Afonso, F., Siapati, E. K. and Bonnet, D. (2004). In vivo contribution of murine mesenchymal stem cells into multiple cell-types under minimal damage conditions. *J. Cell Sci.* **117**, 5655-5664.

Bianco, P., Riminucci, M., Gronthos, S. and Robey, P. G. (2001). Bone marrow stromal stem cells: nature, biology, and potential applications. *Stem Cells* **19**, 180-192.

Charge, S. B. and Rudnicki, M. A. (2004). Cellular and molecular regulation of muscle regeneration. *Physiol. Rev.* **84**, 209-238.

Choi, K. S., Shin, J. S., Lee, J. J., Kim, Y. S., Kim, S. B. and Kim, C. W. (2005). In vitro trans-differentiation of rat mesenchymal cells into insulin-producing cells by rat pancreatic extract. *Biochem. Biophys. Res. Commun.* **330**, 1299-1305.

Colter, D. C., Class, R., DiGirolamo, C. M. and Prockop, D. J. (2000). Rapid expansion of recycling stem cells in cultures of plastic-adherent cells from human bone marrow. *Proc. Natl. Acad. Sci. USA* **97**, 3213-3218.

da Silva Meirelles, L. and Nardi, N. B. (2003). Murine marrow-derived mesenchymal stem cell: isolation, in vitro expansion, and characterization. *Br. J. Haematol.* **123**, 702-711.

De Bari, C., Dell'Accio, F., Tylzanowski, P. and Luyten, F. P. (2001). Multipotent mesenchymal stem cells from adult human synovial membrane. *Arthritis Rheum.* **44**, 1928-1942.

Diaz-Flores, L., Gutierrez, R., Lopez-Alonso, A., Gonzalez, R. and Varela, H. (1992). Pericytes as a supplementary source of osteoblasts in periosteal osteogenesis. *Clin. Orthop. Relat. Res.* **275**, 280-286.

D'Ippolito, G., Diabira, S., Howard, G. A., Menei, P., Roos, B. A. and Schiller, P. C. (2004). Marrow-isolated adult multilineage inducible (MIAMI) cells, a unique population of postnatal young and old human cells with extensive expansion and differentiation potential. *J. Cell Sci.* **117**, 2971-2981.

Doherty, M. J., Ashton, B. A., Walsh, S., Beresford, J. N., Grant, M. E. and Canfield, A. E. (1998). Vascular pericytes express osteogenic potential in vitro and in vivo. *J. Bone Miner. Res.* **13**, 828-838.

Eglitis, M. A. and Mezey, E. (1997). Hematopoietic cells differentiate into both microglia and macroglia in the brains of adult mice. *Proc. Natl. Acad. Sci. USA* **94**, 4080-4085.

Farrington-Rock, C., Crofts, N. J., Doherty, M. J., Ashton, B. A., Griffin-Jones, C. and Canfield, A. E. (2004). Chondrogenic and adipogenic potential of microvascular pericytes. *Circulation* **110**, 2226-2232.

Ferrari, G., Cusella-De Angelis, G., Coletta, M., Paolucci, E., Stornaiuolo, A., Cossu, G. and Mavilio, F. (1998). Muscle regeneration by bone marrow-derived myogenic progenitors. *Science* **279**, 1528-1530.

Fuchs, E., Tumbar, T. and Guasch, G. (2004). Socializing with the neighbors: stem cells and their niche. *Cell* **116**, 769-778.

Herzog, E. L., Chai, L. and Krause, D. S. (2003). Plasticity of marrow-derived stem cells. *Blood* **102**, 3483-3493.

Houghton, J., Stoicov, C., Nomura, S., Rogers, A. B., Carlson, J., Li, H., Cai, X., Fox, J. G., Goldenring, J. R. and Wang, T. C. (2004). Gastric cancer originating from bone marrow-derived cells. *Science* **306**, 1568-1571.

Johnson, R. J., Iida, H., Alpers, C. E., Majesky, M. W., Schwartz, S. M., Pritzi, P., Gordon, K. and Gown, A. M. (1991). Expression of smooth muscle cell phenotype by rat mesangial cells in immune complex nephritis. Alpha-smooth muscle actin is a marker of mesangial cell proliferation. *J. Clin. Invest.* **87**, 847-858.

Kopen, G. C., Prockop, D. J. and Phinney, D. G. (1999). Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. *Proc. Natl. Acad. Sci. USA* **96**, 10711-10716.

Krawisz, B. R. and Scott, R. E. (1982). Coupling of proadipocyte growth arrest and differentiation. I. Induction by heparinized medium containing human plasma. *J. Cell Biol.* **94**, 394-399.

Lange, C., Bassler, P., Lioznov, M. V., Bruns, H., Kluth, D., Zander, A. R. and Fiegel, H. C. (2005). Liver-specific gene expression in mesenchymal stem cells is induced by liver cells. *World J. Gastroenterol.* **11**, 4497-4504.

McKay, R. (1997). Stem cells in the central nervous system. *Science* **276**, 66-71.

Mezey, E., Chandross, K. J., Harta, G., Maki, R. A. and McKercher, S. R. (2000). Turning blood into brain: cells bearing neuronal antigens generated in vivo from bone marrow. *Science* **290**, 1779-1782.

Nardi, N. B. and da Silva Meirelles, L. (2006). Mesenchymal stem cells: isolation, in vitro expansion and characterization. *Handb. Exp. Pharmacol.* **174**, 249-282.

Orlic, D., Kajstura, J., Chimenti, S., Jakoniuk, I., Anderson, S. M., Li, B., Pickel, J., McKay, R., Nadal-Ginard, B., Bodine, D. M. et al. (2001). Bone marrow cells regenerate infarcted myocardium. *Nature* **410**, 701-705.

Owens, G. K. (1995). Regulation of differentiation of vascular smooth muscle cells. *Physiol. Rev.* **75**, 487-517.

Petersen, B. E., Bowen, W. C., Patrene, K. D., Mars, W. M., Sullivan, A. K., Murase, N., Boggs, S. S., Greenberger, J. S. and Goff, J. P. (1999). Bone marrow as a potential source of hepatic oval cells. *Science* **284**, 1168-1170.

Phinney, D. G., Kopen, G., Isaacson, R. L. and Prockop, D. J. (1999). Plastic adherent stromal cells from the bone marrow of commonly used strains of inbred mice: variations in yield, growth, and differentiation. *J. Cell Biochem.* **72**, 570-585.

Pittenger, M. F., Mackay, A. M., Beck, S. C., Jaiswal, R. K., Douglas, R., Mosca, J. D., Moorman, M. A., Simonetti, D. W., Craig, S. and Marshak, D. R. (1999). Multilineage potential of adult human mesenchymal stem cells. *Science* **284**, 143-147.

Prockop, D. J. (1997). Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science* **276**, 71-74.

Reyes, M., Lund, T., Lenvik, T., Aguiar, D., Koodie, L. and Verfaillie, C. M. (2001). Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells. *Blood* **98**, 2615-2625.

Richardson, R. L., Hausman, G. J. and Campion, D. R. (1982). Response of pericytes to thermal lesion in the inguinal fat pad of 10-day-old rats. *Acta Anat. Basel* **114**, 41-57.

Sabatini, F., Petecchia, L., Tavian, M., de Villeroche, V. J., Rossi, G. A. and Brouty-Boye, D. (2005). Human bronchial fibroblasts exhibit a mesenchymal stem cell phenotype and multilineage differentiating potentialities. *Lab. Invest.* **85**, 962-971.

Salingcarnboriboon, R., Yoshitake, H., Tsuji, K., Obinata, M., Amagasa, T., Nifuji, A. and Noda, M. (2003). Establishment of tendon-derived cell lines exhibiting pluripotent mesenchymal stem cell-like property. *Exp. Cell Res.* **287**, 289-300.

Sato, Y., Araki, H., Kato, J., Nakamura, K., Kawano, Y., Kobune, M., Sato, T., Miyanishi, K., Takayama, T., Takahashi, M. et al. (2005). Human mesenchymal stem cells xenografted directly to rat liver are differentiated into human hepatocytes without fusion. *Blood* **106**, 756-763.

Schlondorff, D. (1987). The glomerular mesangial cell: an expanding role for a specialized pericyte. *FASEB J.* **1**, 272-281.

Seo, B. M., Miura, M., Gronthos, S., Bartold, P. M., Batouli, S., Brahim, J., Young, M., Robey, P. G., Wang, C. Y. and Shi, S. (2004). Investigation of multipotent postnatal stem cells from human periodontal ligament. *Lancet* **364**, 149-155.

Shi, S. and Gronthos, S. (2003). Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. *J. Bone Miner. Res.* **18**, 696-704.

Shintani, S., Murohara, T., Ikeda, H., Ueno, T., Honma, T., Katoh, A., Sasaki, K., Shimada, T., Oike, Y. and Imaizumi, T. (2001). Mobilization of endothelial progenitor cells in patients with acute myocardial infarction. *Circulation* **103**, 2776-2779.

Slack, J. M. (2000). Stem cells in epithelial tissues. *Science* **287**, 1431-1433.

Wagers, A. J. and Weissman, I. L. (2004). Plasticity of adult stem cells. *Cell* **116**, 639-648.

Watt, F. M. and Hogan, B. L. (2000). Out of Eden: stem cells and their niches. *Science* **287**, 1427-1430.

Weissman, I. L. (2000). Translating stem and progenitor cell biology to the clinic: barriers and opportunities. *Science* **287**, 1442-1446.

Wexler, S. A., Donaldson, C., Denning-Kendall, P., Rice, C., Bradley, B. and Hows, J. M. (2003). Adult bone marrow is a rich source of human mesenchymal 'stem' cells but umbilical cord and mobilized adult blood are not. *Br. J. Haematol.* **121**, 368-374.

Woodbury, D., Schwarz, E. J., Prockop, D. J. and Black, I. B. (2000). Adult rat and human bone marrow stromal cells differentiate into neurons. *J. Neurosci. Res.* **61**, 364-370.

Zuk, P. A., Zhu, M., Mizuno, H., Huang, J., Futrell, J. W., Katz, A. J., Benhaim, P., Lorenz, H. P. and Hedrick, M. H. (2001). Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng.* **7**, 211-228.

Capítulo 4

Tetraploidy in long-term cultured murine mesenchymal stem cells: a link between cultured mesenchymal stem cells and the perivascular niche?

Lindolfo da Silva Meirelles & Nance Beyer Nardi

Manuscrito em preparação a ser submetido para *Cell and Tissue Research*

Tetraploidy in long-term cultured murine mesenchymal stem cells: a link between cultured mesenchymal stem cells and the perivascular niche?

Lindolfo da Silva Meirelles & Nance Beyer Nardi

Departamento de Genética, Universidade Federal do Rio Grande do Sul
Av. Bento Gonçalves 9500
Porto Alegre, RS, Brazil
91501-970
Telephone: +55 51 33086737

Abstract

Mesenchymal stem cells are adult multipotent cells that are able to differentiate along mesodermal and non-mesodermal pathways. They are distributed throughout the organism, most probably due to their association to blood vessels. Furthermore, they have been recently proposed to be progenitors for the mature cells of the tissues they are in. Here, we analyzed by flow cytometry the DNA content of long-term cultured murine mesenchymal stem cells derived from different anatomical locations. The results suggest that long-term cultured murine MSCs progress to a tetraploid, or nearly tetraploid, state upon extended cultivation *in vitro*. These results provide further evidence of the identity of MSCs as perivascular cells, for which tetraploidization has been described.

Introduction

Nearly 35 years ago, A. Friedenstein and colleagues described fibroblastic precursors present in post-natal bone marrow that could be detected using an *in vitro* colony-forming assay (Friedenstein et al., 1974). The cells that originated such fibroblastic colonies were thus called fibroblast colony-forming units (CFU-Fs). In the subsequent years, it was found that the progeny of CFU-F was able to form bone, cartilage, and adipose tissue cells *in vitro* and *in vivo* (reviewed by Prockop, 1997), and hence the term mesenchymal stem cell (MSC) emerged (Caplan, 1991). Scientific evidence has

accumulated that indicate that the particular cell type currently referred to as MSC can differentiate not only into mesenchymal cell types but also into non-mesenchymal cells such as astrocytes (Kopen et al., 1999) and hepatocytes (Sato et al., 2005). Post-natal MSCs, or MSC-like, cells have also been detected in other organs but the bone marrow (da Silva Meirelles et al, 2006). The anatomical location of MSCs has also been a subject of interest, and some studies have pointed to the perivascular space as their niche (Bianco et al., 2001; Shi and Gronthos, 2003). Consequently, there is a possibility that cells known as pericytes, mural cells, or Rouget cells, represent the *in vivo* counterparts of MSCs. We have recently found evidence indicating that perivascular cells bear MSC properties and, consequently, MSCs exist all over the post-natal organism due to their association with the vasculature (da Silva Meirelles et al., 2006). Furthermore, a hypothesis has been drawn in which MSCs are, in fact, perivascular cells that give rise to the mature cells of the tissues they are embedded in (da Silva Meirelles et al, 2006).

So far, evidence exists indicating that the long-term cultivation of embryonic stem cells (Maitra et al., 2005), and of human MSCs (Rubio et al., 2005), brings about chromosomal alterations that might lead to problems if they were applied *in vivo*. On the other hand, evidence also exists demonstrating that chromosomal anomalies such as aneuploidies do occur naturally *in vivo*, suggesting that gain or loss of chromosomes may have an effect on the differentiation of the resulting cells. To examine the chromosomal integrity of cultured MSCs derived from different organs of mice, we have analyzed their DNA content by flow cytometry. We have found that the cell lines studied were in general tetraploid, or nearly tetraploid, and that tetraploidy came along with prolonged cell cultivation. Furthermore, the MSC populations retained adipogenic differentiation capabilities in spite of their ploidy. Since tetraploidization of rodent perivascular cells has been reported, these observations suggest a link between cultured MSCs to the perivascular niche, and provide further evidence favoring the perivascular stem cell hypothesis.

Material and methods

Mice

Mice used in this study were from C57Bl/6 or eGFP strains. eGFP mice (green mouse FM131), derived from the C57Bl/6 strain, were gently provided by Dr M. Okabe, Osaka University, Japan. The animals were kept under standard conditions (12 hours light/12 hours dark, water and food ad libitum) in our animal house. All the experimental procedures were performed according to institutional guidelines.

Cell culture

The cell populations used in this study are described in Table 1. 001Gvc, 014Ba1, 018Bm and 021Bkg were previously characterized (da Silva Meirelles and Nardi, 2006), and were retrieved from frozen stocks. 004Gbm and 013Cbm were derived as previously described (Meirelles and Nardi, 2003). The cells were cultured in low glucose Dulbecco's Modified Eagle's Medium (Sigma Chemical Co., St Louis, MO) containing 10% fetal bovine serum (Cultilab, Campinas, SP, Brazil) and 10 mM HEPES (free acid; Sigma). Trypsin-EDTA (0.25% and 0.01%, respectively) was used to harvest the cells prior to each passage, after rinsing with hepes-buffered Hank's balanced salt solution.

Table 1 – Description of the MSC cell populations used.

Cell line	***Mouse strain***	***Gender***	***Age***	***Anatomical origin***
001Gvc	eGFP	male	22 weeks	vena cava
004Gbm	eGFP	female	57 weeks	bone marrow
013Cbm	C57Bl/6	female	26 weeks	bone marrow
014Ba1	BALB/c	male	30 weeks	aorta artery
018Bm	BALB/c	female	18 weeks	skeletal muscle
021Bkg	BALB/c	female	27 weeks	kidney glomeruli

Adipogenic differentiation

To induce adipogenic differentiation, MSCs were cultured in Iscove's Modified Dulbecco's Medium added of 20% human platelet-free plasma, supplemented with 10^{-7} M dexamethasone, 2.5 μg/ml insulin, 50 μM indomethacin (all from Sigma), 5 μM rosiglitazone (Avandia; GlaxoSmithKline, Middlesex, United Kingdom) dissolved in

DMSO (Sigma), and 10 units/ml sodium heparin. This protocol was adapted from Krawisz and Scott (1982) and da Silva Meirelles et al. (2006). At the end of the differentiation experiments, cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 1 hour at room temperature (RT), stained with Oil Red O solution for 5 minutes at RT, and counterstained with Harry's hematoxylin for 1 minute at RT.

DNA content analysis

Cells were harvested with trypsin-EDTA, washed twice with PBS and resuspended into 0.5 ml PBS in a 15-ml centrifuge tube. They were chilled in ice, vortexed gently, and two ml of ice-cold methanol were added dropwise. The cells were stored at $4^{o}C$ overnight or for a maximum of 7 days, when they were used for the experiments. Adipocytes differentiated from MSCs were harvested using collagenase type I (0.5 mg/ml in serum-free medium). The whole contents were centrifuged at 400 g for 10 min, and the buoyant fraction was collected using a micropipette. The volume was measured, and ice-cold ethanol was added to it to a proportion of around 80%. The cells were kept at $4^{o}C$ until used. The fixation using methanol was modified from Rousselle et al. (1998). Fresh mouse splenocytes were processed in the same ways as the cultured cells to provide a diploid control for subsequent analyses.

The fixed cells were centrifuged at 400 g for 10 minutes, and washed twice with PBS. They were resuspended into 0.9 ml PBS, and 0.1 ml of 1 mg/ml RNAse A was added. After incubation at 37°C for 25 minutes, 30 µl propidium iodide solution (5 mg/ml in ddH_2O) were added to the cell suspensions, and they were incubated for 30 min at RT. This protocol was modified from Juan and Darzynkiewicz (1998). The cells were washed twice with PBS, and finally resuspended into 0.5 ml PBS.

DNA content was measured using a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA), equipped with a 488 nm laser beam. At least 10,000 events were acquired. Data were collected using CellQuest software (Becton Dickinson), and were analyzed using WinMDI 2.8 (freely available at http://facs.scripps.edu/software.html). Graphics plotting the parameters FL-2 width versus FL-2 area were built. Quadrants were used to mark G_0/G_1 and G_2 regions of the diploid control (intersections), and to compare these with those of the other samples. Images containing both quadrant intersections were

built by overlay using the GNU Image Manipulation Program (freely available at http://www.gimp.org/).

Results

Long-term cultured mMSCs display a tetraploid or nearly tetraploid DNA content

Even though flow cytometry is a good method to analyze DNA content of different types of cells, the application of this methodology on murine MSCs was difficult because these cells are very large (around 20 µm diameter in suspension), and they tend to clog the flow cytometer pipeline when fixed, particularly in the presence of propidium iodide, and when a large number of events is acquired. As a consequence, sometimes the dots in the graphics were dislocated during acquisition. To avoid this problem, the samples were read at intervals, pausing acquisition at times and then allowing large amounts of PBS to flow through the pipeline before continuing.

As shown in Figure 1, MSC populations derived from different organs of adult mice generally display a nearly tetraploid DNA content. The MSC population 021Bkg represented an exception to this general rule, as their DNA content was compatible with that of a diploid cell line. The results indicating a tetraploid, or nearly tetraploid, DNA content are validated by cytogenetic analysis of one bone marrow cell line generated in a previous study (Islam et al., 2006).

The relative number of tetraploid cells increases along passages

We also observed that the proportion of diploid cells in relation to the remainder decreases along the passages, as shown in Figure 2. Since murine bone marrow MSCs may be considered free from hematopoietic contaminants by passage 8 using our standard conditions (Meirelles and Nardi, 2003), the cells had their DNA content first assessed at passage 9. At this point, the proportion of diploid cells in G_0/G_1 plus S phases of cell cycle corresponded to around 37% of the total, whereas this proportion decreased to around 27% at passage 11, and to around 2% and less then 2% at passages 18 and 20, respectively.

Long-term cultured mMSCs retain their adipogenic differentiation characteristics

Since most of the cell populations used had been previously characterized, adipogenic differentiation was performed for two reasons: first, to check if the cell populations retained their capability of differentiation toward at least one mesenchymal lineage; and second, because we wanted to check the ploidy status before and after differentiation, and adipocytes are easier to dissociate than cells subjected to osteogenic differentiation, where a mineralized extracellular matrix forms. As shown in Figure 3, the MSCs retrieved from frozen stocks retained their adipogenic differentiation characteristics previously described (da Silva Meirelles et al., 2006).

Adipogenic differentiation does not affect ploidy status

To check if adipogenic differentiation could somehow interfere with the ploidy status, one MSC population that was known to have adequate adipogenic differentiation capability (004Gbm, as judged from the adipogenic differentiation assay) was set up in two parallel cultures. When these were nearly confluent, one of them had its DNA content analyzed, whereas the other was subjected to adipogenic differentiation. When mature adipocytes were visible (Figure 3E), the cells were harvested and their DNA content compared with that of their undifferentiated counterparts. As seen in Figure 4, no differences in DNA ploidy could be observed between undifferentiated and differentiated cells. Differentiated cells displayed, however, a group of cells with an apparent DNA content below the G_0/G_1 limit, indicating that those were actually apoptotic cells (Figure 4B).

Discussion

In this work, we demonstrated that long-term cultured murine MSCs generally have a tetraploid, or nearly tetraploid, DNA content. The proportion of diploid cells decreased along serial passaging in one of the populations studied, and adipogenic differentiation did not modify the ploidy status when one of the populations used was analyzed.

Even though it is difficult to relate data obtained from experiments *in vitro* with other data from experiments *in vivo*, our results may be interpreted as an indication that cultured murine MSCs undergo, *in vitro*, an increase in their ploidy that is comparable to the tetraploidization that has been described as accompanying the hypertrophy of rodent smooth muscle cells *in vitro* in response to transforming growth factor beta (Owens et al., 1988) or angiotensin II (Geisterfer et al, 1988) and, more importantly, *in vivo*, as a consequence of hypertension (Chobanian et al., 1987) or aging (Jones and Ravid, 2004). In fact, the identity of cultured murine MSCs has been proposed to overlap with that of perivascular cells based in part on the expression of alpha smooth muscle actin, a smooth muscle cell marker (Owens, 1995). We interpret the results presented herein a further evidence indicating the perivascular origin of the cultured cell populations operationally defined as MSCs.

Aknowledgements

The authors are indebted to to Conselho Nacional de Desenvolvimento Cientifico e Tecnologico (CNPq) for financial support. Thanks are due to Dr. Andrés Delgado Cañedo for help with flow cytometry methods, and discussions.

References

Bianco P, Riminucci M, Gronthos S, Robey PG (2005) Bone marrow stromal stem cells: nature, biology, and potential applications. Stem Cells. 2001;19(3):180-192.

Caplan AI. Mesenchymal stem cells (1991) J Orthop Res. 9:641-650.

Chobanian AV, Lichtenstein AH, Schwartz JH, Hanspal J, Brecher P (1987) Effects of deoxycorticosterone/salt hypertension on cell ploidy in rat aortic smooth muscle cells. Circulation. 75:I102-106

da Silva Meirelles L, Chagastelles PC, Nardi NB (2006) Mesenchymal stem cells reside in virtually all post-natal organs and tissues. J Cell Sci. 119:2204-2213.

Friedenstein AJ, Deriglasova UF, Kulagina NN, Panasuk AF, Rudakowa SF, Luria EA, Ruadkow IA (1974) Precursors for fibroblasts in different populations of hematopoietic cells as detected by the in vitro colony assay method. Exp Hematol. 2:83-92.

Geisterfer AA, Peach MJ, Owens GK (1988) Angiotensin II induces hypertrophy, not hyperplasia, of cultured rat aortic smooth muscle cells. Circ Res. 62:749-756.

Islam MQ, Meirelles Lda S, Nardi NB, Magnusson P, Islam K (2006) Polyethylene glycol-mediated fusion between primary mouse mesenchymal stem cells and mouse fibroblasts generates hybrid cells with increased proliferation and altered differentiation. Stem Cells Dev. 15:905-919.

Jones MR, Ravid K (2004) Vascular smooth muscle polyploidization as a biomarker for aging and its impact on differential gene expression. J Biol Chem. 279:5306-5313.

Juan G, Darzynkiewicz Z (1998) Detection of cyclins in individual cells by flow and laser scanning cytometry. Methods Mol Biol. 91:67-75.

Kopen GC, Prockop DJ, Phinney DG (1999) Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. Proc Natl Acad Sci U S A. 96:10711-10706.

Krawisz BR, Scott RE (1982) Coupling of proadipocyte growth arrest and differentiation. I. Induction by heparinized medium containing human plasma. J Cell Biol. 94:394-399.

Maitra A, Arking DE, Shivapurkar N, Ikeda M, Stastny V, Kassauei K, Sui G, Cutler DJ, Liu Y, Brimble SN, Noaksson K, Hyllner J, Schulz TC, Zeng X, Freed WJ, Crook J, Abraham S, Colman A, Sartipy P, Matsui S, Carpenter M, Gazdar AF, Rao M, Chakravarti A (2005) Genomic alterations in cultured human embryonic stem cells. Nat Genet. 37:1099-1103.

Meirelles Lda S, Nardi NB (2003) Murine marrow-derived mesenchymal stem cell: isolation, in vitro expansion, and characterization. Br J Haematol. 123:702-711.

Owens GK, Geisterfer AA, Yang YW, Komoriya A (1988) Transforming growth factor-beta-induced growth inhibition and cellular hypertrophy in cultured vascular smooth muscle cells. J Cell Biol. 107:771-780.

Owens GK (1995) Regulation of differentiation of vascular smooth muscle cells. Physiol Rev. 75:487-517.

Prockop DJ (1997) Marrow stromal cells as stem cells for nonhematopoietic tissues. Science. 276:71-74.

Rousselle C, Robert-Nicoud M, Ronot X (1998) Flow cytometric analysis of DNA content of living and fixed cells: a comparative study using various fixatives. Histochem J. 30:773-781.

Rubio D, Garcia-Castro J, Martin MC, de la Fuente R, Cigudosa JC, Lloyd AC, Bernad A (2005) Spontaneous human adult stem cell transformation. Cancer Res. 65:3035-3039.

Sato Y, Araki H, Kato J, Nakamura K, Kawano Y, Kobune M, Sato T, Miyanishi K, Takayama T, Takahashi M, Takimoto R, Iyama S, Matsunaga T, Ohtani S, Matsuura A, Hamada H, Niitsu Y (2005) Human mesenchymal stem cells xenografted directly to rat liver are differentiated into human hepatocytes without fusion. Blood. 106:756-763.

Shi S, Gronthos S (2003) Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. J Bone Miner Res. 18:696-704.

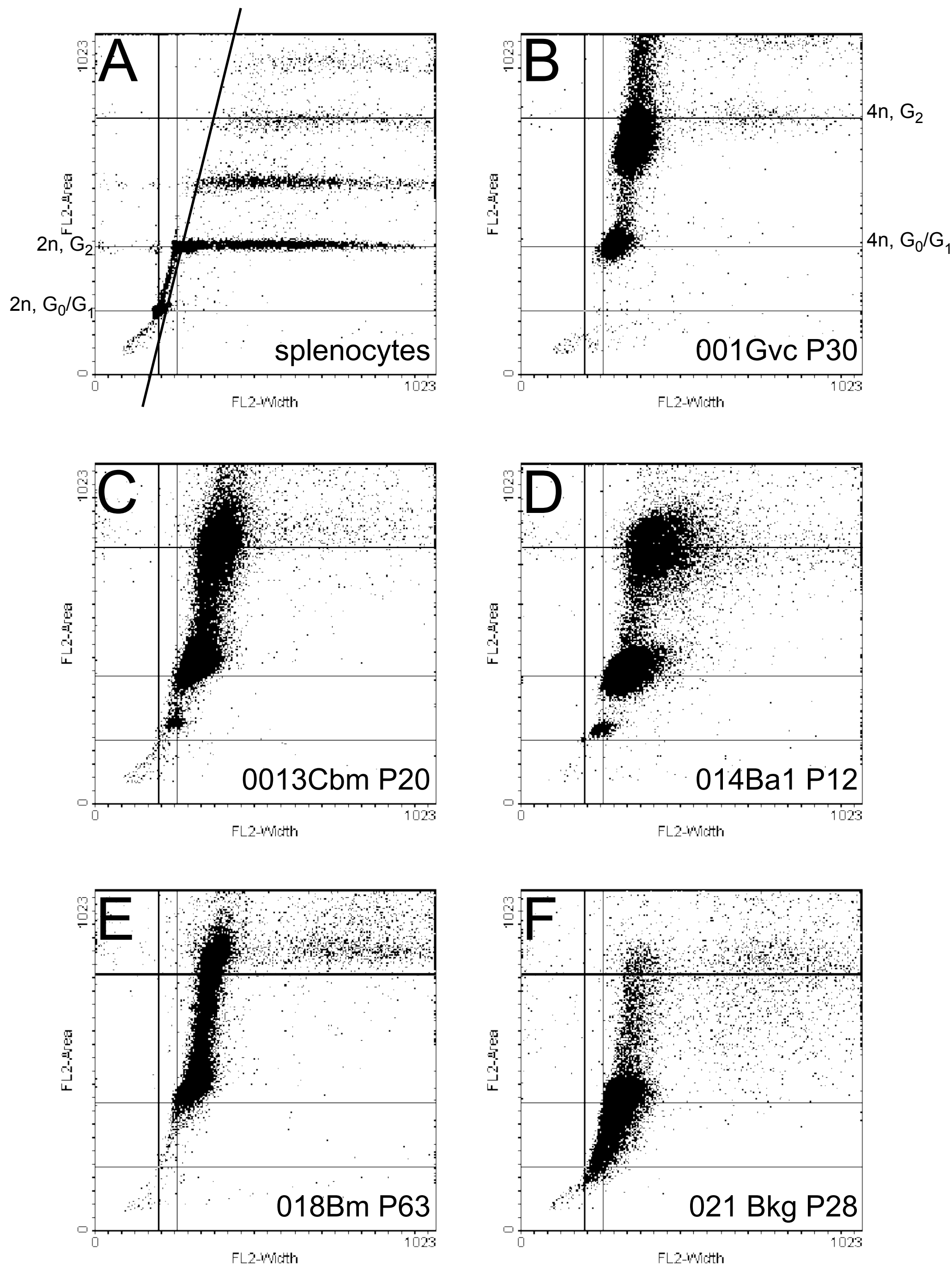

Figure 1 - DNA content of MSCs from different organs. Murine splenocytes (A) were used to draw horizontal lines corresponding to cells in phases G_0/G_1 or G_2 of the cell cycle. A third horizontal line was placed twice the distance between those two lines above the G_2 line, as a marker for 4n cells in the G2 phase. The dots to the right of the line drawn in angle, represent cell clusters. B, vena cava MSCs at passage 30 present a DNA content compatible with the presence of tetraploid and, possibly, cells with even higher DNA content. C and D depict the DNA content of bone marrow and aorta MSCs, respectively, at passages 20 and 12, and indicates that both populations comprise a massive majority of tetraploid cells, and a minority of diploid, or near diploid, cells. E, muscle MSCs at passage 63, displaying a DNA content compatible with a near tetraploid state. F, kidney glomerulus MSCs at passage 28, with a DNA content that indicates that the majority of the cells are diploid, or nearly diploid, while a smaller portion of the population is tetraploid.

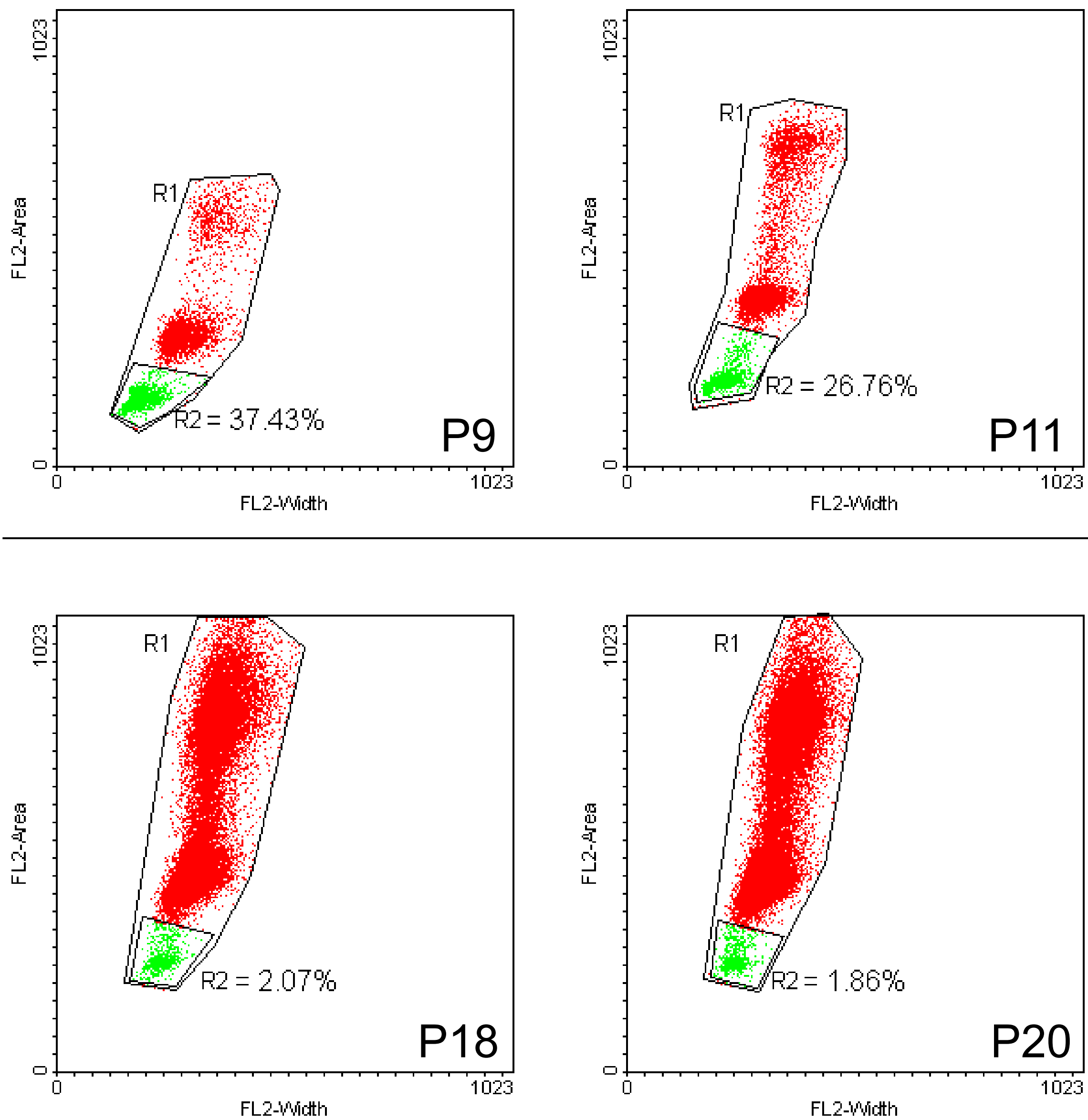

Figure 2 - Reduction of the proportion of diploid cells along passages. The bone marrow MSC population 013Cbm had its DNA content analysed at passages (P) 9, 11, 18 and 20. As an estimate of the proportion of diploid cells, a gate (R2) was drawn that comprehended diploid cells in G_0/G_1 and S phases of the cell cycle, and it was compared with a larger gate (R1) comprehending all non-clustered cells. The proportions are expressed as a percentage of the total number of non-clustered cells.

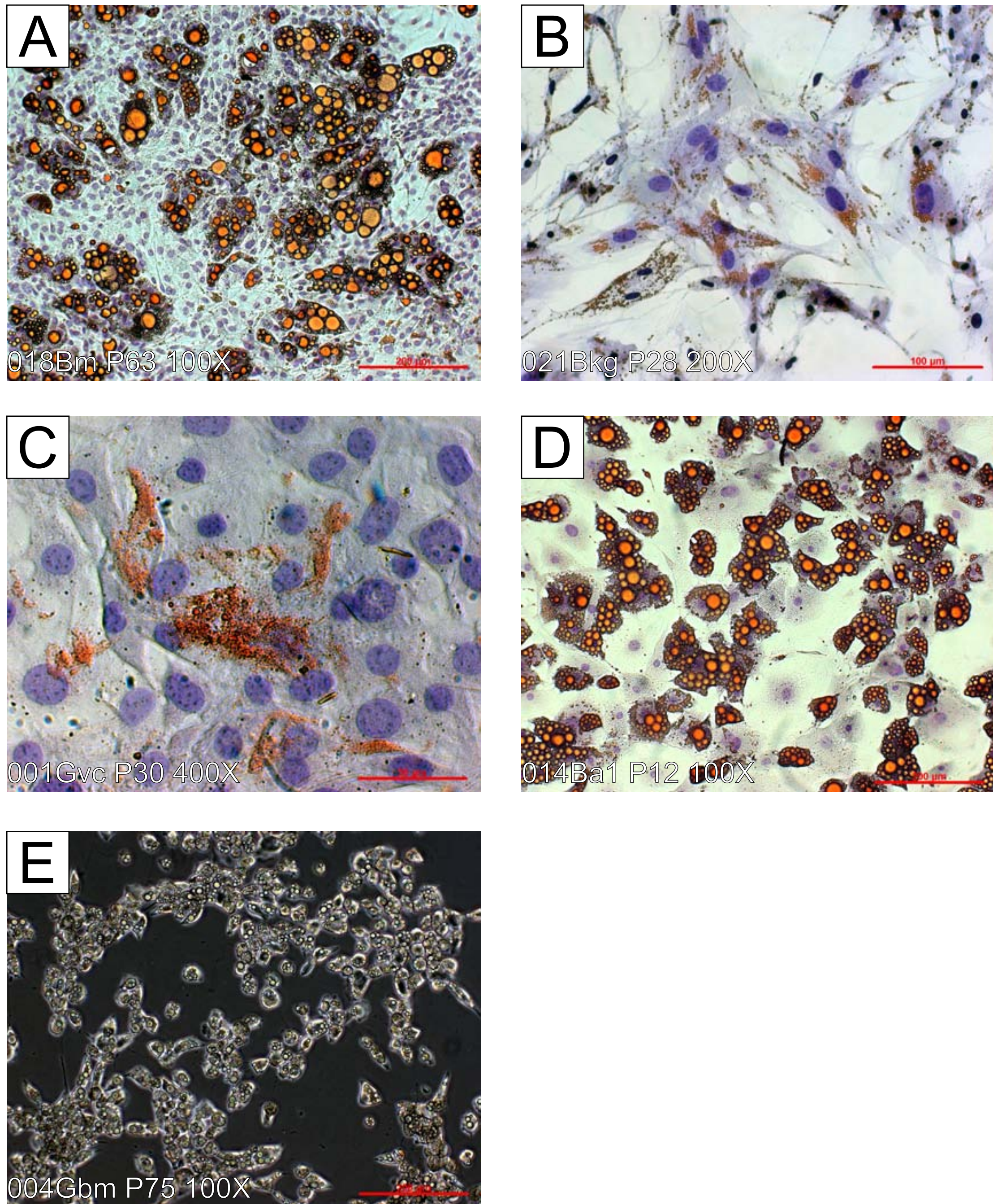

Figure 3 - Adipogenic differentiation of the cell populations used. Adipocytic cells differentiated from MSC populations 018Bm at passage 63 (A), 021Bkg at passage 28 (B), 001Gvc at passage 30 (C), and 014Ba1 at passage 12 (D), as evidenced by staining with Oil Red O. E, phase contrast picture of live adipocytes differentiated from 004Gbm cells at passage 75.

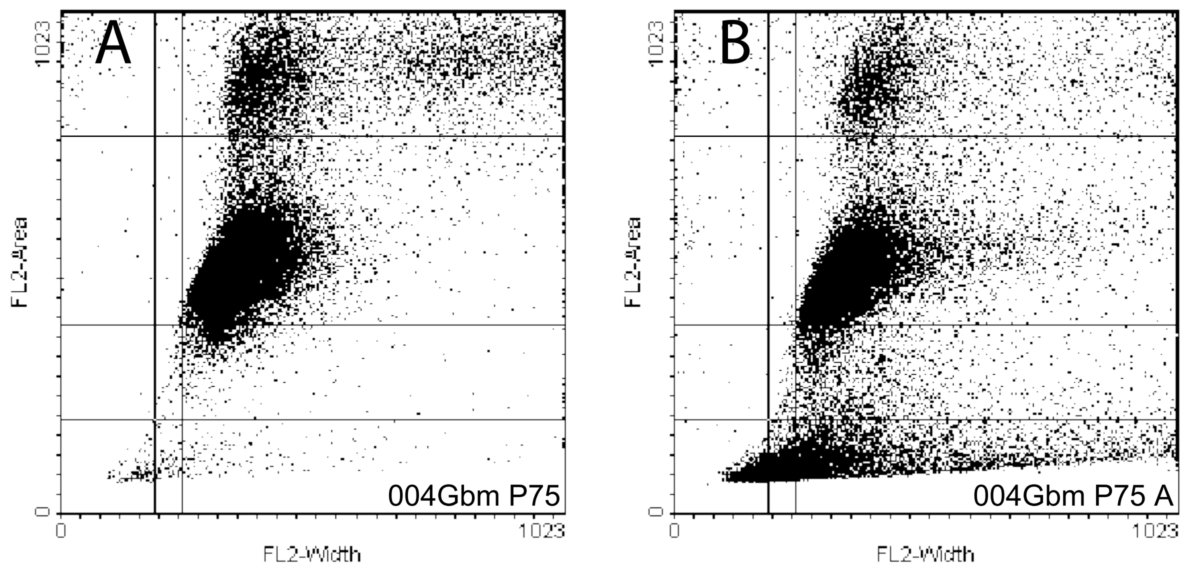

Figure 4 - DNA content in an MSC population subjected to adipogenic differentiation. A, DNA content of the bone marrow MSC cell line 004Gbm at passage 75. B, DNA content of 004Gbm cells subjected to adipogenic differentiation at passage 75 (same as Figure 3E).

Capítulo 5

Towards a unifying concept of the identity and natural distribution of mesenchymal stem cells.

Lindolfo da Silva Meirelles, Arnold I. Caplan & Nance Beyer Nardi

Manuscrito em preparação a ser submetido como artigo de revisão para *Stem Cells and Development*

Manuscript to be submitted as a review to Stem Cells and Development

Towards a unifying concept of the identity and natural distribution of mesenchymal stem cells

Lindolfo da Silva Meirelles[1], Arnold I. Caplan[2], and Nance Beyer Nardi[1]

[1]Departamento de Genetica, Universidade Federal do Rio Grande do Sul, Av Bento Goncalves 9500, 91501-970, Porto Alegre RS, Brazil.

[2]Department of Biology, Skeletal Research Center, Case Western Reserve University, 2080 Adelbert Road, Cleveland, OH 44106-7080, USA.

Address reprint requests to:

Departamento de Genetica, Universidade Federal do Rio Grande do Sul

Av Bento Goncalves 9500

91501-970, Porto Alegre RS, Brazil.

E-mail: nardi@ufrgs.br

Running title: Natural distribution of mesenchymal stem cells.

Lindolfo da Silva Meirelles

Departamento de Genetica, Universidade Federal do Rio Grande do Sul

Av Bento Goncalves 9500

91501-970, Porto Alegre RS, Brazil

Telephone: +55 51 33086737

E-mail: lindolfo_meirelles@hotmail.com

Arnold I. Caplan

Department of Biology, Skeletal Research Center

Case Western Reserve University

2080 Adelbert Road, Cleveland, OH 44106-7080, USA

E-mail: arnold.caplan@case.edu

Nance Beyer Nardi

Departamento de Genetica, Universidade Federal do Rio Grande do Sul

Av Bento Goncalves 9500

91501-970, Porto Alegre RS, Brazil

Telephone: +55 51 33086740

E-mail: nardi@ufrgs.br

ABSTRACT

In spite of the extraordinary advances on the investigation of adult stem cells seen during the past few years, we are still far from understanding their true nature. Most studies are conducted with cells isolated from their natural environment and subject to artificial conditions during in vitro culture. This is more apparent when specific types of adult SCs, such as the mesenchymal stem cells (MSCs), are considered. MSCs present a high degree of plasticity, and have shown promising results in pre-clinical and clinical studies for a number of diseases. These characteristics make them promising tools for cell and cell-based therapies. MSCs, however, are only known *in vitro*, and the definition of their niche in the organism would be of great importance in the rational design of therapeutic approaches. The main cell types they originate – osteoblasts, chondrocytes, and adipocytes – are present through the entire organism, so that their location should allow for their progeny to be distributed to most or all tissues and organs. Multiple evidences now suggest a perivascular location for MSCs. In this review, we focus on evidences showing that MSCs share characteristics with pericytes, and discuss a model in which mesenchymal stem cells have a perivascular niche and lie in the basement, opposed to endothelial cells. According to this model, cues provided by the niche coordinate a gradual transition to progenitor and mature cell phenotypes. This concept has implications for the design of new therapeutic approaches for a range of diseases, targeting the perivascular compartment through cell-mediated therapies.

INTRODUCTION

The past few years have witnessed an outpouring of new studies about the biology of stem cells (SC) and their therapeutic potential. Although it is less than ten years since James Thomson and coworkers derived the first human embryonic stem cell line (1), a huge amount of new information has come to light. Embryo stem cells are collected and cultured with well established techniques; their molecular characterization is well advanced, and research is mainly focused on new methods to differentiate them into specialized cells types. As for adult stem cells, many different types have been identified, and research efforts concentrate on improving methods for their isolation, expansion and characterization, and on investigating their potential for therapeutic uses. Much has already been learned about their basic biology; nevertheless, we are not much closer to understanding their true nature (2).

The main problem lies in the basic approach we use for studying the adult stem cell, which is very similar to the approach used to investigate embryo stem cells – collect, expand and characterize. Actually, however, besides sharing part of their names, there is little in common between these two types of stem cells. Although recent reports show that embryonic stem cells are not "like peas in a pod", since even at the four-cell stage cells from mouse embryos are different from one another (3), it is easy to know which cells to collect from the blastocyst to establish a stem cell culture. The biology of the cells is modified by *in vitro* culture, but the key property of embryo stem cells – pluripotency – is maintained, as shown by their ability to form teratomas when injected into immunodeficient mice (4). As for adult stem cells, one of the key issues is precisely which cells (or cell fraction) to isolate. Since there are no definite markers for adult stem cells, the

cells discarded in the "negative fraction" resulting from isolation processes may contain stem cells not identified by the current methods.

Embryo and adult stem cells are different in two other main points. First, embryo SCs are meant to proliferate, at high rates. It does not go too much against their nature to force them to proliferate *in vitro*. As for the differentiation process, they are also meant to differentiate – more than we want to –, so the problem is to stop them from differentiating too much, and direct the process into the lines we are more interested in (5). One of the key properties of adult SC, on the other hand, is quiescence (6). This is of fundamental importance for species of long life cycle such as ours, to avoid genetic damage to a cell line that is meant to survive for many decades without reposition from external sources. Working with adult SCs *in vitro*, however, means having to force them to proliferate, while keeping their stemness in adequate levels. This difficulty is more apparent for some adult SC types than for others. There is, for instance, a great interest in expanding hematopoietic stem cells (HSCs) *in vitro* to increase their numbers in situation such as the use of cord blood for transplantation (7). Expanding HSCs, while still keeping their undifferentiated state, has proven a difficult goal to achieve, and a great number of studies have suggested different methodologies (reviewed in ref. 8). The same situation applies to other adult stem cells (9).

Second, the inner mass of blastocysts consists of a group of cells sharing the characteristic of being stem cells, and the question of defining the appropriate culture condition for maintaining their proliferation as embryo SCs has been more easily solved (5). Adequate niches for the multiple types of adult stem cells, on the other hand, are proving extremely hard to define, so that added to the difficulty of isolating tissue-specific stem cells we have currently no clearly defined way of maintaining them *in vitro*.

The question then is: how to maintain and expand *ex vivo* cells that fundamentally depend on their niche (that we can, possibly, replace with the adequate signals) and are meant to be quiescent? By forcing them to divide, it is inevitable that their biology will be strongly altered. Although many studies are dedicated to answering this question, what we know about adult stem cells which have been manipulated *ex vivo* is probably very different from their behaviour *in vivo*. This is still more apparent when specific types of adult SCs, such as the mesenchymal stem cells (MSCs), are considered.

MESENCHYMAL STEM CELLS

Mesenchymal stem cells may be defined as cells capable of giving rise to a number of unique, differentiated mesenchymal cell types (10,11). First described as fibroblast precursors from bone marrow by Friedenstein et al. in 1970 (12), MSCs may be also referred to as fibroblast colony-forming units (CFU-Fs) or marrow stromal cells (13). There is evidence that MSCs exist not only in the bone marrow, but in virtually every location of the body (14-16). Although no definitive markers are known for MSCs, their cell-surface antigen profile is well explored (reviewed in ref. 17).

The literature presents evidence that cultured MSCs also exhibit a degree of plasticity that goes beyond the mesenchymal limit, when maintained *in vitro* or implanted *in vivo* (18-22). They have shown promising results in pre-clinical and clinical studies for a number of diseases (reviewed in refs. 23-25), involving very different tissues, such bone or cartilage defects, cardiac disorders, central nervous system or spinal cord injury, and lung diseases. They may also hasten hematopoietic recovery after bone marrow transplantation, and their unique immunologic properties might facilitate engraftment of transplanted

organs and reduce graft-vs-host disease. MSCs have also been proposed to exert paracrine trophic effects through the secretion of bioactive molecules (26). These characteristics make them promising tools for cell and cell-based therapies.

Protocols for the isolation of MSCs involve the selection of plastic-adherent cells (27-29). Our ignorance on the basic biology of MSCs is reflected in the different types of cultures derived from attempts of different groups to isolate them. These cell populations have been named mesenchymal stem cells, marrow stromal cells, marrow-isolated adult multilineage inducible (MIAMI) cells, recycling stem cells (RS-1, RS-2), or multipotent adult progenitor cells (MAPCs) (reviewed in ref. 30). Gregory et al. in 2005 (31) analyzed the multiple factors involved in MSC differentiation *in vitro*, suggesting that it is regulated by a two-stage mechanism: preconditioning by factors in the culture microenvironment, and response to soluble differentiating factors. An attempt to clarify the nomenclature for MSCs has been recently put forward by the International Society for Cellular Therapy (32).

The fact is that MSCs are only known *in vitro*, and our inability to prospectively identify them in their natural location in the organism results in that “MSC biology, at the present time, remains biology out of context” (33). Several papers have described cell populations derived from bone marrow or different locations, from human and other species, that are able to differentiate into mesenchymal cell phenotypes *in vitro* (14,28,34). On the other hand, there is evidence that even cells that have a mature phenotype *in vivo* may be able to dedifferentiate to a more primitive phenotype when cultured (35) and differentiate into other cell types *in vitro* (36,37). As with most other adult stem cells, MSCs are operationally defined, after *in vitro* expansion, by their ability to self-renew and to differentiate – in this case, in mesodermal (28), ectodermal (18) and endodermal (21) cell lineages. How much does the *in vitro* expansion of plastic-adherent, self-renewing

cells modify the biology of what a "mesenchymal stem cell" is *in vivo*? Or: are the cultured cells that display capacity to differentiate into mesenchymal cell types indeed MSCs?

WHERE ARE THE MESENCHYMAL STEM CELLS?

Adult, tissue-specific stem cells are found in specialized niches in the correspondent tissue (38). Hematopoietic stem cells can be found in the bone marrow (reviewed in ref. 39), epidermal SCs in mammalian hair follicles (40), neural SCs in the subventricular zone (41), and so on. Where are the mesenchymal stem cells to be found?

Very little is known about the ontogeny and developmental origin of the MSC (reviewed in ref. 33). The main cell types they originate – osteoblasts, chondrocytes, and adipocytes – are present through the entire organism. There are furthermore, as mentioned above, signs of great plasticity of these cells. Their location in the organism must allow for their progeny to be distributed to most or all tissues and organs. Three main situations could be considered. In the first one, MSCs are located in one specific tissue or organ, from where they circulate to other sites of the organism to replenish cell populations entering apoptosis through physiological processes, or necrosis in case of lesions. Although not formally denied, the great difficulty in establishing conventional MSC cultures from peripheral blood (14) goes against this possibility. Furthermore, post-natal mesenchymal stem cells have been isolated from different sources, besides the bone marrow – e.g. adipose tissue (42), tendon (43), periodontal ligament (22), synovial membrane (44) and lungs (45). We recently showed that MSC cultures with very similar morphologic, immunophenotypic and functional properties can be established from the

brain, spleen, liver, kidney, lung, bone marrow, muscle, thymus, and pancreas of mice (14).

These findings raise a second possibility, in which MSCs are embedded in many different tissues and organs, as resident stem cell populations. The experimental approach to the true location of MSCs within all these tissues is difficult, and it has not been formally tested. However, the absolute dependence of adult stem cells on their niche makes it hard to envisage a situation in which the appropriate microenvironment for MSCs would be similarly available in the core of different types of tissues.

The third possibility, which constitutes the main theme of this work, has been put forward by some authors, and suggests a relationship between MSCs and perivascular cells (46-49). This situation would adequately (a) make MSCs available to all tissues, in their role as a source of new cells for physiological turnover or for the repair of lesions, and (b) explain the establishment of MSC cultures from virtually all tissues examined thus far (14).

THE PERICYTE

Pericytes – also referred to as periendothelial cells, Rouget cells, Ito/stellate cells (in the liver) – are mural cells that lie on the abluminal side of blood vessels, immediately opposed to endothelial cells (50-52). Pericytes are defined morphologically based on their location in relation to the endothelial cells, especially in microvessels (arterioles, capillaries and venules). There is evidence, however, that pericytes are also present in large vessels. By staining tissue sections with the 3G5 antibody, a pericyte marker, Andreeva et al., 1998 (53) could identify pericytes lying adjacent to endothelial cells not only in small,

but also in large vessels. They concluded that pericytes form a continuous subendothelial network which spans the whole vasculature.

Pericyte markers

Whenever considering using marker molecules to detect pericytes, one should be aware of the expression of the same molecules by other cell types, especially cells that are in close proximity such as endothelial cells. Since there is evidence supporting the view that MSCs and pericytes are in fact the same in the bone marrow, knowing what molecules are expressed by MSCs is also highly recommended. A list of markers known to be expressed or not by pericytes, ECs and MSCs is shown in Table 1. Markers ascribed to other cell types, such as smooth muscle cells and hematopoietic cells, are included for comparison whenever possible.

As stated by Armulik et al., 2005 (54), “the heterogeneous morphology and marker expression make unambiguous identification of pericytes a challenge”. The NG2 proteoglycan is one of the molecules used to identify pericytes *in vivo* in rodents (55). In rats, the NG2 proteoglycan has been shown not being consistently expressed by all pericytes under normal circumstances, its expression being preferentially on the venular (56). The human homologue of NG2, termed high molecular weight melanoma associated antigen (HMW-MAA) and also known as human melanoma proteoglycan (57), is a marker for activated (proliferative) human pericytes (58).

Alpha smooth muscle actin (αSMA) may also be used as a pericyte marker in spite of being expressed by smooth muscle cells (59), and displaying expression differences between species (60).

Angiopoietin-1 has also been reported to be expressed by human pericytes *in vitro* and *in vivo* (61). Since this is a secreted factor, using it as a maker for the detection of pericytes *in vivo* by immunohistochemistry might be difficult because it may be also found in the basement membrane, and bound to its receptor on the surface of endothelial cells.

The monoclonal antibody SH-2, which has been raised against human MSCs (27) and later found to recognize CD105 (62), can recognize endothelial and perivascular cells in embryonic and young post-natal dermis. Its expression seems to be developmentally controlled, and is possibly associated with active angiogenesis or neoangiogenesis (63).

The antigen defined by the STRO-1 antibody has been shown to react with stromal and erythroid cells (64), and perivascular and endothelial cells (47) in human bone marrow. The same antigen is expressed by perivascular and endothelial cells in human dental pulp (48). Of interest, CFU-Fs have been observed only in the STRO-1+ positive fraction of human bone marrow (64). In rats, the STRO-1+, adherent fraction of bone barrow can differentiate into chondrocytes, osteoblasts and adipocytes (65).

Using a mouse developmental model, Brachvogel et al., 2005 (66) demonstrated that annexin A5 is present in angioblasts during vasculogenesis, and its expression becomes restricted to perivascular cells from embryonic day 10.5 on. The annexin A5+ cells sorted from adult brain meninges expressed Sca-1, CD34 and CD117, similarly to brain-derived murine MSCs (14). Furthermore, these sorted cells could be induced to differentiate along osteogenic, chondrogenic and adipogenic pathways. Experiments aiming to test if annexin A5 is expressed by human perivascular cells in a similar way it is expressed in mice might be important to define a novel, and perhaps unique, marker for human pericytes.

To date, the ganglioside defined by the antibody 3G5 seems to be the best marker for pericytes. Even though some specialized cell types are also recognized by this antibody, it

is highly specific for pericytes within the microvasculature (67). This same antibody has been used to indicate that equivalent forms of microvascular pericytes are present in the interface between the intima and the media layers of larger vessels, forming a continuous pericytic network throughout the vasculature (53). In the same work, pericytes in the vasa vasora were also found to be $3G5^{+}$. Human cells isolated from skin using the 3G5 antibody express other pericyte-related markers such as HMW-MAA, desmin, α-SMA and angiopoietin-1 when cultured shortly *in vitro* (61). Cell types such as fibroblasts and smooth muscle cells do not express it (68). The 3G5 antibody has been used to identify microvascular human dermal pericytes in skin biopsies (69). This antibody reacts with human, bovine, rabbit and porcine antigens (70), but it does not react with murine cells. Such a characteristic would be of interest for the detection of human pericytes transplanted into mouse hosts.

Considering the information available on pericyte molecules, it seems that a good combination of markers to detect human pericytes would be the 3G5 antibody-defined ganglioside, and HMW-MAA. Endothelial cells can be distinguished from pericytes based on their expression of CD31 (71).

Pericyte – endothelial cell interactions

Pericytes and endothelial cells exhibit an interdependent relationship, wherein soluble factors and physical interactions synergistically contribute for blood vessel structure maintenance (54). The secretion of angiopoietin-1 by pericytes followed by its uptake by endothelial cells via the Tie-2 receptor is one example of soluble factor mediated interaction (72). Endothelial cells secrete PDGF-BB, which becomes associated with

heparan sulfate proteoglycans in the basement membrane they produce. Upon binding to its receptor on the cell surface of pericytes, this interaction provides them proliferation and migration cues (54). Several other cell types express PDGF-BB (73), but in this particular case, endothelial cells provide pericytes this growth factor associated with positional information, similarly to lights in runways during the night. Furthermore, mice lacking basement membrane-retaining motif for PDGF display defective investment of pericytes in the microvasculature (74).

Physical contact also plays an important role in pericyte-endothelial cell communication. The establishment of connexin 43-containing gap junctions between murine mesenchymal precursors and bovine aortic endothelial cells has been shown to be necessary for the production of the active form of TGF-β by the former. As a consequence, the mesenchymal precursors used started expressing the pericyte/vascular smooth muscle-related proteins aSMA, SM22a, and SM-myosin heavy chain (75). Another important physical contact between pericytes and endothelial cells is that mediated by N-cadherin, which provides vessel structure stabilization (54,76,77).

The intimate relationship between pericytes and endothelial cells shown above is something that is often not considered when defining a pericyte. It is possible that what ultimately tells pericytes from other cell types is the nature of their interaction with endothelial cells. To be considered a pericyte, a given cell must a) establish physical contact with endothelial cells by means of gap junctions; b) express at least one marker attributed to pericytes; and c) not express pan-endothelial cell markers.

THE PERICYTE AS A LOCAL PROGENITOR FOR CHONDROCYTES AND OSTEOCYTES IN BONE REPAIR: A POSSIBLE LINK BETWEEN THE *IN VITRO* AND THE *IN VIVO* DIMENSIONS?

Endochondral bone formation has been studied during development, fracture repair, and ectopic bone formation. In every case (see below), vascularization plays a key role in bone formation.

During the formation of long bones, primitive cartilage becomes vascularized before ossification. After birth, vasculature actively invades the growth plate at the level of the hypertrophic chondrocytes, which express VEGF and undergo apoptosis. VEGF recruits blood vessels to the site formerly occupied by the hypertrophic chondrocytes, and osseous tissue formation takes place (78).

Aiming to establish an experimental model for the study of bone formation, early experiments using demineralized bone matrices implanted subcutaneously in rats have determined a sequence of stages that occur during ectopic bone formation. Such a sequence comprises encystment by host's cells, cartilage formation, cartilage hypertrophy and first bone formation, vascular invasion, and finally massive bone formation (79). The chronological histological characteristics, as described by Reddi and Anderson, 1976 (80), are summarized in Table 2. According to this sequence of events, vascularization of the demineralized bone matrices occurs after the appearance of hypertrophic chondrocytes, and precedes bone formation. Hence, this model is compatible with the events described above for post-natal bone formation in the growth plate.

Osseous tissue formation during fracture repair of long bones differs from the processes described above in that there is no pre-existing cartilage. After fracture, and if it

is mechanically unstable, a blood clot forms, followed by an inflammatory response and angiogenic activity. Fibro-cartilaginous tissue forms in the internal callus, while direct osseous neoformation occurs in the external callus. The internal callus eventually becomes mineralized and is replaced by osseous tissue. Finally, a remodelling process takes place, replacing the callus with secondary lamellar bone. Notably, the hematoma that forms initially has a strong angiogenic effect, and its removal attenuates fracture healing (81).

The role of pericytes as progenitors for cartilage and bone has been previously suggested. Diaz-Flores et al., 1991, 1992 (82,83) tracked the fate of vascular and perivascular cells labelled with Monastral Blue A during periosteal bone healing, and in grafted perichondrium. As a result, mature osteocytes and chondrocytes displaying cytoplasmic inclusions of the dye could be observed by means of light and electron microscopy. The authors concluded that pericytes can give rise to osteocytes and chondrocytes *in vivo*.

Brighton and Hunt, 1997 (84) studied the early the behaviour of periosteal vascular and perivascular cells in response to bone fracture by means of light and electron microscopy. They found that 24 hours after the fracture, both endothelial cells and pericytes were hypertrophied and, at 48 hours, putative pericytes had divided and formed layers of stacked cells. Five days after the fracture, chondroblasts that retained remnants of the basal lamina could be observed in close proximity to the hypertrophied pericytes. At six days post-fracture, hypertrophic chondrocytes, some of them showing signs of degeneration, could be observed in the proximal zone of the inner layer of the periosteal callus. At this time, woven bone was being replaced by lamellar bone in the distal zone of the inner layer. On the seventh day, woven bone had been almost completely replaced by lamellar bone in the distal zone of the inner layer of the callus, while endochondral bone formation was

occurring in the proximal zone. These results indicate that pericytes give rise to chondrocytes, and that osteocytes are observed after that.

A PERIVASCULAR NICHE FOR MESENCHYMAL STEM CELLS

The components of the microenvironment with which stem cells interact are under intense scrutiny, and much has been found about cells, extracellular matrix components, and soluble factors that develop a two-way interaction with different types of adult stem cells (6). As for mesenchymal stem cells as we know them (*in vitro*), very little is needed for their maintenance in a state that allows them to be referred to as stem cells – with the capacity of self-renewal and differentiation. All they need is culture medium, such as Dulbecco's modified Eagle's medium supplemented with fetal calf serum, and a plastic surface to attach to (85). Very little is known about their niche in the organism, yet to comply with the current belief that links stemness to an adequate niche, they must have their own location and microenvironment.

Multiple evidences now suggest a perivascular location for MSCs. Conventional MSC cultures may be established from artery or vein walls (14,86,87). Cells with surface profile similar to MSCs (positive for Stro-1 and CD146) were observed lining blood vessels in the bone marrow and dental pulp (48). A functional relationship between MSCs and mural cells has also become apparent. Pericytes are able to differentiate into different mesenchymal cell types *in vitro* (46,49). Studies *in vivo* have suggested that pericytes can give rise to adipocytes (66,88), chondrocytes (66,82,84), and osteoblasts (83,84). Vascular smooth muscle cells have also been shown to differentiate into other mesenchymal lineages such as osteoblasts, chondrocytes and adipocytes (reviewed in ref. 89).

Pericytes could thus be the *in vivo* representatives of *in vitro* MSCs. On the other hand, MSCs could be a precursor for pericytes and vascular smooth muscle cells, or even to other types of vascular cells. Recent evidences, for instance, show that MAPCs differentiate into arterial and venous endothelial cells, with involvement of sonic hedgehog (Shh) and its receptors, as well as Notch 1 and 3 receptors and some of their ligands in the process (90). Although not completely identified with MSCs, MAPCs share a number of characteristics with them, and further investigation should determine whether more conventional MSCs show this differentiation potential.

Further information on the cellular and soluble components of the microenvironment supporting MSCs *in vivo* are slowly gathering, as the studies progress (reviewed in ref. 17). Based on these evidences as a whole, we suggested a model in which mesenchymal stem cells have a perivascular niche and lie in the basement, opposed to endothelial cells (14). According to this model, cues provided by the niche, composed by other cells, extracellular matrix and signaling molecules including autocrine, paracrine, and endocrine factors (17), coordinate a gradual transition to progenitor and mature cell phenotypes.

CONCLUSION

It is not yet possible to define if MSCs are identical to pericytes or are more primitive cells which originate pericytes and vascular smooth muscle cells. Although still lacking formal proof, however, evidences are advanced enough to give consistency to the concept of a perivascular niche for the adult stem cell we call “mesenchymal stem cell”. The elements of the niche include endothelial cells, soluble factors such as angiopoietin-1 and PDGF-BB, and extracellular matrix components not well known as yet. As known for the

interaction of other types of adult stem cells and their niches, close contact with the basal membrane keeps the cells undifferentiated. When detached from their niche, due to signs received from the microenvironment and/or to internal programming, MSCs differentiate into committed progenitors that gradually integrate into the tissue. According to this model (14), a portion of the apparent post-natal stem cell diversity may be attributed to local MSCs behaving as tissue-specific stem cells.

This concept makes the term “mesenchymal” stem cell seem inappropriate, and possibly the term “perivascular stem cell” might best represent this cell type. The name is however too well established to be changed, and may thus be maintained even if the concept of the nature of the cell is modified. Finally, the concept has implications for the design of new therapeutic approaches for a range of diseases, targeting the perivascular compartment through cell-mediated therapies.

ACKNOWLEDGEMENTS

Thanks are due to Conselho Nacional de Desenvolvimento Cientifico e Tecnologico (CNPq) for financial support, and to Dr. J. Michael Sorrell for discussions.

REFERENCES

1. Thomson JA, J Itskovitz-Eldor, SS Shapiro, MA Waknitz, JJ Swiergiel, VS Marshall and JM Jones. (1998). Embryonic stem cell lines derived from human blastocysts. Science 282:1145-1147.
2. Nardi NB. (2005). All the adult stem cells, where do they all come from? An external source for organ-specific stem cell pools. Med Hypotheses 64:811-817.
3. Piotrowska-Nitsche K, A Perea-Gomez, S Haraguchi and M Zernicka-Goetz. (2005). Four-cell stage mouse blastomeres have different developmental properties. Development 132:479-490.
4. Asano T, K Sasaki, Y Kitano, K Terao and Y Hanazono. (2006). In vivo tumor formation from primate embryonic stem cells. Methods Mol Biol 329:459-467.
5. Spagnoli FM and A Hemmati-Brivanlou. (2006). Guiding embryonic stem cells towards differentiation: lessons from molecular embryology. Curr Opin Genet Dev 16:469-475.
6. Fuchs E, T Tumbar and G Guasch. (2004). Socializing with the neighbors: stem cells and their niche. Cell 116:769-778.
7. Pranke P, J Hendrikx, G Debnath, G Alespeiti, P Rubinstein, N Nardi and J Visser. (2005). Immunophenotype of hematopoietic stem cells from placental/umbilical cord blood after culture. Braz J Med Biol Res 38:1775-1789.
8. Mikkola HK and SH Orkin. (2006). The journey of developing hematopoietic stem cells. Development 133:3733-3744.
9. Pare JF and JL Sherley. (2006). Biological principles for ex vivo adult stem cell expansion. Curr Top Dev Biol 73:141-171.

10. Caplan AI. (1991). Mesenchymal stem cells. J Orthop Res 9:641-650.

11. Prockop DJ. (1997). Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 276:71-74.

12. Friedenstein AJ, RK Chailakhjan and KS Lalykina. (1970). The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. Cell Tissue Kinet 3:393-403.

13. Phinney DG. (2002). Building a consensus regarding the nature and origin of mesenchymal stem cells. J Cell Biochem Suppl 38:7-12.

14. da Silva Meirelles L, PC Chagastelles and NB Nardi. (2006). Mesenchymal stem cells reside in virtually all post-natal organs and tissues. J Cell Sci 119:2204-2213.

15. Prunet-Marcassus B, B Cousin, D Caton, M Andre, L Penicaud and L Casteilla. (2006). From heterogeneity to plasticity in adipose tissues: site-specific differences. Exp Cell Res 312:727-736.

16. Krampera M, S Marconi, A Pasini, M Galie, G Rigotti, F Mosna, M Tinelli, L Lovato, E Anghileri, A Andreini, G Pizzolo, A Sbarbati and B Bonetti. (2007). Induction of neural-like differentiation in human mesenchymal stem cells derived from bone marrow, fat, spleen and thymus. Bone 40:382-390.

17. Kolf CM, E Cho and RS Tuan. (2007). Mesenchymal stromal cells. Biology of adult mesenchymal stem cells: regulation of niche, self-renewal and differentiation. Arthritis Res Ther 9:204-213.

18. Kopen GC, DJ Prockop and DG Phinney. (1999). Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. Proc Natl Acad Sci USA 96:10711-10716.

19. Woodbury D, EJ Schwarz, DJ Prockop and IB Black. (2000). Adult rat and human bone marrow stromal cells differentiate into neurons. J Neurosci Res 61:364-370.

20. Lange C, P Bassler, MV Lioznov, H Bruns, D Kluth, AR Zander and HC Fiegel. (2005). Liver-specific gene expression in mesenchymal stem cells is induced by liver cells. World J Gastroenterol 11:4497-4504.

21. Sato Y, H Araki, J Kato, K Nakamura, Y Kawano, M Kobune, T Sato, K Miyanishi, T Takayama, M Takahashi, R Takimoto, S Iyama, T Matsunaga, S Ohtani, A Matsuura, H Hamada and Y Niitsu. (2005). Human mesenchymal stem cells xenografted directly to rat liver are differentiated into human hepatocytes without fusion. Blood 106:756-763.

22. Seo BM, M Miura, S Gronthos, PM Bartold, S Batouli, J Brahim, M Young, PG Robey, CY Wang and S Shi. (2004). Investigation of multipotent postnatal stem cells from human periodontal ligament. Lancet 364:149-155.

23. Le Blanc K. (2006) Mesenchymal stromal cells: Tissue repair and immune modulation. Cytotherapy 8:559-561.

24. Bobis S, D Jarocha and Majka. (2006). Mesenchymal stem cells: characteristics and clinical applications. Folia Histochem Cytobiol 44:215-230.

25. Beyer Nardi N and L da Silva Meirelles. (2006). Mesenchymal stem cells: isolation, in vitro expansion and characterization. Handb Exp Pharmacol 174:249-282.

26. Caplan AI and JE Dennis. (2006). Mesenchymal stem cells as trophic mediators. J Cell Biochem 98:1076-1084.

27. Haynesworth SE, J Goshima, VM Goldberg and AI Caplan. (1992). Characterization of cells with osteogenic potential from human marrow. Bone 13:81-88.

28. Pittenger MF, AM Mackay, SC Beck, RK Jaiswal, R Douglas, JD Mosca, MA Moorman, DW Simonetti, S Craig and DR Marshak. (1999). Multilineage potential of adult human mesenchymal stem cells. Science 284:143-147.

29. Lennon DP and AI Caplan. (2006). Mesenchymal Stem Cells for Tissue Engineering. In: Vunjak-Novakovic G, Freshney RI (eds). Culture of Cells for Tissue Engineering. Indianapolis: Wiley, pp. 23 – 59.

30. Baksh D, L Song and RS Tuan. (2004). Adult mesenchymal stem cells: characterization, differentiation, and application in cell and gene therapy. J Cell Mol Med 8:301-316.

31. Gregory CA, J Ylostalo and DJ Prockop. (2005). Adult bone marrow stem/progenitor cells (MSCs) are preconditioned by microenvironmental "niches" in culture: a two-stage hypothesis for regulation of MSC fate. Sci STKE 2005:pe37.

32. Horwitz EM, K Le Blanc, M Dominici, I Mueller, I Slaper-Cortenbach, FC Marini, RJ Deans, DS Krause and A Keating; the International Society for Cellular Therapy. (2005). Clarification of the nomenclature for MSC: The International Society for Cellular Therapy position statement. Cytotherapy 7:393-395.

33. Javazon EH, KJ Beggs and AW Flake. (2004). Mesenchymal stem cells: paradoxes of passaging. Exp Hematol 32:414-425.

34. Martin DR, NR Cox, TL Hathcock, GP Niemeyer and HJ Baker. (2002). Isolation and characterization of multipotential mesenchymal stem cells from feline bone marrow. Exp Hematol 30:879-886.

35. Diaz-Romero J, JP Gaillard, SP Grogan, D Nesic, T Trub and P Mainil-Varlet. (2005). Immunophenotypic analysis of human articular chondrocytes: changes in surface

markers associated with cell expansion in monolayer culture. J Cell Physiol 202:731-742.

36. Barbero A, S Ploegert, M Heberer and I Martin. (2003). Plasticity of clonal populations of dedifferentiated adult human articular chondrocytes. Arthritis Rheum 48:1315-1325.
37. Tallheden T, JE Dennis, DP Lennon, E Sjogren-Jansson, AI Caplan and A Lindahl. (2003). Phenotypic plasticity of human articular chondrocytes. J Bone Joint Surg Am 85A Suppl 2:93-100.
38. Watt FM and BL Hogan. (2000). Out of Eden: stem cells and their niches. Science 287:1427-1430.
39. Verfaillie CM. (2000). Anatomy and physiology of hematopoiesis. In: Hoffman R, Benz EJ, Shattil SJ, Furie B, Cohen HJ, Silberstein LE, et al. (Editors), Hematology - basic principles and practice. 3rd edn. New York: Churchill Livingstone.
40. Braun KM, C Niemann, UB Jensen, JP Sundberg, V Silva-Vargas and FM Watt. (2003). Manipulation of stem cell proliferation and lineage commitment: visualisation of label-retaining cells in wholemounts of mouse epidermis. Development 130:5241-5255.
41. Gould E, AJ Reeves, MS Graziano and CG Gross. (1999). Neurogenesis in the neocortex of adult primates. Science 286:548-552.
42. Zuk PA, M Zhu, H Mizuno, J Huang, JW Futrell, AJ Katz, P Benhaim, HP Lorenz and MH Hedrick. (2001). Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 7:211-228.

43. Salingcarnboriboon R, H Yoshitake, K Tsuji, M Obinata, T Amagasa, A Nifuji and M Noda. (2003). Establishment of tendon-derived cell lines exhibiting pluripotent mesenchymal stem cell-like property. Exp Cell Res 287:289-300.
44. De Bari C, F Dell'Accio, P Tylzanowski and FP Luyten. (2001). Multipotent mesenchymal stem cells from adult human synovial membrane. Arthritis Rheum 44:1928-1942.
45. Sabatini F, L Petecchia, M Tavian, V Jodon de Villeroche, GA Rossi and D Brouty-Boye. (2005). Human bronchial fibroblasts exhibit a mesenchymal stem cell phenotype and multilineage differentiating potentialities. Lab Invest 85:962-971.
46. Doherty MJ, BA Ashton, S Walsh, JN Beresford, ME Grant, AE Canfield. (1998). Vascular pericytes express osteogenic potential in vitro and in vivo. J Bone Miner Res 13:828-838.
47. Bianco P, M Riminucci, S Gronthos and PG Robey. (2001). Bone marrow stromal stem cells: nature, biology, and potential applications. Stem Cells 19:180-192.
48. Shi S and S Gronthos. (2003). Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. J Bone Miner Res 18:696-704.
49. Farrington-Rock C, NJ Crofts, MJ Doherty, BA Ashton, C Griffin-Jones and AE Canfield. (2004). Chondrogenic and adipogenic potential of microvascular pericytes. Circulation 110:2226-2232.
50. Sims DE. (1986). The pericyte – a review. Tissue Cell 18:153-174.
51. Shepro D and NM Morel. (1993). Pericyte physiology. FASEB J 7:1031-1038.
52. Sims DE. (2000). Diversity within pericytes. Clin Exp Pharmacol Physiol 27:842-846.

53. Andreeva ER, IM Pugach, D Gordon and AN Orekhov. (1998). Continuous subendothelial network formed by pericyte-like cells in human vascular bed. Tissue Cell 30:127-135.
54. Armulik A, A Abramsson and C Betsholtz. (2005). Endothelial/pericyte interactions. Circ Res 97:512-523.
55. Ozerdem U, E Monosov and WB Stallcup. (2002). NG2 proteoglycan expression by pericytes in pathological microvasculature. Microvasc Res 63:129-134.
56. Murfee WL, TC Skalak and SM Peirce. (2005). Differential arterial/venous expression of NG2 proteoglycan in perivascular cells along microvessels: identifying a venule-specific phenotype. Microcirculation 12:151-160.
57. Stallcup WB. (2002). The NG2 proteoglycan: past insights and future prospects. J Neurocytol 31:423-435.
58. Rajkumar VS, C Sundberg, DJ Abraham, K Rubin and CM Black. (1999). Activation of microvascular pericytes in autoimmune Raynaud's phenomenon and systemic sclerosis. Arthritis Rheum 42:930-941.
59. Skalli O, MF Pelte, MC Peclet, G Gabbiani, P Gugliotta, G Bussolati, M Ravazzola and L Orci. (1989). Alpha-smooth muscle actin, a differentiation marker of smooth muscle cells, is present in microfilamentous bundles of pericytes. J Histochem Cytochem 37:315-321.
60. Gerhardt H and C Betsholtz. (2003). Endothelial-pericyte interactions in angiogenesis. Cell Tissue Res 314:15-23.
61. Sundberg C, M Kowanetz, LF Brown, M Detmar and HF Dvorak. (2002). Stable expression of angiopoietin-1 and other markers by cultured pericytes: phenotypic

similarities to a subpopulation of cells in maturing vessels during later stages of angiogenesis in vivo. Lab Invest 82:387-401.

62. Barry FP, RE Boynton, S Haynesworth, JM Murphy and J Zaia. (1999). The monoclonal antibody SH-2, raised against human mesenchymal stem cells, recognizes an epitope on endoglin (CD105). Biochem Biophys Res Commun 265:134-139.
63. Fleming JE Jr, SE Haynesworth, P Cassiede, MA Baber and AI Caplan. (1998). Monoclonal antibody against adult marrow-derived mesenchymal stem cells recognizes developing vasculature in embryonic human skin. Dev Dyn 212:119-132.
64. Simmons PJ and B Torok-Storb. (1991). Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody, STRO-1. Blood 78:55-62.
65. Dennis JE, JP Carbillet, AI Caplan and P Charbord. (2002). The STRO-1+ marrow cell population is multipotential. Cells Tissues Organs 170:73-82.
66. Brachvogel B, H Moch, F Pausch, U Schlotzer-Schrehardt, C Hofmann, R Hallmann, K von der Mark, T Winkler and E Poschl. (2005). Perivascular cells expressing annexin A5 define a novel mesenchymal stem cell-like population with the capacity to differentiate into multiple mesenchymal lineages. Development 132:2657-268.
67. Nayak RC, AB Berman, KL George, GS Eisenbarth and GL King. (1988). A monoclonal antibody (3G5)-defined ganglioside antigen is expressed on the cell surface of microvascular pericytes. J Exp Med 167:1003-1015.
68. Helmbold P, RC Nayak, WC Marsch and IM Herman. (2001a). Isolation and in vitro characterization of human dermal microvascular pericytes. Microvasc Res 61:160-165.
69. Helmbold P, J Wohlrab, WC Marsch and RC Nayak RC. (2001b). Human dermal pericytes express 3G5 ganglioside--a new approach for microvessel histology in the skin. J Cutan Pathol 28:206-210.

70. Stramer BM, MG Kwok, PJ Farthing-Nayak, JC Jung, ME Fini and RC Nayak. (2004). Monoclonal antibody (3G5)-defined ganglioside: cell surface marker of corneal keratocytes. Invest Ophthalmol Vis Sci 45:807-812.
71. Pusztaszeri MP, W Seelentag and FT Bosman. (2006). Immunohistochemical expression of endothelial markers CD31, CD34, von Willebrand factor, and Fli-1 in normal human tissues. J Histochem Cytochem 54:385-395.
72. Folkman J and PA D'Amore. (1996). Blood vessel formation: what is its molecular basis? Cell 87:1153-1155.
73. Heldin CH and B Westermark. (1999). Mechanism of action and in vivo role of platelet-derived growth factor. Physiol Rev 79:1283-1316.
74. Lindblom P, H Gerhardt, S Liebner, A Abramsson, M Enge, M Hellstrom, G Backstrom, S Fredriksson, U Landegren, HC Nystrom, G Bergstrom, E Dejana, A Ostman, P Lindahl and C Betsholtz. (2003). Endothelial PDGF-B retention is required for proper investment of pericytes in the microvessel wall. Genes Dev 17:1835-1840.
75. Hirschi KK, JM Burt, KD Hirschi KD and C Dai. (2003). Gap junction communication mediates transforming growth factor-beta activation and endothelial-induced mural cell differentiation. Circ Res 93:429-437.
76. Gerhardt H, H Wolburg and C Redies. (2000). N-cadherin mediates pericytic-endothelial interaction during brain angiogenesis in the chicken. Dev Dyn 218:472-479.
77. Tillet E, D Vittet, O Feraud, R Moore, R Kemler and P Huber. (2005). N-cadherin deficiency impairs pericyte recruitment, and not endothelial differentiation or sprouting, in embryonic stem cell-derived angiogenesis. Exp Cell Res 310:392-400.

78. Gerber HP and N Ferrara. (2000). Angiogenesis and bone growth. Trends Cardiovasc Med 10:223-228.
79. Caplan AI. (1990). Cartilage begets bone versus endochondral myelopoiesis. Clin Orthop Relat Res 261:257-267.
80. Reddi AH and WA Anderson. (1976). Collagenous bone matrix-induced endochondral ossification hemopoiesis. J Cell Biol 69:557-572.
81. Carano RA and EH Filvaroff. (2003). Angiogenesis and bone repair. Drug Discov Today 8:980-989.
82. Diaz-Flores L, R Gutierrez, P Gonzalez and H Varela. (1991).Inducible perivascular cells contribute to the neochondrogenesis in grafted perichondrium. Anat Rec 229:1-8.
83. Diaz-Flores L, R Gutierrez, A Lopez-Alonso, R Gonzalez and H Varela. (1992). Pericytes as a supplementary source of osteoblasts in periosteal osteogenesis. Clin Orthop Relat Res 275:280-286.
84. Brighton CT and RM Hunt. (1997). Early histologic and ultrastructural changes in microvessels of periosteal callus. J Orthop Trauma 11:244-253.
85. Meirelles L da S and NB Nardi. (2003). Murine marrow-derived mesenchymal stem cell: isolation, in vitro expansion, and characterization. Br J Haematol 123:702-711.
86. Abedin M, Y Tintut and LL Demer. (2004). Mesenchymal stem cells and the artery wall. Circ Res 95:671-676.
87. Covas DT, CE Piccinato, MD Orellana, JL Siufi, WA Silva Jr, R Proto-Siqueira, EG Rizzatti, L Neder, AR Silva, V Rocha and MA Zago. (2005). Mesenchymal stem cells can be obtained from the human saphena vein. Exp Cell Res 309:340-344.
88. Richardson RL, GJ Hausman, DR Campion. Response of pericytes to thermal lesion in the inguinal fat pad of 10-day-old rats. Acta Anat (Basel) 114:41-57.

89. Iyemere VP, D Proudfoot, PL Weissberg and CM Shanahan. (2006). Vascular smooth muscle cell phenotypic plasticity and the regulation of vascular calcification. J Intern Med 260:192-210.

90. Aranguren XL, A Luttun, C Clavel, C Moreno, G Abizanda, MA Barajas, B Pelacho, M Uriz, M Arana, A Echavarri, M Soriano, EJ Andreu, J Merino, JM Garcia-Verdugo, CM Verfaillie and F Prosper. (2007). In vitro and in vivo arterial differentiation of human multipotent adult progenitor cells. Blood 109:2634-242.

Table 1. Markers expressed by pericytes and other cell types.

Molecule	*CD*	*Pericytes*	*SMCS*	*MSCs*	*ECs*	*HCs*	*Reference*
3G5 antibody-defined ganglioside		+			-		67,70
angiopoietin-1		+			-		61
angiopoietin-2		-			+		61
annexin A5		+			-		65
calponin		+					
desmin		+	+				
EGF receptor		+		+			
nestin		+			+		
NG-2 proteoglycan		+					55,56
PAL-E					+		
PDGF-R		+		+			55
Sca-1 (mouse only)		+		+	+	+	
SM22-α		+					
SM-myosin		+	+	+	+	+	
stem cell factor				+			
STRO-1		+		+	+		
Tie-1		-			+		61
Tie-2		-			+		61
VE-cadherin					+		
VEGFR1		-; +			+		61
vWF		-	-	-	+	-	

α-SMA		+	+				
	9			+			
	10			+			
	11a			-			
	11b			-		+	
aminopeptidase N	13	+		+	+	+	
	14			-			
	15			-			
	18			-			
	25			-			
	29			+			
PECAM	31	-	-	-	+	-	
	34			-		+	85
	36				+	+	
	44			+			
	45	-	-	-		+	
	49a			+			
	49b			+			
	49c			+			
	49d			-			
	49e			+			
	50			-			
	51			+			
ICAM-1	54			+			
	58			+			

	61		+			
	62E		-	+		
	62L		+		+	
	62P		-			
	71		+			
	73		+			
Thy-1	90	+	+		+	
	102		+			
TGFβRIII	105		+	+		
	106		+			
	117		-		+	85
	119		+			
	120a		+			
	120b		+			
	121		+			
	123		+			
	124		+			
	126		+			
	127		+			
prominin	133				+	PROW
PDGFRa	140a	+	+			
PDGFRb	140b	+	+			55

CD, Cluster of Differentiation number; ECs, endothelial cells; HCs, hematopoietic cells; MSCs, mesenchymal stem cells; PROW - Protein Reviews On The Web. http://mpr.nci.nih.gov/prow; SMCs, smooth muscle cells. Empty cells indicate that marker expression is not known.

Table 2. Histological observations at different time points during the course of bone formation in demineralized bone matrices.

Day	*Histological characteristics*
1	Polymorphonuclear leukocytes +++
3	Polymorphonuclear leukocytes + Fibroblasts ++++
5	Fibroblasts +++ Chondroblasts +
7	Chondrocytes +++
9	Hypertrophy and calcification of chondrocytes Capillary ingrowth
10	Chondrolysis Osteoblasts +++
11	Bone +++
14	Bone +++ Early hemocytoblasts
18	Bone ++++ Bone marrow ++
21	Bone ++++ Bone marrow ++++

Capítulo 6

Cultured human mesenchymal stem cells take up perivascular locations when implanted in ceramic cubes *in vivo*.

Lindolfo da Silva Meirelles1, Donald P. Lennon, J. Michael Sorrell, Marilyn Baber, Nance Beyer Nardi & Arnold I. Caplan.

Manuscrito em preparação a ser submetido para *Cell and Tissue Research*

Cultured human mesenchymal stem cells take up perivascular locations when implanted in ceramic cubes *in vivo*.

Lindolfo da Silva Meirelles[1]*, Donald P. Lennon[2], J. Michael Sorrell[2], Marilyn Baber[2], Nance Beyer Nardi[1] and Arnold I. Caplan[2].

1 – Departamento de Genética, Universidade Federal do Rio Grande do Sul, Brazil
Av Bento Goncalves 9500
Porto Alegre, RS, Brazil
91501-970
Telephone: +55 51 33086737

2 – Department of Biology, Skeletal Research Center
Case Western Reserve University
2080 Adelbert Road, Cleveland, OH, USA
44106-7080

* Bolsista do CNPq - Brasil

Abstract

Mesenchymal stem cells (MSCs) are defined as able to differentiate into mature mesenchymal cells *in vitro* and *in vivo*. While much of the knowledge on MSC biology derives from *in vitro* approaches, understanding their behavior *in vivo* is still a matter for study. Experiments using injury models indicate that perivascular cells behave as local progenitors during wound healing and, in addition, results from *in vitro* experiments indicate that the cultured cells defined as MSCs derive from cells located in a perivascular niche *in vivo*. We hypothesized that, if that is true, short-term cultured human MSCs would take up a perivascular location when loaded into ceramic cubes and implanted subcutaneously into immunocompromised mice. Using this approach, MSCs could be detected around blood vessels from periods of time between 15 to 6 weeks, some of them

expressing the pericyte marker 3G5. These results strengthen the hypothesis that cultured MSCs are perivascular cells *in vivo*.

Introduction

Mesenchymal stem cells (MSCs) may be defined as cells capable of giving rise to a number of unique, differentiated mesenchymal cell types (Caplan, 1991; Prockop, 1997). First described as fibroblast precursors from bone marrow by Friedenstein et al. (1970), MSCs may be also referred to as fibroblast colony-forming units (CFU-Fs) or marrow stromal cells (Phinney, 2002). To date, there is evidence that suggests MSCs may exist not only in bone marrow, but in virtually every location of the body (da Silva Meirelles et al., 2006; Prunet-Marcassus et al., 2006; Krampera et al., 2006). MSCs from human bone marrow can be isolated and cultivated using standard methods (Haynesworth et al., 1992; Pittenger et al., 1999; Lennon and Caplan, 2006). A variety of molecules is known to be expressed by cultured MSCs (Beyer Nardi and da Silva Meirelles, 2006). The literature presents evidence that cultured MSCs also exhibit a high degree of plasticity *in vitro*, and when implanted *in vivo*, such plasticity going beyond the mesenchymal limit (Kopen et al., 1999; Woodbury et al., 2000; Lange et al., 2005; Sato et al., 2005; Seo et al. 2005). MSCs have also been proposed to exert paracrine trophic effects through the secretion of bioactive molecules (Caplan and Dennis, 2006). These characteristics make them promising tools for cell and cell-based therapies.

Although a growing body of information regarding the characteristics of cultured MSCs has accumulated over the years, their exact identity *in vivo* is still a matter for study. A careful examination of the literature indicates an identity overlap between MSCs and pericytes because a) evidence indicates that pericytes can give rise to differentiated cells in mesenchymal tissues *in vivo* (Richardson et al. 1982; Diaz-Flores et al., 1991; Diaz-Flores et al., 1992; Brighton and Hunt, 1997); b) cultured pericytes can differentiate into cells of mesenchymal lineages *in vitro* and when implanted *in vivo* (Doherty et al., 1998; Farrington-Rock et al., 2004); c) MSCs have been traced to the perivascular space in studies using defined MSC markers (Fleming et al., 1998; Bianco et al., 2001; Shi and Gronthos, 2003; Brachvogel et al., 2005); and d) cells bearing MSC characteristics can be

isolated from virtually all tissues, as explained by a model that conceives that those are perivascular cells *in vivo* (da Silva Meirelles et al., 2006).

Taking the above into account, we hypothesized that, if cultured human MSCs are in fact perivascular cells *in vivo*, they would take up a perivascular location when loaded into ceramic cubes and implanted subcutaneously into immunocompromised mice. To test that hypothesis, the investment of ceramic cubes loaded with MSCs at early time points was examined, and the implanted human cells could be observed throughout the pores of the cubes, many of them in perivascular locations. Cultured human MSCs were found to express the pericyte markers 3G5 and HMW-MAA *in vitro*, and 3G5-positive MSCs could be detected in ceramic cubes implanted *in vivo*. Human brain vascular pericytes (HBVPs), used as a positive control for immunofluorescence, were also found to express the human MSC markers CD13, CD44 and CD105. These results are consistent with the hypothesis that cultured MSCs are perivascular cells *in vivo*, and provide additional evidence of identity overlap between MSCs and pericytes.

Materials and methods

Cell culture.

Bone marrow aspirates were obtained from healthy volunteers after informed consent. MSCs were isolated and cultured as described elsewhere (Lennon and Caplan, 2006). Briefly, bone marrow was mixed with one volume of low glucose Dulbecco's Modified Eagle's Medium (Sigma Chemical, Saint Louis, MO) added of 10% fetal bovine serum (Gibco/Invitrogen, Carlsbad, CA) from selected lots. After centrifugation on a Percoll gradient, the fraction above the density cut was cultured in the same medium composition. For passaging, cells were rinsed with Tyrode's Balanced Salt Solution (TBSS, Sigma), and incubated with 0.25% trypsin-EDTA (Gibco). Cells from first or second passages were used for the experiments. HBVPs were acquired from ScienCell (San Diego, CA), and were cultured according to the supplier instructions.

Ceramic cube implantation

Implantation of MSC-loaded ceramic cubes into syngeneic or immunocompromised host animals as an in vivo assay for the osteochondrogenic potential of these cells has been described previously (Dennis et al., 1992, Dennis and Caplan, 1993). Briefly, blocks of porous ceramic (mean pore size of 200 mm), consisting of 60% tricalcium phosphate and 40% hydroxyapatite, were cut into cubes measuring 3 mm per side. The ceramic cubes were washed with water to remove ceramic dust, dried under a heating lamp, and sterilized in an autoclave. To improve cell attachment, ceramic cubes were combined with a 100 mg/ml solution of human fibronectin (Collaborative Biomedical, Bedford,MA)in Tyrode's salt solution in a 12x75-mm sterile, capped polystyrene tube (Becton Dickinson, Franklin Lakes, NJ). A 20-gauge needle attached to a 30-ml syringe was inserted through the cap of the tube, and the syringe plunger was retracted to evacuate air from the tube, thus generating a partial vacuum and permitting the fibronectin solution to enter the pores of the cubes. Cubes remained in the fibronectin solution for 2 h at room temperature (RT) and were then air dried overnight in a laminar flow hood.

First or second-passage MSCs were tripsinized and resuspended at a concentration of $5x10^6$ cells/ml in serum-free medium, and transferred to a 12x75-mm sterile tube. Fibronectin-coated ceramic cubes were introduced in the tube, and air was withdrawn with a 20-ml syringe as for fibronectin coating.

Cell-loaded cubes were incubated at 37°C for 2h and then implanted subcutaneously into pockets created by blunt dissection on the dorsal surface of SCID mice (Charles River Laboratories, Wilmington, MA) anesthetized with a mixture of Ketamine and Xylazine (100 and 10 mg/kg body weight, respectively). The incision on the skin was closed with a steel wound clip. At defined times post-implant, the animals were euthanized by anesthetic overdose, and the cubes were harvested. This protocol was approved by the Institutional Animal Care and Use Committee.

Ceramic cube processing

After removal, the ceramic cubes were fixed with 10% neutral buffered formalin overnight. The cubes were washed once in TBSS, and decalcified in a solution containing tris (0.1M) and 10% (w/v) EDTA disodium salt, pH 7.0, for 2 weeks at 4°C, with 3 solution changes per week. This method was chosen because it has been shown to preserve

tissue antigens well as compared with others (Jonsson et al., 1986). For some experiments, cubes were fixed in JB fixative (Beckstead, 1994), and then decalcified using the same solution described above. Some cubes were included in paraffin. 5 μm sections were prepared, and stained with Mallory-Heidenhain. For immunostaining, cubes were embedded in optimal cutting temperature compound (OCT) and snap-frozen in liquid nitrogen. 7 μm cryosections were prepared and transferred to gelatin-coated slides.

Antibodies and immunostaining

Commercially available primary antibodies used were anti-HMW-MAA (AbD Serotec, Raleigh, NC), diluted 1:100, and anti-human CD31 (Chemicon, Temecula, CA), also diluted 1:100. Antibodies against CD13 and CD44, and the antibodies SH-2 (Haynesworth et al., 1992b) and 6E2 were present in the supernatant of their respective hybridomas, developed in this laboratory. The supernatants were used with no further dilution for immunostaining. The SH-2 antibody has been shown to recognize CD105 (Barry et al., 1999). The antibody 6E2 recognizes an antigen present on the surface of most human cells, and does not cross-react with murine cells. The 3G5 hybridoma was purchased from American Type Culture Collection (Manassas, VA), and its supernatant was used with no further dilution. A fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Ig (SouthernBiotech, Birmingham, AL) was used as secondary antibody.

For immunostaining, cryosections were rinsed in TBSS at room temperature to remove traces of OCT, washed once with phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA), and incubated with the primary antibodies for one hour at RT. The slides were then washed with PBS-BSA, and incubated with the secondary antibody for one hour at RT. Some slides were incubated with the secondary antibody only to provide a negative control. For immunocytochemistry, cells were plated on 35-mm culture dishes or chamber slides at leas 24 hour before the experiment, and processed in the same way as described for cryosections.

The samples were observed using either an upright microscope (Leica) or an inverted microscope (Olympus), both equipped with filter sets for the detection of FITC. Images were acquired using digital cameras attached to the microscopes, using software

provided by the respective manufacturers. Images were sharpened using Adobe Photoshop CS2 software.

Results

Blood vessels invade MSC-loaded ceramic cubes

One of the first steps to examine if MSCs take up perivascular locations was to observe vascular invasion in the MSC-loaded cubes. As shown in Figure 1A, cubes were already invaded by the host's vasculature at the first time point studied (8 days post-transplant). Empty cubes, however, had undergone nearly no vascular invasion (Figure 1B). Many vessels could be observed near the osteoblastic layer that forms in the walls of the pores at all time points analyzed (Figure 1). A noteworthy feature regarding the spatial relation of blood vessels to the bone layer that forms inside the cube pores is that the vasculature was never found inside the osseous tissue.

MSCs and HBVPs share surface markers

Since the molecules 3G5 and HMW-MAA are expressed in resting (Helmbold et al, 2004) and proliferative (Rajkumar et al., 1999) pericytes, respectively, *in vivo*, we hypothesized that, if cultured MSCs are actually pericytes, only a small fraction of them would express 3G5 after one passage, while a higher proportion would express HMW-MAA. HBVPs were used as a positive control for the immunostaining. HBVPs had their expression of some MSC markers checked to evaluate if they share some identity with MSCs.

Virtually all cultured MSCs and HBVPs expressed HMW-MAA in a uniform fashion (Figure 2A and B). Both populations presented subpopulations of 3G5-positive cells (Figure 2C and D). 3G5-positive cells were also present as subpopulations among the cells of the first colonies in primary culture (Figure 2E). All HBVPs expressed SH-2 (Figure 2F), CD13 (Figure 2G) and CD44 (Figure 2H).

Effect of fixation on the epitope defined by the 3G5 antibody

Locating 3G5-positive cells in the MSC-loaded cubes was of interest because the 3G5 antibody does not recognize murine antigens (Stramer et al. 2004), meaning that 3G5-positive cells found would be human in origin. Furthermore, 3G5 expression *in vivo* was expected only in pericytes (Andreeva, et al., 1998; Helmbold et al., 2004), meaning that 3G5-positive cells would represent human MSCs that became pericytes *in vivo*.

As a first step to check if 3G5-positive cell could be observed in cube cryosections, the effect of formalin – the fixative routinely used – on the 3G5 antigen was assessed by immunofluorescence on cultured HBVPs. As shown in Figure 3A and B, fixation with formalin abolished the reactivity of the 3G5 antibody with its epitope. As an alternative, JB fixative was tested with the same purpose, and was found to be effective (Figure 3C and D). Ethanol was also tested not only because it might work as a fixative, but also because embedding JB-fixed cubes in paraffin could be of interest. Ethanol also abolished 3G5 reactivity (Figure 3E).

The majority of cells inside the MSC-loaded cubes are human in origin

To observe what cells inside the ceramic cube were derived from the MSCs implanted, cryosections were immunostained with the 6E2 antibody. As seen in Figure 4A and B, most cells inside the ceramic cubes were human in origin. Immunolocalization of 3G5-positive cells was attempted in cryosections from cubes fixed with formalin and subjected decalcification using Tris-EDTA because there was a possibility that this process could work as an antigen retrieval protocol, since Tris-EDTA may be used as an antigen retrieval method under other conditions (Torlakovic et al. 2005). Figure 4C shows a 3G5-positive cell in a perivascular location.

MSCs take up perivascular locations

Even though the immunostainings performed using the 3G5 antibody allowed for the visualization of some 3G5-positive cells, the signal was usually weak and sometimes telling whether or not it was positive or artifactual was difficult. To seek for 3G5 positive cells, cubes were fixed in JB fixative instead of formalin, and cryosectioned. To observe

human cells inside the cubes, cryosections were stained with the 6E2 antibody. In those preparations, erythrocytes displaying some background fluorescence helped to locate the blood vessels. Cells displaying a fluorescence level clearly higher than control sections or erythrocytes could be identified around many blood vessels (Figure 5A). Cells expressing human CD31 could not be located inside the cubes (Figure 5B). Immunoreactivity of the anti-CD31 antibody used was checked on live and JB-fixed cultured human umbilical vein endothelial cells (not shown). Finally, 3G5-expressing cells could also be located inside JB-fixed ceramic cubes, but their frequency seemed to be far lower than the frequency of human cells around blood vessels (not shown).

Discussion

This study differs from other where MSCs shown to engraft across the blood vessels (Schmidt et al., 2006) in that here, MSCs were present in the target site prior to vascularization and tissue formation, leading to a process that involves the interaction of the pre-existing MSCs with the ceramic environment, and with the yet-to-come host vasculature. In this regard, MSCs displayed the property of attracting the host vasculature, as evidenced by the poor investment of empty ceramic cubes by blood vessels (Figure 1B).

The majority of the cells inside the pores of the cubes were found to be derived from the MSCs at 15 days post-implant (Figure 4A), indicating that the osseous tissue formed inside the cube was formed by a portion of the MSCs, and not by host's cells taken there along with, or by the blood vessels. Human cells could be detected also 6 weeks post-implant, and in this case perivascular human cells were commonly observed (Figure 5A). 3G5 positive cells could be observed in the cubes 15 days (Figure 4C) and 6 weeks (Figure 5D-F) post-implant, but their frequency was very low as compared with the amount of human cells present. Even though this may indicate that the frequency of MSCs that in fact became pericytes was low, the possibility that this reflects the low frequency of 3G5-positive cells in the short-term MSC cultures cannot be discarded. Since no CD31-positive human cells could be found inside the cubes, the MSCs found around the blood vessels may be considered pericytes on the basis of their location, independently of their expression of 3G5.

The high frequency of MSC-derived pericytes may be considered as evidence that the MSCs that were not in contact with the osteoinductive ceramic surface have a natural tendency to remain as perivascular cells, which is consistent with the hypothesis that cultured MSCs descend from pericytes (da Silva Meirelles et al., 2006). In addition to that, the fact that both cultured MSCs and pericytes share markers associated with pericytes and MSCs, respectively (Figure 2), reinforce this view.

References

Andreeva ER, Pugach IM, Gordon D, Orekhov AN (1998) Continuous subendothelial network formed by pericyte-like cells in human vascular bed. Tissue Cell. 30:127-135.

Barry FP, Boynton RE, Haynesworth S, Murphy JM, Zaia J (1999) The monoclonal antibody SH-2, raised against human mesenchymal stem cells, recognizes an epitope on endoglin (CD105). Biochem Biophys Res Commun. 265:134-139.

Beckstead JH (1994) A simple technique for preservation of fixation-sensitive antigens in paraffin-embedded tissues. J Histochem Cytochem. 42:1127-1134.

Beyer Nardi N, da Silva Meirelles L (2006) Mesenchymal stem cells: isolation, in vitro expansion and characterization. Handb Exp Pharmacol. (174):249-282.

Bianco P, Riminucci M, Gronthos S, Robey PG (2001) Bone marrow stromal stem cells: nature, biology, and potential applications. Stem Cells. 19:180-192.

Brachvogel B, Moch H, Pausch F, Schlotzer-Schrehardt U, Hofmann C, Hallmann R, von der Mark K, Winkler T, Poschl E (2005) Perivascular cells expressing annexin A5 define a novel mesenchymal stem cell-like population with the capacity to differentiate into multiple mesenchymal lineages. Development. 132:2657-2668.

Brighton CT, Hunt RM (1997) Early histologic and ultrastructural changes in microvessels of periosteal callus. J Orthop Trauma. 11:244-253.

Caplan AI (1991) Mesenchymal stem cells. J Orthop Res. 9:641-650.

Caplan AI, Dennis JE (2006) Mesenchymal stem cells as trophic mediators. J Cell Biochem. 98:1076-1084.

da Silva Meirelles L, Chagastelles PC, Nardi NB (2006) Mesenchymal stem cells reside in virtually all post-natal organs and tissues. J Cell Sci. 119:2204-2213.

Dennis JE, Caplan AI (1993) Porous ceramic vehicles for rat-marrow-derived (Rattus norvegicus) osteogenic cell delivery: effects of pre-treatment with fibronectin or laminin. J Oral Implantol. 19:106-115.

Dennis JE, Haynesworth SE, Young RG, Caplan AI (1992) Osteogenesis in marrow-derived mesenchymal cell porous ceramic composites transplanted subcutaneously: effect of fibronectin and laminin on cell retention and rate of osteogenic expression. Cell Transplant. 1:23-32.

Diaz-Flores L, Gutierrez R, Gonzalez P, Varela H (1991) Inducible perivascular cells contribute to the neochondrogenesis in grafted perichondrium. Anat Rec. 229:1-8.

Diaz-Flores L, Gutierrez R, Lopez-Alonso A, Gonzalez R, Varela H (1992) Pericytes as a supplementary source of osteoblasts in periosteal osteogenesis. Clin Orthop Relat Res. (275):280-286.

Doherty MJ, Ashton BA, Walsh S, Beresford JN, Grant ME, Canfield AE (1998) Vascular pericytes express osteogenic potential in vitro and in vivo. J Bone Miner Res. 13:828-838.

Farrington-Rock C, Crofts NJ, Doherty MJ, Ashton BA, Griffin-Jones C, Canfield AE (2004) Chondrogenic and adipogenic potential of microvascular pericytes. Circulation. 110:2226-2232.

Fleming JE Jr, Haynesworth SE, Cassiede P, Baber MA, Caplan AI (1998) Monoclonal antibody against adult marrow-derived mesenchymal stem cells recognizes developing vasculature in embryonic human skin. Dev Dyn. 212:119-132.

Friedenstein AJ, Chailakhjan RK, Lalykina KS (1970) The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. Cell Tissue Kinet. 3:393-403.

Haynesworth SE, Baber MA, Caplan AI (1992b) Cell surface antigens on human marrow-derived mesenchymal cells are detected by monoclonal antibodies. Bone. 13:69-80.

Haynesworth SE, Goshima J, Goldberg VM, Caplan AI (1992) Characterization of cells with osteogenic potential from human marrow. Bone. 13:81-88.

Helmbold P, Wohlrab J, Marsch WC, Nayak RC (2001) Human dermal pericytes express 3G5 ganglioside - a new approach for microvessel histology in the skin. J Cutan Pathol. 28:206-210.

Jonsson R, Tarkowski A, Klareskog L (1986) A demineralization procedure for immunohistopathological use. EDTA treatment preserves lymphoid cell surface antigens. J Immunol Methods. 88:109-114.

Kopen GC, Prockop DJ, Phinney DG (1999) Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. Proc Natl Acad Sci U S A. 96:10711-10716.

Krampera M, Marconi S, Pasini A, Galie M, Rigotti G, Mosna F, Tinelli M, Lovato L, Anghileri E, Andreini A, Pizzolo G, Sbarbati A, Bonetti B (2007) Induction of neural-like differentiation in human mesenchymal stem cells derived from bone marrow, fat, spleen and thymus. Bone. 40:382-390.

Lange C, Bassler P, Lioznov MV, Bruns H, Kluth D, Zander AR, Fiegel HC (2005) Liver-specific gene expression in mesenchymal stem cells is induced by liver cells. World J Gastroenterol. 11:4497-4504.

Lennon DP, Caplan AI (2006) Mesenchymal Stem Cells for Tissue Engineering. In: Vunjak-Novakovic G, Freshney RI (eds). Culture of Cells for Tissue Engineering. Wiley, Indianapolis, pp. 23–59.

Phinney DG (2002) Building a consensus regarding the nature and origin of mesenchymal stem cells. J Cell Biochem Suppl. 38:7-12.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR (1999) Multilineage potential of adult human mesenchymal stem cells. Science. 284:143-147.

Prockop DJ (1997) Marrow stromal cells as stem cells for nonhematopoietic tissues. Science. 276:71-74.

Prunet-Marcassus B, Cousin B, Caton D, Andre M, Penicaud L, Casteilla L (2006) From heterogeneity to plasticity in adipose tissues: site-specific differences. Exp Cell Res. 312:727-736.

Rajkumar VS, Sundberg C, Abraham DJ, Rubin K, Black CM (1999) Activation of microvascular pericytes in autoimmune Raynaud's phenomenon and systemic sclerosis. Arthritis Rheum. 42:930-941.

Richardson RL, Hausman GJ, Campion DR (1982) Response of pericytes to thermal lesion in the inguinal fat pad of 10-day-old rats. Acta Anat (Basel). 114:41-57.

Sato Y, Araki H, Kato J, Nakamura K, Kawano Y, Kobune M, Sato T, Miyanishi K, Takayama T, Takahashi M, Takimoto R, Iyama S, Matsunaga T, Ohtani S, Matsuura A, Hamada H, Niitsu Y (2005) Human mesenchymal stem cells xenografted directly to rat liver are differentiated into human hepatocytes without fusion. Blood. 106:756-763.

Schmidt A, Ladage D, Steingen C, Brixius K, Schinkothe T, Klinz FJ, Schwinger RH, Mehlhorn U, Bloch W (2006) Mesenchymal stem cells transmigrate over the endothelial barrier. Eur J Cell Biol. 85:1179-1188.

Seo MJ, Suh SY, Bae YC, Jung JS (2005) Differentiation of human adipose stromal cells into hepatic lineage in vitro and in vivo. Biochem Biophys Res Commun. 328:258-264.

Shi S, Gronthos S (2003) Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. J Bone Miner Res. 18:696-704.

Stramer BM, Kwok MG, Farthing-Nayak PJ, Jung JC, Fini ME, Nayak RC (2004) Monoclonal antibody (3G5)-defined ganglioside: cell surface marker of corneal keratocytes. Invest Ophthalmol Vis Sci. 45:807-812.

Torlakovic E, Nielsen S, Vyberg M (2005) Antibody selection in immunohistochemical detection of cyclin D1 in mantle cell lymphoma. Am J Clin Pathol. 124:782-789.

Woodbury D, Schwarz EJ, Prockop DJ, Black IB (2000) Adult rat and human bone marrow stromal cells differentiate into neurons. J Neurosci Res. 61:364-370.

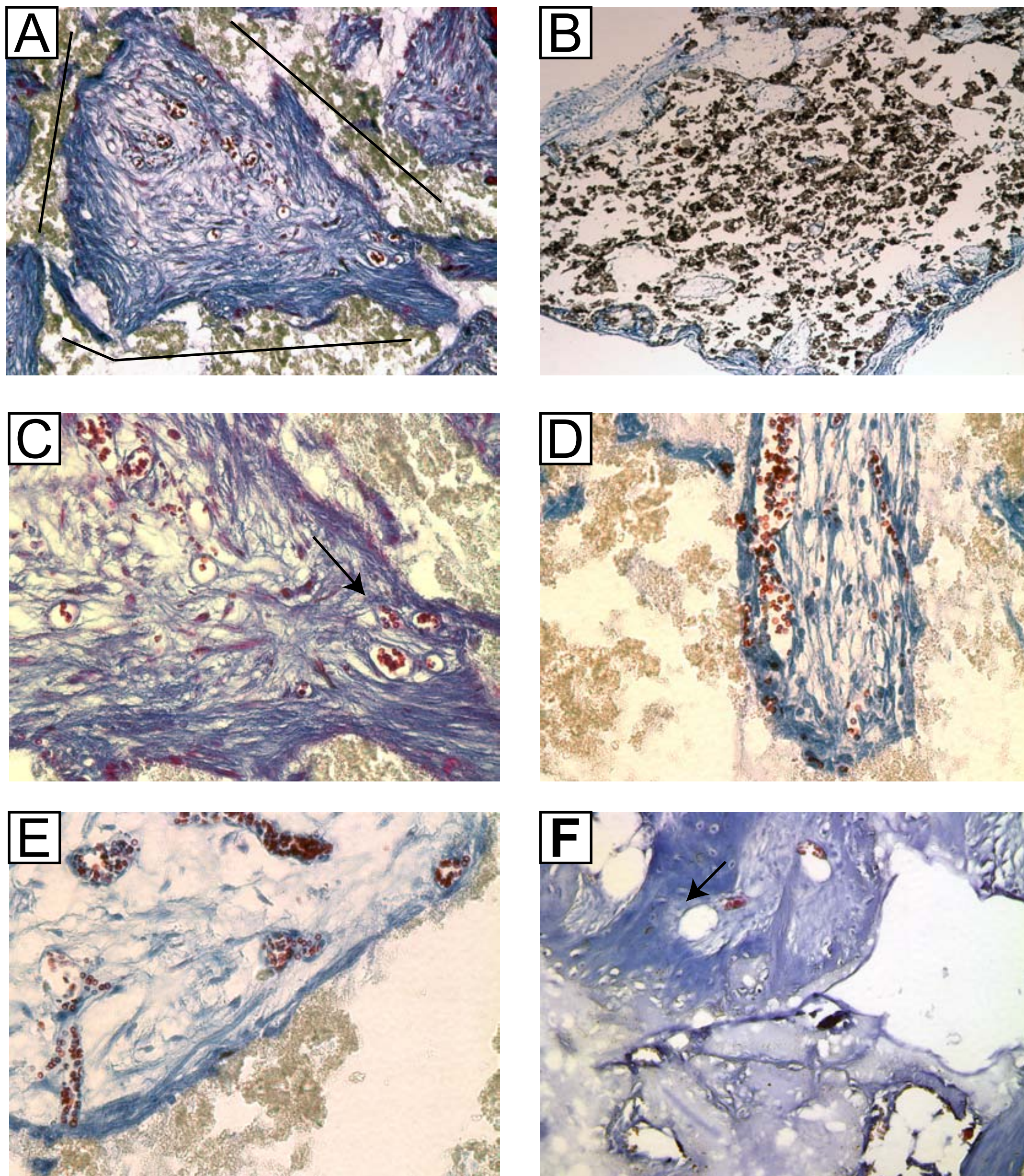

Figure 1 - Aspect of blood vessels inside ceramic cubes at different time points. A, section from a cube harvested 8 days post-implant. Lines indicate the areas formerly occupied by ceramic, and define the limits of the contents of a pore (200X). B, section from an empty cube harvested 15 days post-implant. Note the lack of vasculature and tissue in the center (50X). C, same as A, shown in a higher magnification, evidencing an area where the blood vessels are in close proximity with the osteoblastic layer on the pore wall (arrow) (400X). D and E, cubes harvested 15 days post-implant. F, cube harvested 7 weeks post-implant. The arrow on the osseous layer points to a transitional area between the perivascular zone of a vessel and the osseous tissue (200X).

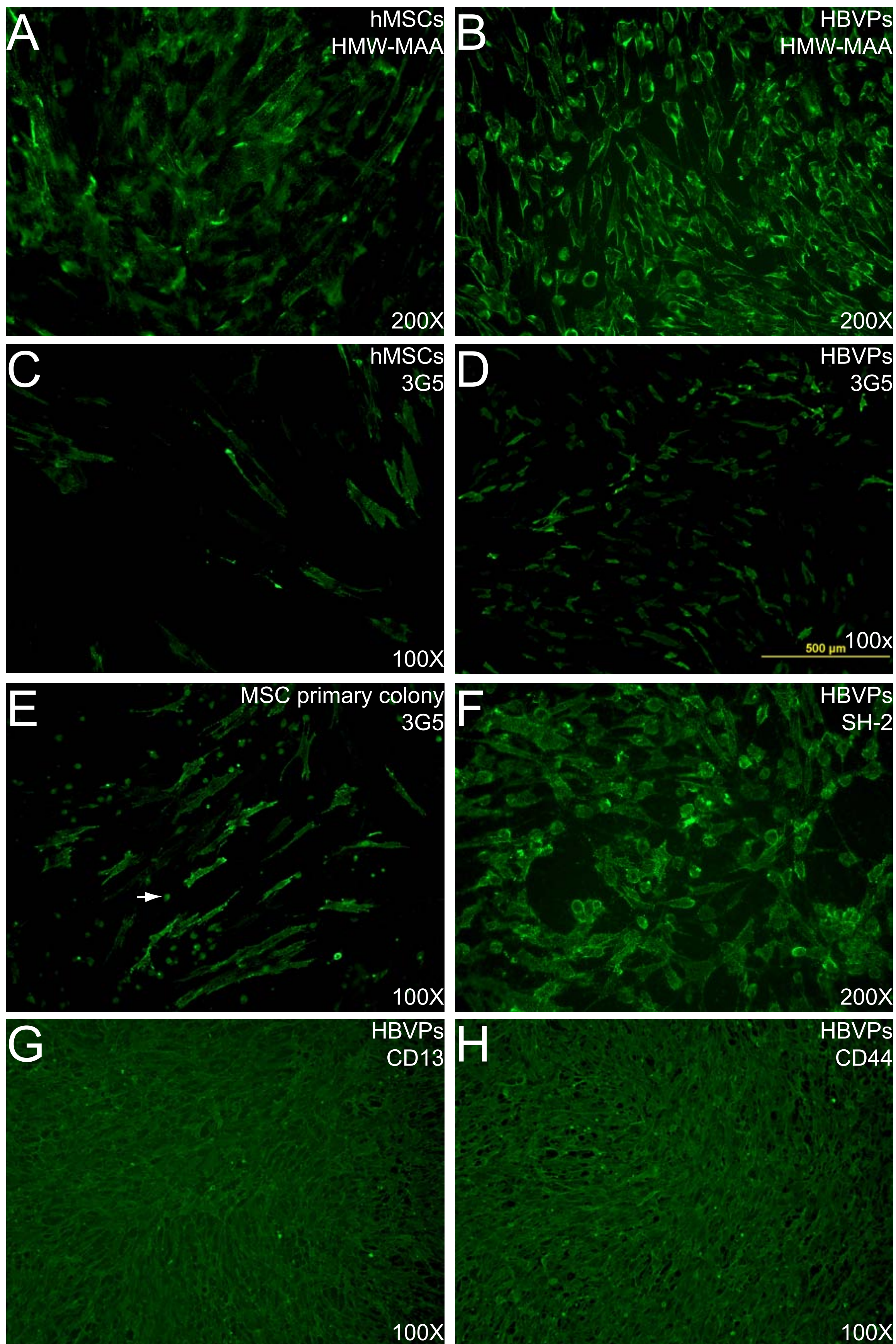

Figure 2 - Pericyte markers expression on MSCs, and MSC markers expression on HBVPs. A and B, cultured MSCs and HBVPs uniformly express the activated pericyte marker, HMW-MAA. C and D, fractions of cultured MSCs and HBVPs express the pericyte marker 3G5. E, 3G5-positive cells are also present on the first MSC colonies in primary culture. Round cells (one pointed by an arrow) represent false positives as compared with the immunostaining control slide (not shown). F, G and H, cultured HBVPs express the human MSC markers SH-2 (F), CD13 (G), and CD44 (H).

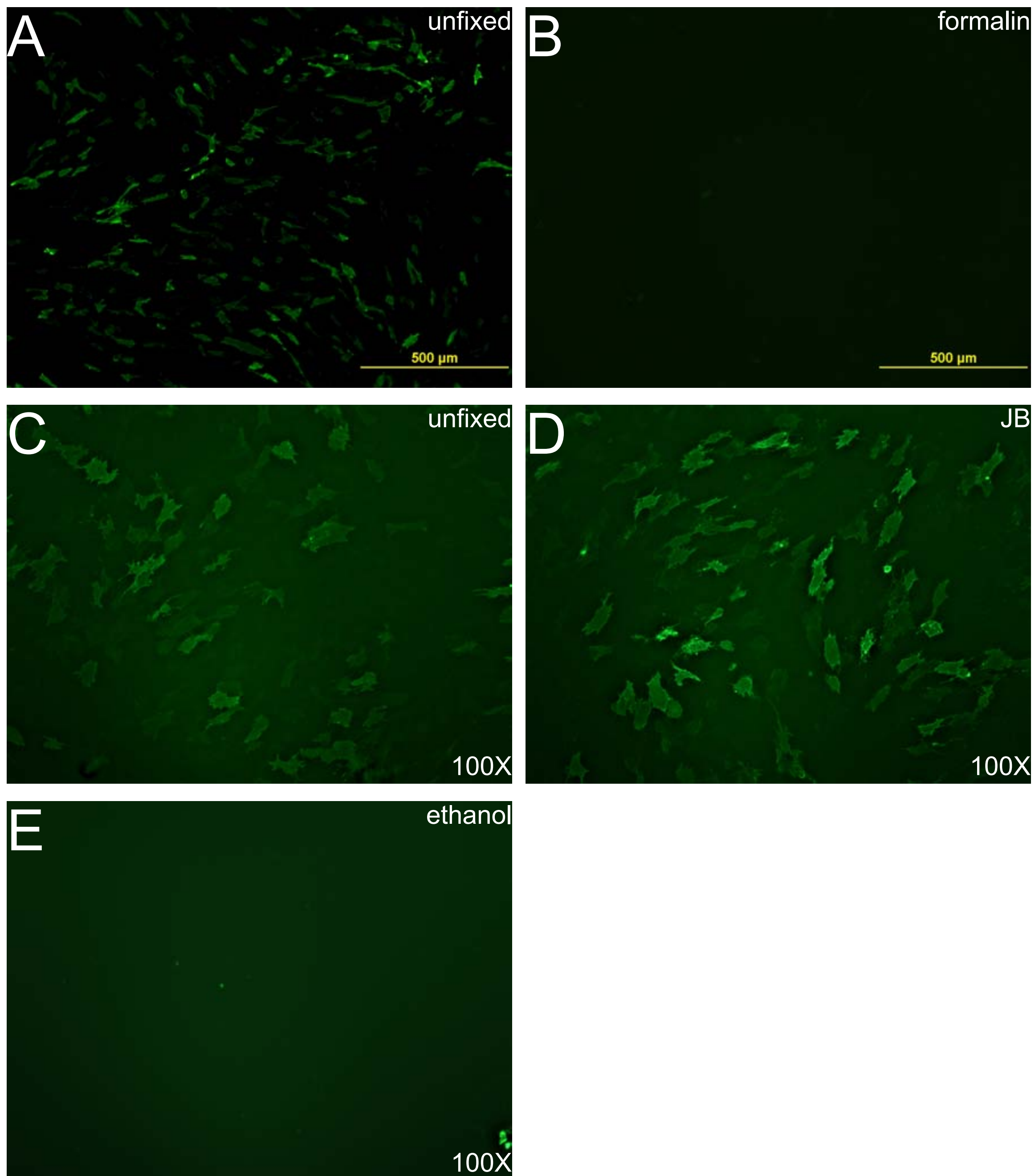

Figure 3 - Effect of different fixatives on the epitope defined by the 3G5 antibody. A and B, pictures of a first experiment in which unfixed (A) and formalin-fixed (B) HBVPs were immunostained with the 3G5 antibody. C, E and F, pictures from a second experiment in which unfixed (C), JB-fixed (E) and ethanol-fixed (F) HBVPs were immunostained with the same antibody.

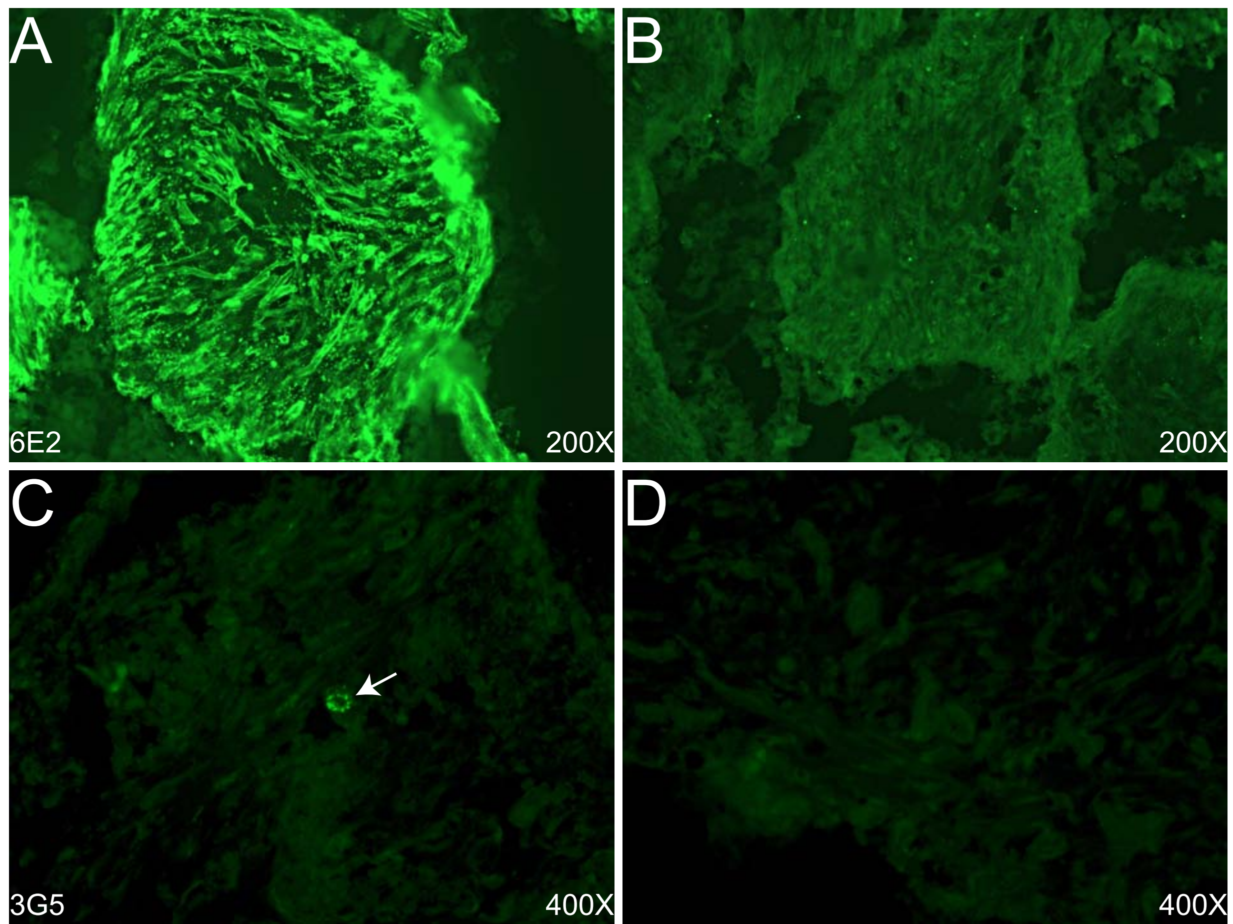

Figure 4 - Immunohistochemistry on formalin-fixed cubes decalcified using tris-EDTA. A, detail of one of the pores of a cube processed 15 days post-implant, after immunostaining using the 6E2 antibody. B, negative control for A. C, immunolocalization of a 3G5-positive cell (arrow) adjacent to a blood vessel, in a section of a cube harvested 3 weeks post-implant. D, negative control for C.

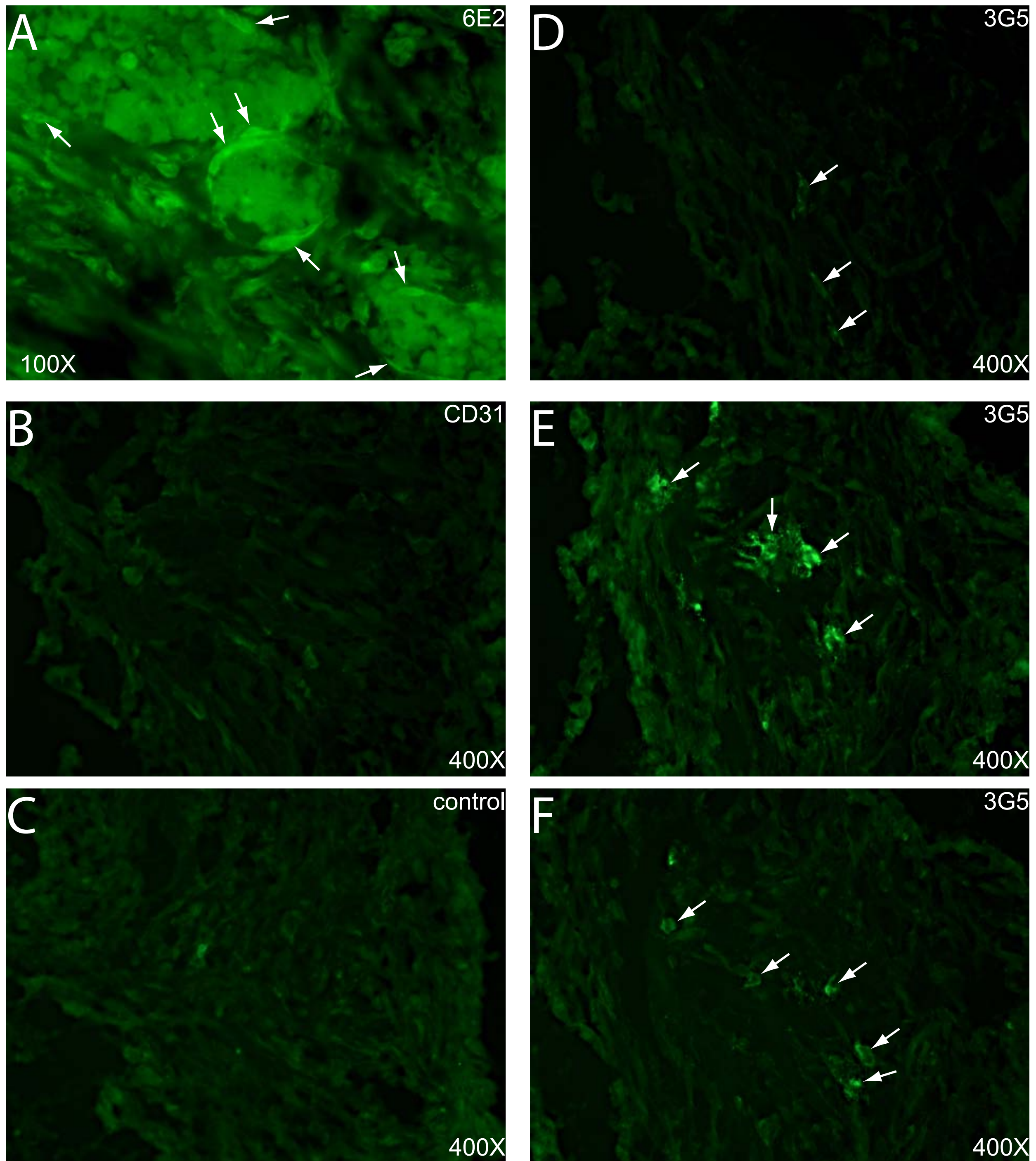

Figure 5 - Immonohistochemistry on JB-fixed cube cryosections. A, immunostaining using the 6E2 antibody. Background fluorescence from erythrocytes make blood vessels distinguishable, and human cells can be seen around them (arrows). B, cryosection stained with anti-human CD31 antibody. C, negative control. D - F, pictures of the same region on consecutive sections subjected to immunostaining with the 3G5 antibody. Positive cells can be observed near the osteoblastic layer on the pore wall (arrows).

Capítulo 7

Discussão

A MSC, por suas características de obtenção reprodutível por métodos padronizados e alta plasticidade *in vitro*, vem atraindo a atenção de pesquisadores que visam a sua utilização como agente terapêutico para o tratamento de várias doenças, ou como componente de materiais biológicos que possam repor tecidos perdidos em acidentes. É relevante, portanto, que se estudem as características de sua biologia, a fim de que se obtenha maior sucesso nessas aplicações.

O primeiro artigo apresentado nesta tese (Capítulo 3) demonstra que células que apresentam características de tronco mesenquimal *in vitro* podem ser obtidas de diferentes órgãos e tecidos de camundongo. Nele, descartou-se a hipótese de que tais células eram originárias do sangue circulante, e reforçou-se a hipótese de que as mesmas são provenientes dos vasos sangüíneos, mais especificamente da região perivascular. Com base também em outros dados da literatura, postulou-se um modelo no qual células perivasculares seriam as responsáveis pela reposição de células dos órgãos em que estão localizadas, podendo assumir um papel mais ativo no caso de lesão tecidual.

O segundo artigo (Capítulo 4) abordou o ponto específico do conteúdo de DNA das mMSCs cultivadas *in vitro*, indicando que este encontra-se duplicado nas mesmas. Isso foi considerado mais uma evidência na direção da origem perivascular das mesmas, posto que a duplicação do conteúdo de DNA é uma característica de células perivasculares *in vivo*. A condição tetraplóide de mMSCs cultivadas *in vitro* também levou a uma colaboração com grupo estrangeiro, a fim de estudar-se a influência da fusão de tipos celulares maduros com MSCs *in vitro*, uma vez que uma das hipóteses que poderiam explicar o surgimento de células tetraplóides multipotentes seria a fusão, em cultura, de células-tronco com células proliferativas indiferenciadas. (Anexos 2 e 3).

As conclusões do primeiro artigo, e também do segundo artigo, levaram a uma reflexão a respeito de qual poderia ser o papel das células perivasculares *in vivo*, além de sua função estrutural conhecida, uma vez que a abordagem utilizada no primeiro artigo não permite uma conclusão definitiva acerca do comportamento de células perivasculares como células-tronco *in vivo*. O terceiro artigo desta tese (Capítulo 5) sintetiza esta reflexão, ressaltando aspectos de MSCs e de pericitos que apontam para uma sobreposição de identidade entre ambos. O Capítulo 5 pode ser considerado, também, uma conseqüência direta do primeiro trabalho resultante desta tese, apresentado como Anexo 1.

Já o quarto artigo (Capítulo 6) demonstra que MSCs cultivadas podem assumir uma localização perivascular quando implantadas *in vivo*, utilizando-se um modelo experimental de osteogênese. Essa característica foi interpretada como mais uma evidência indicando a relação próxima entre MSCs definidas como tal *in vitro* e o espaço perivascular.

Tomados em conjunto, os artigos apresentados indicam que o nicho perivascular é, certamente, um objeto de estudo muito interessante para a obtenção de conhecimento básico sobre a MSC. Além disso, o panorama que se vislumbra quando se pensa na região perivascular como um nicho de células-tronco indica que não são MSCs que se encontram distribuídas por todo o organismo pós-natal, mas sim um conjunto heterogêneo de células-tronco associadas ao nicho perivascular e que, sob certas condições *in vitro*, podem assumir fenótipos compatíveis com aqueles atribuídos à progênie da MSC. Uma evidência que aponta nesta direção é o fato de que mMSCs derivadas de diferentes tecidos apresentam propriedades de diferenciação distintas (Capítulo 3). O conceito de heterogeneidade na tronquicidade das células perivasculares ao longo da vasculatura é também coerente face à heterogeneidade no compartimento endotelial (Garlanda e Dejana, 1997).

As semelhanças entre MSCs obtidas de diferentes órgãos (Capítulo 3), por sua vez, poderiam ser explicadas por suas origens embrionárias a partir de células derivadas da região dorsal da aorta no período embrionário (dia 9,5 em camundongos) chamadas mesoangioblastos (Minasi et al., 2002). Mesoangioblastos foram assim denominados por darem origem a tecidos mesodérmicos não-hematopoiéticos, em contraste com hemangioblastos, que são células derivadas da região ventral da aorta e que dão origem a células hematopoiéticas e endoteliais; ambos seriam descendentes de um angioblasto primitivo (Cossu e Bianco, 2003). Isso indica que células perivasculares, e por extensão MSCs, têm muito em comum com células endoteliais, embora sejam tipos celulares distintos. E, de fato, uma relação íntima entre células perivasculares e endoteliais, remanescente de interações entre ambas durante o desenvolvimento embrionário, persiste durante a vida adulta (Armulik et al., 2005).

O modelo proposto em que células perivasculares atuam como células-tronco nos diferentes tecidos parece, em um primeiro momento, contrário a outro modelo que propõe que células circulantes atuam como progenitoras para diferentes tecidos (Ratajczak et al.,

2004), exibido na Figura 1. A este modelo, somam-se evidências de que células circulantes possuem potencial de diferenciação semelhante ao da MSC *in vitro* (Kuznetsov et al., 2001; Kuwana et al., 2003) e também de que células da linhagem dos macrófagos enxertam-se em áreas de angiogênese ativa (Rajantie et al., 2004).

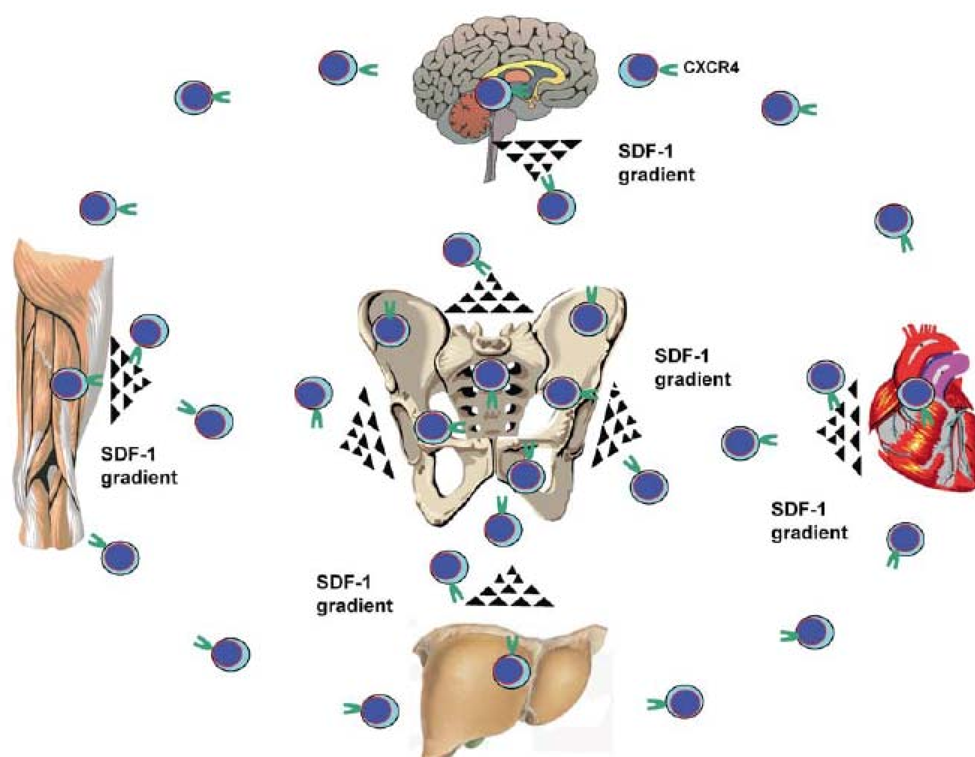

Figura 1 – Modelo de células tronco/progenitoras circulantes. Células originárias da medula óssea encontram-se na circulação, a partir da qual podem ser mobilizadas para diferentes tecidos através de gradientes quimiotáticos, em determinadas situações como, por exemplo, lesão tecidual. Retirado de Ratajczak et al. (2004).

Entretanto, dados de um estudo recente (Kajikawa et al., 2007) indicam que ambos modelos relatados no parágrafo anterior não são mutuamente excludentes, mas sim complementares. Naquele estudo, os autores utilizaram dois modelos para verificar a contribuição de células circulantes e de células locais para o processo de reparo de lesão no tendão patelar. Em um dos modelos (rato com células circulantes expressando GFP),

células circulantes foram encontradas no local da lesão 24 horas após a cirurgia, e a quantidade de células derivadas da circulação reduziu-se para níveis muito baixos após 7 dias. No outro modelo (enxerto de tendão patelar de rato GFP em rato não-transgênico), verificou-se que células locais tornaram-se proliferativas 3 dias após a lesão no tendão, e que o número destas aumentou grandemente após 7 dias, mantendo sua proliferação. Os autores concluíram que o reparo da lesão no tendão patelar ocorre em duas fases: primeiramente, células circulantes migram para o local da lesão; em um segundo momento, células locais passam a proliferar, e o número de células originárias da circulação é reduzido a níveis muito baixos.

No primeiro artigo desta tese (Capítulo 3), utilizou-se o cultivo de glomérulos renais de camundongo para a obtenção de células com características de MSC. Conclui-se que as culturas derivadas dos glomérulos eram originárias das células mesangiais, que são consideradas um tipo especializado de pericito (Schlondorff, 1987). Uma característica marcante do estabelecimento de culturas a partir dos glomérulos isolados é o fato de que as células aderentes derivadas dos glomérulos nunca foram observadas antes de 3 dias a partir do início do cultivo. Acrescenta-se a isto uma proliferação impressionante a partir de então. Em verdade, a transferência de glomérulos do organismo para condições artificiais *in vitro* poderia ser considerada uma indução de lesão tecidual no glomérulo, mas sem a presença de células circulantes.

A obtenção de uma resposta proliferativa de células glomerulares *in vitro* sem a presença de células circulantes não descarta a hipótese de que as células circulantes desempenham um papel importante neste processo *in vivo*. No entanto, essa informação, em conjunto com os dados que apontam para as células mesangiais como as que originam as culturas de células com características de MSC derivadas dos glomérulos, indicam que células perivasculares podem estar envolvidas no processo de reparo tecidual bifásico descrito por Kajikawa et al. (2007).

Assim sendo, considera-se que o modelo proposto no Capítulo 3 não se encontra em oposição com o modelo proposto por Ratajczak et al. (2004), mas sim em harmonia. Propõe-se, então, uma extensão do primeiro, através da incorporação do último (Figura 2).

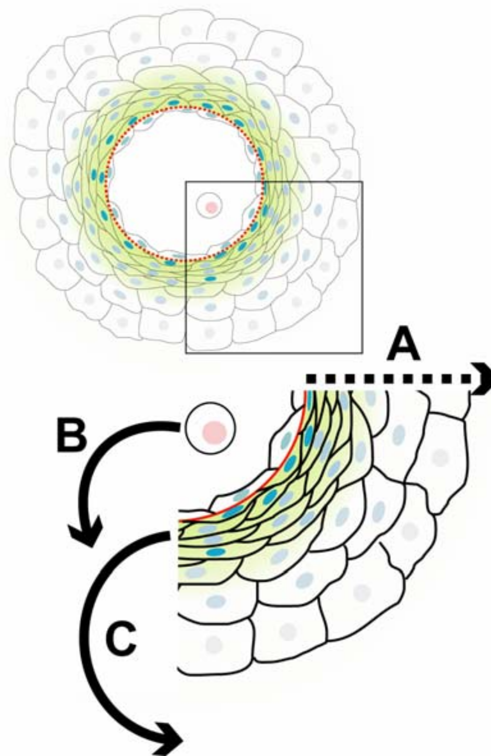

Figura 2 – Modelo proposto de contribuição de MSCs para manutenção tecidual. Nesta representação esquemática de uma secção transversal de um vaso simples, as MSCs situam-se sobre a membrana basal (linha vermelha), opostas às células endoteliais. Sinais dados pelo micro-ambiente tecido-específico coordenam uma transição gradual (representada por um gradiente de cor verde) de células indiferenciadas para fenótipos celulares progenitores e maduros. Esse processo pode ocorrer naturalmente conforme representado pela seta pontilhada (A). Em caso de lesão tecidual, e após a colonização da área da lesão por progenitores circulantes (B), MSCs indiferenciadas podem ser mobilizadas diretamente para dentro do tecido sem a transição por células progenitoras, conforme representado pela seta curva (C).

Capítulo 8

Referências bibliográficas

Alfonso ZZC, Forneck ED and Nardi NB (2000) Establishment of an adherent cell layer from human umbilical cord blood. Genet Mol Biol 23:519-522.

Armulik A, Abramsson A and Betsholtz C (2005) Endothelial/pericyte interactions. Circ Res 97:512-523.

Baddoo M, Hill K, Wilkinson R, Gaupp D, Hughes C, Kopen GC and Phinney DG (2003) Characterization of mesenchymal stem cells isolated from murine bone marrow by negative selection. J Cell Biochem 89:1235–1249.

Beyer Nardi N and da Silva Meirelles L (2006) Mesenchymal stem cells: isolation, in vitro expansion and characterization. Handb Exp Pharmacol. (174):249-282.

Bianchi G, Banfi A, Mastrogiacomo M, Notaro R, Luzzatto L, Cancedda R and Quarto R (2003) Ex vivo enrichment of mesenchymal cell progenitors by fibroblast growth factor 2. Exp Cell Res 287:98–105.

Bjornson CRR, Rietze RL, Reynolds BA, Magli MC and Vescovi AL (1999) Turning brain into blood: a hematopoietic fate adopted by adult neural stem cells in vivo. Science 283:534-537.

Breems DA, Blokland EAW, Siebel KE, Mayen AEM, Engels LJA and Ploemacher RE (1998) Stroma-contact prevents loss of hematopoietic stem cell quality during ex vivo expansion of CD34+ mobilized peripheral blood stem cells. Blood 91:111-117.

Bruder SP, Fink DJ and Caplan AI (1994) Mesenchymal stem cells in bone development, bone repair, and skeletal regeneration therapy. J Cell Biochem 56:283-294.

Campagnoli C, Roberts IAG, Kumar S, Bennett PR, Bellantuono I and Fisk NM (2001) Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow. Blood 98:2396-2402.

Caplan AI (1991) Mesenchymal stem cells. J Orthop Res 9:641-650.

Caplan AI and Bruder SP (2001) Mesenchymal stem cells: building blocks for molecular medicine in the 21st century. Trends Mol Med 7:259-264.

Caplan AI and Dennis JE (2006) Mesenchymal stem cells as trophic mediators. J Cell Biochem. 98:1076-1084.

Choi KS, Shin JS, Lee JJ, Kim YS, Kim SB and Kim CW (2004) In vitro trans-differentiation of rat mesenchymal cells into insulin-producing cells by rat pancreatic extract. Biochem Biophys Res Commun. 330:1299-1305.

Cossu G and Bianco P (2003) Mesoangioblasts - vascular progenitors for extravascular mesodermal tissues. Curr Opin Genet Dev 13:537-542.

de Bari C, Dell'Accio F, Vandenabeele F, Vermeesch JR, Raymackers J-M and Luyten FP (2003) Skeletal muscle repair by adult human mesenchymal stem cells from synovial membrane. J Cell Biol 160:909-918.

Deans RJ and Moseley AB (2000) Mesenchymal stem cells: biology and potential clinical uses. Exp Hematol 28:875-884.

Dexter TM (1982) Stromal cell associated haemopoiesis. J Cell Physyol Suppl 1:87-94.

Elenkov IJ, Wilder RL, Chrousos GP and Vizi ES (2000) The sympathetic nerve - an integrative interface between two supersystems: the brain and the immune system. Pharmacol Rev 52:595-638.

Friedenstein AJ, Gorskaja UF and Julagina NN (1976) Fibroblast precursors in normal and irradiated mouse hematopoietic organs. Exp Hematol 4:267-274.

Gage FH (2000) Mammalian Neural Stem Cells. Science 287:1433-1438.

Garlanda C and Dejana E (1997) Heterogeneity of endothelial cells. Specific markers. Arterioscler Thromb Vasc Biol. 17:1193-1202.

Haynesworth SE, Goshima J, Goldberg VM and Caplan AI (1992) Characterization of cells with osteogenic potential from human marrow. Bone. 13:81-88.

Horwitz EM, Prockop DJ, Fitzpatrick LA, Koo WWK, Gordon PL, Neel M, Sussman M, Orchard P, Marx JC, Pyeritz RE and Brenner MK (1999) Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. Nat Med 5:309-313.

Horwitz EM, Prockop DJ, Gordon PL, Koo WWK, Fitzpatrick LA, Neel MD, McCarville ME, Orchard PJ, Pyeritz RE and Brenner MK (2001) Clinical responses to bone marrow transplantation in children with severe osteogenesis imperfecta. Blood 97:1227-1231.

Houghton J, Stoicov C, Nomura S, Rogers AB, Carlson J, Li H, Cai X, Fox JG, Goldenring JR and Wang TC (2004) Gastric cancer originating from bone marrow-derived cells. Science. 306:1568-1571.

Hu Y, Liao L, Wang Q, Ma L, Ma G, Jiang X, Zhao RC (2003) Isolation and identification of mesenchymal stem cells from human fetal pancreas. J Lab Clin Med 141:342-349.

in ‘t Anker PS, Scherjon SA, van der Keur CK, Noort WA, Claas FHJ, Willemze R, Fibbe WE and Kanhai HHH (2003) Amniotic fluid as a novel source of mesenchymal stem cells for therapeutic transplantation. Blood 102:1548-1549 (carta ao editor).

Kajikawa Y, Morihara T, Watanabe N, Sakamoto H, Matsuda K, Kobayashi M, Oshima Y, Yoshida A, Kawata M and Kubo T (2007) GFP chimeric models exhibited a biphasic pattern of mesenchymal cell invasion in tendon healing. J Cell Physiol. 210:684-691.

Kopen GC, Prockop DJ and Phinney DG (1999) Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. Proc Natl Acad Sci U S A 96:10711–10716.

Kuwana M, Okazaki Y, Kodama H, Izumi K, Yasuoka H, Ogawa Y, Kawakami Y and Ikeda Y (2003) Human circulating CD14+ monocytes as a source of progenitors that exhibit mesenchymal cell differentiation. J Leukoc Biol 74:833-845.

Kuznetsov SA, Mankani MH, Gronthos S, Satomura K, Bianco P and Robey PG (2001) Circulating skeletal stem cells. J Cell Biol. 153:1133-1140.

Lange C, Bassler P, Lioznov MV, Bruns H, Kluth D, Zander AR and Fiegel HC (2005) Liver-specific gene expression in mesenchymal stem cells is induced by liver cells. World J Gastroenterol. 11:4497-4504.

Le Blanc K, Rasmusson I, Sundberg B, Gotherstrom C, Hassan M, Uzunel M and Ringden O (2004) Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. Lancet. 363:1439-1441.

Long, MW (2001) Osteogenesis and bone-marrow-derived cells. Blood Cells Mol Dis 27:677–690.

Mackay AM, Beck SC, Murphy JM, Barry FP, Chichester CO and Pittenger MF (1998) Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. Tissue Eng 4:415-428.

Majumdar MK, Thiede MA, Mosca JD, Moorman M and Gerson SL (1998) Phenotypic and functional comparison of cultures of marrow-derived mesenchymal stem cells (MSCs) and stromal cells. J Cell Physiol 176:57-66.

Makino S, Fukuda K, Miyoshi S, Konishi F, Kodama H, Pan J, Sano M, Takahashi T, Hori S, Abe H, Hata J-i, Umezawa A and Ogawa S (1999) Cardiomyocytes can be generated from marrow stromal cells in vitro. J Clin Invest 103:697–705.

McKay R (1997) Stem cells in the central nervous system. Science 276:66-71.

Meirelles Lda S and Nardi NB (2003) Murine marrow-derived mesenchymal stem cell: isolation, in vitro expansion, and characterization. Br J Haematol 123:702-711.

Mihara K, Imai C, Coustan-Smith E, Dome JS, Dominici M, Vanin E and Campana D (2003) Development and functional characterization of human bone marrow mesenchymal cells immortalized by enforced expression of telomerase Br J Haematol 120:846 - 849.

Minasi MG, Riminucci M, De Angelis L, Borello U, Berarducci B, Innocenzi A, Caprioli A, Sirabella D, Baiocchi M, De Maria R, Boratto R, Jaffredo T, Broccoli V, Bianco P and Cossu G (2002) The meso-angioblast: a multipotent, self-renewing cell that originates from the dorsal aorta and differentiates into most mesodermal tissues. Development 129:2773-2783.

Morrison SJ, Shah NM and Anderson DJ (1997) Regulatory mechanisms in stem cell biology. Cell 88:287-298.

Muraglia A, Cancedda R and Quarto R (2000) Clonal mesenchymal progenitors from human bone marrow differentiate in vitro according to a hierarchical model. J Cell Sci 113:1161-1166.

Nardi NB and Alfonso ZZC (1999) The hematopoietic stroma. Braz J Med Biol Res 32:601-609.

Odorico JS, Kaufman DS and Thomson JA (2001) Multilineage differentiation from human embryonic stem cell lines. Stem cells 19:193-204.

Okamoto T, Aoyama T, Nakayama T, Nakamata T, Hosaka T, Nishijo K, Nakamura T, Kiyono T and Toguchida J (2002) Clonal heterogeneity in differentiation potential of immortalized human mesenchymal stem cells. Biochem Biophys Res Commun 295:354–361.

Owen M (1985) Lineage of osteogenic cells and their relationship to the stromal system. In: Peck WA (ed) Bone and mineral research. Elsevier, New York NY, pp 1-25.

Phinney DG (2002) Building a consensus regarding the nature and origin of mesenchymal stem cells. J Cell Biochem Suppl 38:7-12.

Phinney DG, Kopen G, Isaacson RL and Prockop DJ (1999) Plastic adherent stromal cells from the bone marrow of commonly used strains of inbred mice: variations in yield, growth, and differentiation. J Cell Biochem 72:570-585.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S and Marshak DR (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284:143-147.

Prockop DJ (1997) Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 276:71-74.

Rajantie I, Ilmonen M, Alminaite A, Ozerdem U, Alitalo K and Salven P (2004) Adult bone marrow-derived cells recruited during angiogenesis comprise precursors for periendothelial vascular mural cells. Blood. 104:2084-2086.

Ratajczak MZ, Kucia M, Reca R, Majka M, Janowska-Wieczorek A and Ratajczak J (2004) Stem cell plasticity revisited: CXCR4-positive cells expressing mRNA for early muscle, liver and neural cells 'hide out' in the bone marrow. Leukemia. 18:29-40.

Safford KM, Hicok KC, Safford SD, Halvorsen Y-DC, Wilkison WO, Gimble JM and Rice HE (2002) Neurogenic differentiation of murine and human adipose-derived stromal cells. Biochem Biophys Res Commun 294:371-379.

Salingcarnboriboon R, Yoshitake H, Tsuji K, Obinata M, Amagasa T, Nifuji A and Noda M (2003) Establishment of tendon-derived cell lines exhibiting pluripotent mesenchymal stem cell-like property. Exp Cell Res 287:289-300.

Sato Y, Araki H, Kato J, Nakamura K, Kawano Y, Kobune M, Sato T, Miyanishi K, Takayama T, Takahashi M, Takimoto R, Iyama S, Matsunaga T, Ohtani S, Matsuura A, Hamada H and Niitsu Y (2005) Human mesenchymal stem cells xenografted directly to rat liver are differentiated into human hepatocytes without fusion. Blood. 15;106:756-763.

Schlondorff D (1987) The glomerular mesangial cell: an expanding role for a specialized pericyte. FASEB J. 1987 1:272-281.

Sekiya I, Larson BL, Smith JR, Pochampally R, Cui J-g and Prockop DJ (2002) Expansion of human adult stem cells from bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality. Stem cells 20:530-541.

Seshi B, Kumar S and Sellers D (2000) Human bone marrow stromal cell: coexpression of markers specific for multiple mesenchymal cell lineages. Blood Cells Mol Dis 26:234–246.

Slack JMW (2000) Stem cells in epithelial tissues. Science 287:1431-1433.

van der Kooy D and Weiss S (2000) Why stem cells? Science 287:1439-1441.

Verfaillie CM (1992) Direct contact between human primitive hematopoietic progenitors and bone marrow stroma is not required for long-term in vitro hematopoiesis. Blood 79:2821-2826.

Verfaillie, CM (2002) Adult stem cells: assessing the case for pluripotency. Trends Cell Biol 12:502-508.

Wakitani S, Saito T and Caplan AI (1995) Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine. Muscle Nerve 18:1417-1426.

Watt FM and Hogan BLM (2000) Out of eden: stem cells and their niches. Science 287:1427-1430.

Weissman IL (2000) Translating stem and progenitor cell biology to the clinic: barriers and opportunities. Science 287:1442-1446.

Wexler SA, Donaldson C, Denning-Kendall P, Rice C, Bradley B and Hows JM (2003) Adult bone marrow is a rich source of human mesenchymal 'stem' cells but umbilical cord and mobilized adult blood are not. Br J Haematol 121:368-74.

Whetton AD and Grahan GJ (1999) Homing and mobilization in the stem cell niche. Trends Cell Biol 9:233-238.

Wieczorek G, Steinhoff C, Schulz R, Sheller M, Vingron M, Ropers H-H and Nuber UA (2003) Gene expression profile of mouse marrow stromal cells determined by cDNA microarray analysis. Cell Tissue Res 311:227-237.

Woodbury D, Schwarz EJ, Prockop DJ and Black IB (2000) Adult rat and human bone marrow stromal cells differentiate into neurons. J Neurosci Res 61:364-370.

Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P and Hedrick MH (2002) Human adipose tissue is a source of multipotent stem cells. Mol Bio Cell 13:4279-4295.

Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz P and Hedrick MH (2001). Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 7:211-228.

Zvaifler NJ, Marinova-Mutafchieva L, Adams G, Edwards CJ, Moss J, Burger JA and Maini RN (2000) Mesenchymal precursor cells in the blood of normal individuals. Arthritis Res 2:477–488.

Capítulo 9

Anexos

Esta seção contém produção bibliográfica em que houve participação do autor durante o período de seu Doutorado, e que estão relacionados com o tema desta tese.

1. *Mesenchymal stem cells: isolation, in vitro expansion and characterization* é uma revisão publicada no *Handbook of Experimental Pharmacology*, abordando aspectos gerais do isolamento, cultivo e caracterização de MSCs. Esta foi baseada no projeto de Doutorado apresentado pelo autor desta tese

2. *Functional characterization of cell hybrids generated by induced fusion of primary porcine mesenchymal stem cells with an immortal murine cell line* é resultante da interação com grupo sueco que tinha como objetivo investigar os efeitos da fusão de células-tronco mesenquimais porcinas com fibroblastos de camundongo sobre o potencial de diferenciação dos híbridos resultantes. Cultivo, diferenciação e caracterização de marcadores de superfície foram realizados pelo autor desta tese no Brasil.

3. *Polyethylene glycol-mediated fusion between primary mouse mesenchymal stem cells and mouse fibroblasts generates hybrid cells with increased proliferation and altered differentiation* também é resultante da interação com o grupo sueco mencionado anteriormente, e analisou as conseqüências da fusão de células-tronco mesenquimais murinas, isoladas e caracterizadas em nosso laboratório, com fibroblastos de camundongo, em termos de potencial de diferenciação e marcadores de superfície. Cultivo, diferenciação e caracterização de marcadores de superfície foram realizados pelo autor desta tese no Brasil.

Mesenchymal stem cells: isolation, in vitro expansion and characterization.

Nance Beyer Nardi & Lindolfo da Silva Meirelles

Handb Exp Pharmacol. 2006;(174):249-82.

HEP (2006) 174:249–282
© Springer-Verlag Berlin Heidelberg 2006

Mesenchymal Stem Cells: Isolation, In Vitro Expansion and Characterization

N. Beyer Nardi (✉) · L. da Silva Meirelles

Genetics Department, Universidade Federal do Rio Grande do Sul, Av Bento Gonçalves 9500, Porto Alegre RS, CEP 91540–000, Brazil
nardi@ufrgs.br

1 Introduction . . . 250

2 The Identity of the Mesenchymal Stem Cell . . . 251
2.1 The Colony-Forming Unit-Fibroblast . . . 251
2.2 The Bone Marrow Stroma . . . 252
2.3 The Mesenchymal Stem Cell . . . 253
2.4 Other Cell Populations Related to the Mesenchymal Stem Cell . . . 254

3 Distribution of the Mesenchymal Stem Cell . . . 255

4 Isolation and Culture of Mesenchymal Stem Cells . . . 256

5 Homing and Engraftment of Transplanted Mesenchymal Stem Cells . . . 259

6 Characterization of Mesenchymal Stem Cells . . . 260

7 Differentiation of Mesenchymal Stem Cells . . . 262

8 Applications of Mesenchymal Stem Cells in Cell and Gene Therapy . . . 263
8.1 Study of Cancer Biology . . . 264
8.2 Cell Therapy . . . 264
8.2.1 Fibrosis . . . 265
8.2.2 Cardiovasculogenesis . . . 265
8.2.3 Arteriogenic Effects . . . 266
8.2.4 Immunosuppressive Effects . . . 267
8.3 Mesenchymal Stem Cells and Tissue Engineering . . . 267
8.4 Genetic Therapy . . . 268
8.4.1 Correction of Genetic Disorders . . . 269
8.4.2 Cancer Suppression . . . 270

9 Pharmacologic Aspects of Mesenchymal Stem Cell Biology . . . 271

10 Conclusions . . . 272

References . . . 274

Abstract Mesenchymal stem cells (MSC), one type of adult stem cell, are easy to isolate, culture, and manipulate in ex vivo culture. These cells have great plasticity and the potential for therapeutic applications, but their properties are poorly understood. MSCs can be found in bone marrow and in many other tissues, and these cells are generally identified through a combination of poorly defined physical, phenotypic, and functional properties; consequently, multiple names have been given to these cell populations. Murine MSCs have been directly applied to a wide range of murine models of diseases, where they can act as therapeutic agents per se, or as vehicles for the delivery of therapeutic genes. In addition to their systemic engraftment capabilities, MSCs show great potential for the replacement of damaged tissues such as bone, cartilage, tendon, and ligament. Their pharmacological importance is related to four points: MSCs secrete biologically important molecules, express specific receptors, can be genetically manipulated, and are susceptible to molecules that modify their natural behavior. Due to their low frequency and the lack of knowledge on cell surface markers and their location of origin, most information concerning MSCs is derived from in vitro studies. The search for the identity of the mesenchymal stem cell has depended mainly on three culture systems: the CFU-F assay, the analysis of bone marrow stroma, and the cultivation of mesenchymal stem cell lines. Other cell populations, more or less related to the MSC, have also been described. Isolation and culture conditions used to expand these cells rely on the ability of MSCs, although variable, to adhere to plastic surfaces. Whether these conditions selectively favor the expansion of different bone marrow precursors or cause similar cell populations to acquire different phenotypes is not clear. The cell populations could also represent different points of a hierarchy or a continuum of differentiation. These issues reinforce the urgent need for a more comprehensive view of the mesenchymal stem cell identity and characteristics.

Keywords Mesenchymal stem cell · Bone marrow stroma · Differentiation · Stem cell niche · Cell therapy · Genetic therapy

1 Introduction

Stem cells present in early embryonic stages are pluripotent and can generate all of the cell types found in adult organisms, whereas, adult stem cells exhibit a continuum of plasticity or multipotency. In adult humans, the first and one of the best-known stem cells to be described is the hematopoietic stem cell (HSC). A great variety of other stem/precursor cell types have also been described, but much less is known about their origin and maintenance in vivo as organ-specific stem cell pools (Nardi 2005).

The mesenchymal stem cell (MSC) is one of the most interesting of the adult stem cell types. These cells are easily isolated, cultured, and manipulated ex vivo. MSCs exhibit great plasticity and harbor the potential for therapeutic applications, but these cells are poorly defined. This has led to a heterogeneity of names and phenotypes ascribed by different groups to this cell population. MSCs are present in the bone marrow and in many other tissues, and these cells are presently identified through a combination of poorly defined physical, phenotypic, and functional properties. A number of recent reviews have adequately described the nature of MSCs (Short et al. 2003; Zipori 2004; Barry

and Murphy 2004; Kassem et al. 2004; Baksh et al. 2004; Javazon et al. 2004), and the focus of this review will be to describe experimental approaches for their isolation, in vitro expansion, and characterization. We will also discuss the cellular therapeutic potentials of MSCs and place a special emphasis on the pharmacological prospects of these cells in vitro and in vivo.

2 The Identity of the Mesenchymal Stem Cell

For most cells present in adult organisms, mitosis is accompanied by differentiation. Stem cells are defined as those cells with the ability to proliferate without differentiating. At the moment, the very existence of a mesenchymal stem cell in vivo is not completely understood, since it is based on indirect evidence derived mainly from the in vitro cultivation of bone marrow and other tissues. This is true, but only to a point, because most types of adult stem cells can only be identified after isolation, which can then be examined through in vitro or in vivo assays to determine if they have the two main characteristics of stem cells: the ability to proliferate and to differentiate into mature cell types. Most of the information for MSCs, which are present at a low frequency, are derived from in vitro studies, due to a lack of information with respect to specific surface markers and their location in vivo. In vitro studies, by their very nature, may, however, introduce experimental artifacts (Javazon et al. 2004). This possibility is clearly described in several studies, including one reported by Rombouts and Ploemacher (2003), who compared the homing abilities of primary and culture-expanded MSCs in a syngeneic mouse model. Uncultured MSCs demonstrated highly efficient homing to bone marrow, but the infusion of immortalized multipotent syngeneic stromal cells, or even primary MSCs that had been cultured for only 24 h, were rarely if ever seen in the lymphohematopoietic organs. Murine MSCs were also reported to have a deficient capacity to home to bone marrow by Anjos-Afonso et al. (2004).

The identification of the mesenchymal stem cell has thus far depended on in vitro culture systems, which have provided very heterogeneous information and made the characterization of MSCs even more difficult. Three in vitro systems are generally employed to examine these cells: the CFU-F assay, the analysis of the bone marrow stroma, and the cultivation of mesenchymal stem cell lines. Other cell populations, more or less related to the MSC, have also been described.

2.1 The Colony-Forming Unit-Fibroblast

The first direct evidence that nonhematopoietic, mesenchymal precursor cells were present in the bone marrow originated from the work conducted in

Moscow during the 1960s and 1970s of Friedenstein and colleagues (reviewed in Phinney 2002). These pioneering experiments involved the incubation of bone marrow samples in tissue culture flasks. The presence of an adherent fraction could be seen within a few days, which proved highly heterogeneous. Around the 3rd–5th days, individual foci of two to four fibroblasts were observed among the histiocytes and mononuclear cells, which could differentiate into cells that could form small deposits of bone or cartilage (Friedenstein et al. 1976). These cells were termed colony forming unit-fibroblasts or CFU-F.

During the 1980s, several studies showed that cells isolated by the Friedenstein method were multipotent and could differentiate into osteoblasts, chondroblasts, adipocytes, and even myoblasts (reviewed in Prockop 1997). The frequency of CFU-F in bone marrow suspensions is very different among species, and the results are influenced by the culture conditions (reviewed in Short et al. 2003). Growth factors stimulating the proliferation of CFU-F include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), basic fibroblast growth factor, transforming growth factor-β, and insulin-like growth factor-1 (Gronthos and Simmons 1995; Kuznetsov et al. 1997a; Baddoo et al. 2003; Bianchi et al. 2003). In contrast, cytokines such as interleukin 4 (IL-4) and interferon-alpha can inhibit the establishment of CFU-F (Wang et al. 1990; Gronthos and Simmons 1995). The formation of CFU-F has been considered indicative of mesenchymal stem cells, but a direct relationship between the two has not been clearly established, probably because of the great heterogeneity in morphology, cell size and differentiation potential observed among species and between colonies (Javazon et al. 2004).

2.2 The Bone Marrow Stroma

Stromal cells, along with extracellular matrix (ECM) components and soluble regulatory factors, have until recently been thought of as secondary components of a microenvironment required for sustained hematopoiesis (Nardi and Alfonso 1999). The stroma, studied both in vitro and in vivo, is composed of a very heterogeneous population of cells, which includes macrophages, fibroblasts, adipocytes, and endothelial cells (Dexter et al. 1976; Ogawa 1993). Adventitial reticular cells branch through the medullary cavity and provide a reticular network that supports hematopoietic cells. Marrow adipocytes control hematopoietic volume, such that impaired hematopoiesis is associated with increased accumulation of fat inclusions, and accelerated hematopoiesis is associated with loss of fat vacuoles. These processes determine the space available for hematopoietic cells (Tavassoli 1984). Adipocytes may also act as a reservoir for lipids needed during proliferation. Macrophages are important in the clean-up of ineffective erythropoiesis and in the removal of the nuclear pole that is produced during this process.

Stromal cells produce ECM components and both soluble and membrane-associated growth factors to form a dynamic structure that plays an active role in hematopoiesis, i.e., the hematopoietic stem cell niche. Matrix proteins in this microenvironment include fibronectin, collagen, vitronectin, and tenascin, and some of the most relevant soluble factors include stem cell factor (SCF), granulocyte-macrophage colony-stimulating factor (GM-CSF) and the granulocyte colony-stimulating factor (G-CSF). Representative adhesion molecules include members of the integrin superfamily (VLA-1, VLA-2, VLA-3, VLA-4, VLA-5, VLA-6) (reviewed in Whetton and Grahan 1999). Physical contact among the stromal cells is important for the regulation of this microenvironment. Although it has been shown that contact is not fundamental for the hematopoietic process to occur (Verfaillie 1992), it seems to be related to the quality of the hematopoietic cells produced (Breems et al. 1998). The stromal compartment has implications for human health, since abnormalities in the stromal compartment may represent a possible mechanism implicated in aplastic anemia (Kojima 1998) and in the abnormal behavior of Ph^+ cells in chronic myeloid leukemia (Cordero et al. 2004).

Based on the well-established generation of multiple types of mesenchymal cell from bone marrow, stromal stem cells were additionally proposed to exist by Owen (1985). Analogous to the hematopoietic system, they proposed that stromal stem cells reside in the bone marrow in their own niche, where the cells were able to self-renew and generate mature conjunctive/stromal cell types. The identity of this stem cell—which is now almost universally termed mesenchymal stem cell—is still, as stated above, poorly understood.

2.3 The Mesenchymal Stem Cell

The lack of consensus about the proper nomenclature needed to describe these cells has resulted in an incorrect, but synonymous use of the terms “marrow stromal cell” and “mesenchymal stem cell.” Actually, stromal cells encompass all cells present in the bone marrow that are not part of the hematopoietic system. MSCs, on the other hand, correspond to that rare cell population that can form other MSCs and generate mature cells of mesenchymal tissues. Protocols involving the isolation of bone marrow cells based on their adherence to plastic surfaces result in the immediate establishment of stromal cell cultures, and not of MSC cultures. A more adequate term for the large number of cell types with the potential to differentiate into mesenchymal tissues would be “mesenchymal progenitor cell” (MPC), which would include cell types from a hierarchy immediately above the pluripotent MSC but intermediate to that represented by mature mesenchymal cell types.

Another point of debate is the fact that the HSC is itself of mesodermic origin, hence a type of MSC. For this reason, some authors prefer the term “nonhematopoietic mesenchymal stem cell.” The fact that these cells, which are

described below, may have alternative differentiation pathways that go beyond the normal limits of mesoderm and ectoderm formation renders the term "mesenchymal" inadequate. Probably, the best nomenclature to define this cell type would be "adult nonhematopoietic stem cell," followed by "plastic-adherent, bone-marrow derived stem cells". All these concepts, however, are already included when the term "mesenchymal stem cell" is used, and there is a tendency to accept this terminology, even though it is inadequate.

2.4
Other Cell Populations Related to the Mesenchymal Stem Cell

In addition to the heterogeneity, which characterizes MSC cultures established from various species or in different laboratories, some groups have described cell populations that are very similar to MSCs, but which have a different nomenclature. Bone marrow stromal (stem) cells (BMSSCs), stromal precursor cells (SPCs), and recycling stem cells (RS-1, RS-2) are some of these variations (Baksh et al. 2004 and references therein). More recently, D'Ippolito et al. (2004) described the marrow-isolated adult multilineage inducible (MIAMI) cells which, although isolated from humans, can proliferate extensively without showing signs of senescence or loss of differentiation potential (which, as described below, is not usual for human cells). These cells may represent a more primitive subset of bone marrow stem cells. Higher proliferative and differentiation potential has also been reported for the multipotential adult progenitor cell (MAPC) described by Catherine Verfaillie's group (Reyes et al. 2001; Reyes and Verfaillie 2001). Young et al. (2001) described human reserve pluripotent mesenchymal stem cells, present in the connective tissues of skeletal muscle and dermis.

Isolation and culture conditions used by the different groups are also variable, and probably represent the main factor responsible for the phenotype and function of the resulting cell populations. Whether these conditions selectively favor the expansion of different bone marrow precursors or cause similar cell populations to acquire different phenotypes is not clear. The cell populations could also represent different points in a hierarchy (Caplan 1994), and some studies suggest that this second alternative might be more realistic. Lodie et al. (2002), for instance, systematically compared different protocols used to isolate/expand human bone marrow adherent cells and concluded that the cell populations isolated by these various techniques are virtually indistinguishable. We have recently observed that the maintenance of MSC lines generated according to established protocols (Meirelles and Nardi 2003) but grown under MAPC conditions for 4 weeks induced changes in the immunophenotypic profile of the cells to become more similar to that of MAPCs (N. Nardi and L. Meirelles, unpublished results). In either case and as reported for hematopoietic stem cells (Pranke et al. 2001), these results demonstrate that the mesenchymal stem cell compartment is heterogeneous and that cultivation

conditions can alter some of their basic properties. These points reinforce the urgent need for a more comprehensive view of the mesenchymal stem cell identity and its characteristics.

3
Distribution of the Mesenchymal Stem Cell

Although very poorly understood, the interaction of MSCs with their niche is as essential for their existence and function as it is for any other of the adult stem cells (Watt and Hogan 2000; Fuchs et al. 2004). The primary source of MSCs in adult individuals is the bone marrow, where they are immersed in the stroma (Pittenger et al. 1999). They are present at a low frequency in bone marrow, and recent studies employing the CFU-F assay suggest that in humans there is one MSC per 34,000 nucleated cells (Wexler et al. 2003). In mice, the frequency was estimated to be one for 11,300–27,000 nucleated cells (Meirelles and Nardi 2003). Once again, the heterogeneity of this microenvironment hampers the unraveling of its components and their relationship, and basic questions remain unanswered. What is the niche for the MSC? Do hematopoietic and mesenchymal stem cells share the same niche and exchange signals to drive proliferation and differentiation?

MSCs have been found in several other tissues and in ontogeny (Table 1). In mice, they were isolated from the brain, thymus, liver, spleen, kidney, muscle, and lungs of adult mice (L. Meirelles and N. Nardi, unpublished results), and other MSC-related populations such as MAPCs have been observed in different organs as well (Jiang et al. 2002). This distribution could be explained by different scenarios: (a) adult tissues contain independent reservoirs of similar

Table 1 Distribution of MSCs in different organs/tissues and ontogeny stages

Site	Species	Ontogeny stage	Reference
Adipose tissue	Human	Post-natal	Zuk et al. 2001, 2002
Adipose tissue	Mouse	Post-natal	Safford et al. 2002
Pancreas	Human	Fetal	Hu et al. 2003
Bone marrow	Human	Fetal	Campagnoli et al. 2001
Liver	Human	Fetal	Campagnoli et al. 2001
Blood	Human	Fetal	Campagnoli et al. 2001
Tendon	Mouse	Postnatal	Salingcarnboriboon et al. 2003
Synovial membrane	Mouse	Postnatal	de Bari et al. 2003
Amniotic liquid	Human	Fetal	in't Anker et al. 2003
Peripheral blood	Human	Postnatal	Zvaifler et al. 2000; Kuwana et al. 2003
Umbilical cord blood	Human	Fetal/postnatal	Alfonso et al. 2000

stem cells, whose characteristic traits are determined by signals released by each niche; (b) MSCs exist as a reservoir in one specific location, from which they circulate through the organism to colonize different tissues/organs; or (c) MSCs originate from cell populations belonging to blood vessels, and are, as a consequence, present through the whole organism. Since Bianco and Cossu (1999) suggested that MSCs originate from marrow pericytes, this third possibility has received experimental support (reviewed in Short et al. 2003). The issue is, however, still unclear.

4
Isolation and Culture of Mesenchymal Stem Cells

Few adult stem cell populations can be unequivocally identified, and isolation of these cells requires in vitro or in vivo experimentation and characterization based on immunophenotypic or functional traits. Hematopoietic stem cells, for instance, can be enriched through the selection of cells expressing surface markers such as CD34 in humans and Sca-1 in mice, or by their ability to exclude the DNA-binding dye Hoechst 33342 (Goodell et al. 1996). Mesenchymal stem cells lack clearly defined surface markers, so that the most widely used approach to isolate them relies on their ability to adhere to plastic surfaces (Wakitani et al. 1995; MacKay et al. 1998; Makino et al. 1999; Muraglia et al. 2000).

For the selective isolation of bone marrow MSCs, total cells are washed, counted, resuspended in culture medium, and plated in six-well tissue culture dishes at approximately 1.94×10^6 cells/cm^2. Nonadherent cells are removed 24–72 h later by changing the medium. After 1 week, a heterogeneous culture develops, which is generally referred to as bone marrow stroma (Fig. 1a). Maintenance of the culture with a twice-weekly medium change and removal of nonadherent cells results, after 2 or 3 weeks, in a relatively homogeneous culture of morphologically and immunophenotypically similar mesenchymal stem cells (Fig. 1b,c). Our experience shows that the identification of MSCs depends on the availability of a good inverted microscope with phase contrast, since they are difficult to visualize otherwise.

Cultures can be maintained for variable periods, depending on the species and organ of origin, by passaging and subculturing the adherent cells which are detached by trypsinization. Although Dulbecco's modified Eagle's medium (DMEM) is frequently employed for the culture of MSCs, other media have also been shown to be appropriate (reviewed by Otto and Rao 2004). The presence of HEPES buffer is also important in our experience.

In an attempt to further enrich the frequency of MSCs in the initial cell population, other methods have been developed, such as the immunodepletion of hematopoietic contaminants identified, for instance, by the molecules CD34, CD45, and CD11b (Kopen et al. 1999; Badoo et al. 2003; Ortiz et al. 2003).

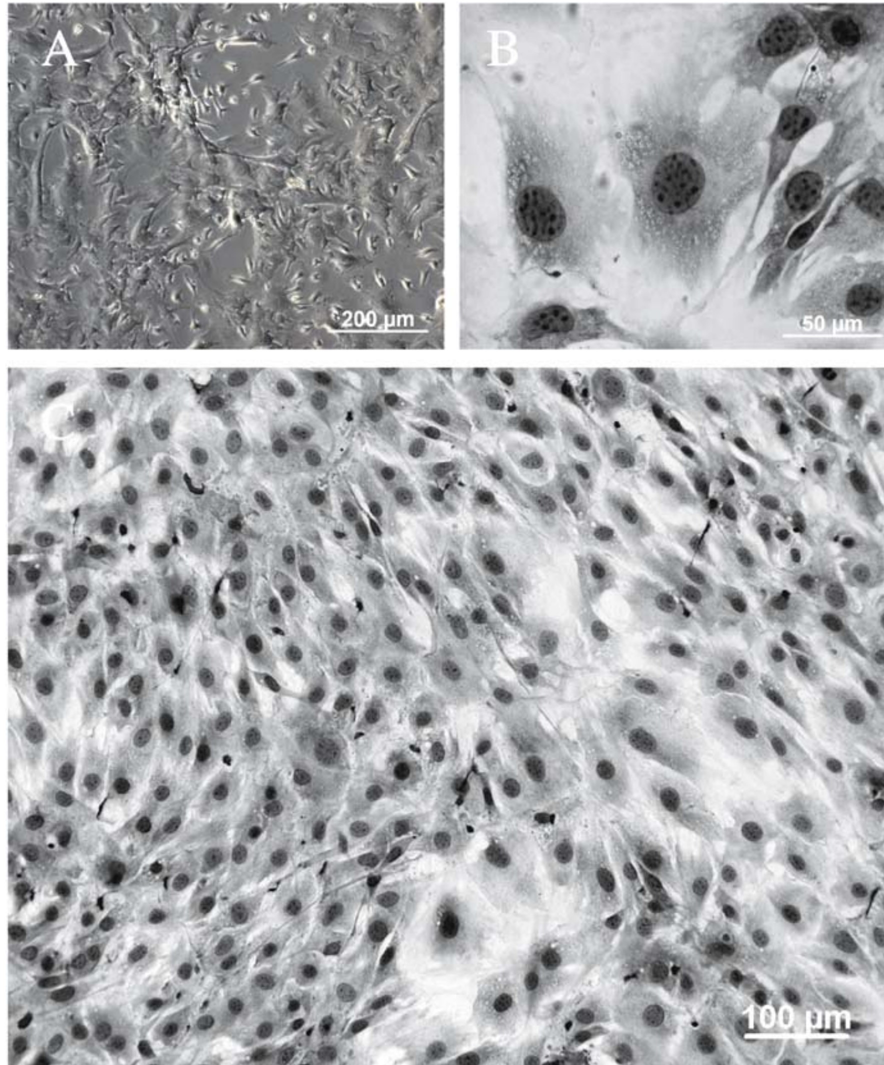

Fig. 1 a BALB/c bone marrow cells cultured for 1 week in DMEM with 10% FCS generate heterogeneous cell populations, referred to as bone marrow stromal cells (×100). Maintenance of the adherent cell population results in a homogeneous culture of mesenchymal stem cells of a flat-type morphology (**b**, ×400; **c**, ×100)

Other techniques involved cell size-based enrichment, involving the filtration of bone marrow cells through a 3-μm seive (Hung et al. 2002; Tuli et al. 2003), or changing plating densities (Colter et al. 2000; Sekiya et al. 2002). Since none of these approaches results in the establishment of homogeneous cell cultures, the development of efficient—and particularly reproducible—methods for the

isolation and expansion of MSCs remains an important goal of this research field. It is possible that only when the true origin and nature of MSCs is better understood will we be able to confidently work with them in vitro.

Following removal of nonadherent cells 1–4 days after the establishment of the culture, cells are maintained with periodic passages until a relatively homogeneous population is established. Culture media may vary, but the most frequently used are Dulbecco's modified Eagle's medium (DMEM) and α-minimum essential medium (reviewed in Otto and Rao 2004). The batch of fetal calf serum employed to cultivate these cells may introduce phenotypic variations, which show that unknown factors influence the selection and expansion of these cells. The addition of specific growth factors is also important in defining the final characteristics of MSC cultures, and these are probably the main reasons for the heterogeneity observed in the mesenchymal stem cell types described in the literature. The growth of murine MAPCs, for instance, depends on the supplementation of leukemia inhibitory factor (LIF) and the use of fibronectin-coated surfaces (Jiang et al. 2002). The persistence of hematopoietic contaminants, shown by the presence of $CD45^+$ and $CD11b^+$ cells in the cultures, has also been reported (Phinney et al. 1999).

Ideal culture conditions would maintain mesenchymal stem cells with (a) phenotypic and functional characteristics similar to those exhibited in their original niche, (b) indefinite proliferation, and (c) a capacity to differentiate into multiple lineages. Since the in situ characteristics of MSCs are not known, efforts have concentrated in the last two objectives. The self-renewal potential of MSCs is not definitely established and can vary greatly according to the methodology used and the species (Bianco et al. 2001), but cells can be expected to expand for at least 40 population doublings (PDs) before their growth rate decreases significantly, as seen with human MSCs (Bruder et al. 1997). Supplementation of growth factors can also modify these results. Fibroblast growth factor-2 (FGF-2), for instance, was shown to increase the lifespan of human MSCs to more than 70 PDs (Bianchi et al. 2003). Murine MSCs, on the other hand, show apparently unlimited in vitro growth capacity (Meirelles and Nardi 2003 and unpublished observations). The high self-renewal capacity shown by murine MSCs without evidence of replicative senescence is probably related to that of rat oligodendrocyte precursor cells (Tang et al. 2001).

Cell seeding density may also influence the expansion capacity of mesenchymal stem cells. Human MSCs, for instance, expand to much higher PDs when plated at low density than at high density, with an increase of total cells from 60- to 2,000-fold (Colter et al. 2000). On the other hand, the establishment of long-term cultures of murine mesenchymal stem cells is dependent on a minimal cell density of 2×10^6 bone marrow cells/cm^2 (Meirelles and Nardi 2003). Long-term culture and high cell density are also determinants of loss of differentiation potential for human cells, another indication that the conditions for the in vitro maintenance of MSCs differ from those provided by their natural microenvironment.

5
Homing and Engraftment of Transplanted Mesenchymal Stem Cells

In vivo tracking of implanted MSCs is very important, because the success of cell and gene therapy protocols with these cells depends on their engraftment abilities. The analysis of MSC engraftment is also related to the exact nature of the grafted cells. Heterogeneous populations of adherent cells derived from mouse bone marrow engraft in multiple organs after systemic infusion (Pereira et al. 1995, 1998), and although there is strong evidence showing that the grafted cells are comprised of mainly MSCs, engraftment of other cell types can not be excluded.

More recently, studies using a well characterized and relatively homogeneous cell population comprised of murine bone marrow devoid of hematopoietic cells, were consistent with a successful in vivo engraftment of candidate MSCs in the central nervous system (Kopen et al. 1999; McBride et al. 2003). This cell population, in contrast to other murine MSCs, was not expandable in vitro (Baddoo et al. 2003; Meirelles and Nardi 2003; Gojo et al. 2003; Fang et al. 2004), and interspecies experiments were used to study MSC systemic engraftment in mice. For instance, when human MSCs were injected intraperitoneally into 13-day-old mouse embryos in uterus, multiorgan engraftment was detected 8 weeks after birth by real-time PCR (McBride et al. 2003). The sites analyzed included femur, heart, brain, liver, kidney, spleen, and lungs, where the highest level of human DNA was detected.

When cells obtained from bleomycin-resistant BALB/c mice, essentially as described by Kopen et al. (1999), were injected systemically into bleomycin-sensitive C57BL/6 mice, engraftment in the lungs was considerably higher in animals with lung injury than in animals without injury. Tissue damage, therefore, enhances MSC engraftment (Ortiz et al. 2003); however, murine MSC engraftment has also been demonstrated in noninjured animals. Gojo et al. (2003) reported the engraftment of in vitro-proliferative murine MSCs in the heart, lung, spleen, stomach, small intestine, and skeletal muscle of non-injured mice, where they differentiated locally into cardiomyocytes, vascular endothelial cells, and possibly vascular luminal cells. Selective sorting of an adherent fraction of passage two or three bone marrow cultures yielded non-hematopoietic cells that engrafted into several organs after systemic infusion (Anjos-Afonso et al. 2004). Engraftment in some organs was infrequent (brain, bone marrow), but in others (liver, lung, kidney), it exhibited higher levels of engrafted MSCs, and the presence of donor cells in circulating blood was also observed.

The deposition of MSCs in the lungs may represent a significant hurdle for engraftment therapies that employ systemic delivery of MSCs. This approach caused some animals to develop fibrosis and subsequent breathing difficulties. Gao et al. (2001) observed this phenomenon following systemic infusion of MSCs in rats, perhaps because the MSC diameter was larger than that of lung

capillaries (20–24 μm vs 10–15 μm, respectively). The use of the vasodilator sodium nitroprusside at the time of injection, however, reduced entrapment of MSCs in the lungs. Murine MSCs, when detached from the dish, are also 20–25 μm wide (L. Meirelles, unpublished results), and if administered in large doses are likely to be trapped in lung capillaries before reaching important organs such as the brain and bone marrow.

In 2002, two groups (Ying et al. 2002; Terada et al. 2002) showed that stem cells can fuse with other cells in vitro and can acquire the characteristics of these cells, thus raising the possibility that the stem cell contribution to target tissues might be due to cell fusion rather than to (trans) differentiation. Fusion was also demonstrated between MSCs and epithelial cells in vitro (Spees et al. 2002); however, chromosomal analysis of xenografts indicate that fusion is not the principle mechanism responsible for the MSC contribution to multiple tissues in vivo (Pochampally et al. 2004; Sato et al. 2005). In a study describing the role of MSCs in the generation of gastric cancer in a mouse model, for example, it was observed that the percentage of tetraploid cells in affected and unaffected animals remained at the same levels (Houghton et al. 2004; see Sect. 8.1 for further information).

While the experimental study of MSC engraftment in humans is elusive, a recent paper describing microchimerism, possibly due to circulating fetal mesenchymal stem cells in pregnancy, in bone marrow and bone of women decades after giving birth to male fetuses has provided some insights into human MSC engraftment properties in vivo (O'Donoghue et al. 2004). A combined approach including immunocytochemistry, FISH, and PCR using rib sections and cultured MSCs derived from rib bone marrow was used. The results strongly suggest that circulating MSCs present in fetal blood (Campagnoli et al. 2001) crossed the placenta during pregnancy, entered the maternal circulation (O'Donoghue et al. 2003) and grafted with maternal bone and bone marrow. Khosrotehrani et al. (2004) also reported the detection of grafted male fetal cells in thyroid, cervix, intestine, liver, and lymph nodes when analyzing biopsy material of women who have had male pregnancies. The male cells were shown to express liver, hematopoietic, or epithelial markers, indicating tissue-specific incorporation.

Although fetal stem cells entering the maternal circulation include other cell populations, the results of experiments in animals suggest that MSCs are the main cell type that can engraft in maternal tissues. Human adult MSCs, therefore, can be expected to have multisite engraftment capabilities as well.

6
Characterization of Mesenchymal Stem Cells

Cultured MSCs have been extensively analyzed both morphologically and with respect to surface and molecular markers. None of these characteristics, how-

ever, is specific enough to adequately define this cell type, and mesenchymal stem cells are still operationally defined by the ultimate criteria used for identifying stem cells: prolonged proliferation and the potential to originate differentiated cell types. To abandon the term "operational MSC," it is necessary to show that these cells contribute to the formation of mesenchymal tissues after in vivo infusion (Verfaillie 2002).

A great number of surface markers have been described for committed mesenchymal progenitors (Otto and Rao 2004), and Deans and Moseley (2000) have compiled a long list of candidate markers, including CD44, CD29, and CD90, to define human MSCs. The expression of CD34 is not clearly defined in murine MSCs, but the marker is known to be absent from human and rat cells. More specific antigens such as Stro-1, SH2, SH3, and SH4 are also important markers for MSCs (reviewed in Barry and Murphy 2004), which are also positive for MHC-1 and Sca-1. None of these markers, however, seems to be a reliable parameter for the analysis of culture purity, since on the one hand even long-term cultures may exhibit some heterogeneity (maybe due to cell cycle-related marker expression) and, on the other, functionally different cultures may have similar immunophenotypic profiles.

Relatively little attention has been given to the morphology of the MSCs originating in culture. Two types of morphology can be observed—large, flat cells or elongated, fibroblastoid cells. The derivation of two types of adherent cell cultures from cord blood has already been pointed out by our group (Alfonso et al. 2000). The functional significance of these differences remains to be established.

In an earlier report (Meirelles and Nardi 2003), we described the isolation and long-term culture of murine MSCs without the need for any other medium supplementation than fetal calf serum. The cells exhibited a constant flat-type morphology (see Fig. 1), even when originating from other tissues such as spleen, lungs and brain (not published). In most publications, it is difficult to adequately assess cell morphology, but a review of the literature shows that in many cases the cells maintained a flattened shape, while others exhibited an elongated, fibroblastic phenotype (Table 2). The morphology of MAPCs also seems to be relatively flat (Reyes et al. 2001).

Still little is known about the profile of gene expression in mesenchymal stem cells. Tremain et al. (2001), in a study that also emphasized the heterogeneity of MSC cultures, reported over 2,000 expressed transcripts in a clone that originated from a stromal cell culture. In two recent studies, gene expression of bone marrow (Silva et al. 2003) and cord blood-derived MSCs (Panepucci et al. 2004; Jeong et al. 2005) was analyzed by serial analysis of gene expression (SAGE). A great number of genes were identified in the cultured cells, and an important contribution of extracellular protein products, adhesion molecules, cell motility, TGF-beta signaling, growth factor receptors, DNA repair, protein folding, and ubiquination as part of their transcriptome was observed.

Table 2 Reports of flat or elongated/fibroblastic morphology of cultured mesenchymal stem cells from different origins

Morphology	Species	Reference
Flat	Mouse	Meirelles and Nardi 2003 (Fig. 2)
	Human	Azizi et al. 1998 (Fig. 2a)
	Human	D'Ippolito et al. 2004 (Fig. 1)
	Human	Gronthos et al. 2003 (Fig. 2G)
	Human	Stute et al. 2004 (Fig. 6B)
	Human	Hung et al. 2004 (Fig. 1)
	Rat	Azizi et al. 1998 (Fig. 2c)
	Rat	Davani et al. 2003 (Fig. 1A)
	Rat	Kobayashi et al. 2004 (Fig. 1)
Fibroblastic	Human	Azizi et al. 1998 (Fig. 2b)
	Human	Koç et al. 2000 (Fig. 1)
	Human	Campagnoli et al. 2001 (Fig. 1)
	Human	Pittenger et al. 1999 (Fig. 1)
	Human	Pittenger and Martin 2004 (Fig. 1)
Other	Human	Seshi et al. 2000 (Fig. 1)

7
Differentiation of Mesenchymal Stem Cells

Although considered nondifferentiated cells, MSCs are nevertheless capable of performing at least one specialized function: they support hematopoiesis. This function is generally attributed to the bone marrow stroma, which is frequently confused with MSCs. Apparently homogeneous cultures of MSCs support hematopoietic stem cells with greater efficiency than conventionally established bone marrow stroma (Meirelles and Nardi 2003).

The in vitro differentiation of MSCs into several lineages is easily achieved. Representative examples of the protocols employed in a number of studies are shown in Table 3. Determination of the phenotype of differentiated cells depends on morphological, immunophenotypic, and functional criteria. The differentiation of osteoblasts, for instance, is determined by upregulation of alkaline phosphatase activity and deposition of a mineralized extracellular matrix in the culture plates that can be detected with Alizarin Red or other stains. Adipocytes are easily identified by their morphology and staining with Oil Red O. For identification of myocytes or neuronal cells, immunocytochemistry is performed with antibodies specific for antigens such as myosin and dystrophin, or Tau and GFAP, respectively.

Table 3 Examples of the culture protocols used for inducing in vitro differentiation of MSCs

Tissue	Species	Culture medium complement	Reference
Bone	Mouse	10^{-8} M dexamethasone, 5 μg/ml ascorbic acid 2-phosphate and 10 mM β-glycerophosphate	Meirelles and Nardi 2003
Cartilage	Human	Transforming growth factor-β3 in serum-free medium, added to three-dimensional cultures	Pittenger et al. 1999
Fat	Human	1-methyl-3-isobutylxanthine, dexamethasone, insulin, and indomethacin	Pittenger et al. 1999
	Mouse	10^{8} M dexamethasone and 5 μg/ml insulin	Meirelles and Nardi 2003
Neuron-like	Human	Isobutylmethylxanthine and dibutyryl cyclic AMP	Deng et al. 2001
	Mouse	50 ng/ml of basic fibroblast growth factor (bFGF) and 20 ng/ml of epidermal growth factor (EGF)	Anjos-Afonso et al. 2004
Muscle	Mouse	Amphotericin B	Phinney et al. 1999
	Rat	5-azacytidine	Wakitani et al. 1995
	Pig	5-azacytidine	Moscoso et al. 2005

In vitro cultured MSCs show great heterogeneity in their differentiation potential. Although the analysis of established MSC cultures show them to be pluripotent, with a tri-lineage (osteo/chondro/adipo, De Ugarte et al. 2003) or even higher (Anjos-Afonso et al. 2004) differentiation potential, clonal assays have shown that only one-third of the MSC clones derived from established cultures are pluripotent (Pittenger et al. 1999; Muraglia et al. 2000). Within established cultures, thus, a minority of cells seem to be pluripotent, with most of them having bi- or only uni-lineage differentiation capacity (Digirolamo et al. 1999). Models have been proposed to explain these results (Baksh et al. 2004), and it is possible that cultures are composed of a mixture of cells with different differentiation potentials. A small portion may correspond to authentic stem cells, whereas most may be committed to more differentiated phenotypes.

8 Applications of Mesenchymal Stem Cells in Cell and Gene Therapy

Although human MSCs can be immortalized through genetic modification using expression vectors carrying the catalytic subunit of human telomerase (Mihara et al. 2003), the study of murine MSCs in vitro is more attractive since they represent an unmodified natural population. They can also be promptly

obtained by researchers who do not have access to a source of human cells or do not have adequate facilities for their manipulation. More importantly, murine MSCs can be directly applied to a wide range of murine models of diseases, where they can act as therapeutic agents per se or as vehicles for the delivery of therapeutic genes. Finally, MSCs obtained from rats, rabbits, pigs, and sheep will also be useful for the development of engineered tissues using autologous cells.

8.1 Study of Cancer Biology

The sustained proliferation of murine MSCs provides an interesting model for the evaluation of genetic and epigenetic factors involved in the maintenance of stemness, as well as the components responsible for the generation of tumors. Murine MSC self-renewal is not linked to neoplasia: experiments in which mice received intravenous or intraperitoneal MSC infusion do not develop donor-derived tumors (L. Meirelles and N. Nardi, unpublished results). Unraveling the genetic determinants of self-renewal may lead to the identification of candidate genes involved in tumorigenesis and to the development of drugs that can act specifically on their products. The fusion of murine MSCs and nonproliferative cells, for instance, would help with the mapping of genes involved in proliferation to specific chromosomes and chromosomal regions.

While studying the behavior of marrow-derived cells in a mouse model of gastric cancer induced by *Helicobacter pylori*, cells bearing the marker TFF2 could be associated with the predominant cell type present in this cancer (Houghton et al. 2004). In vitro studies using purified marrow-derived hematopoietic stem cells or MSCs exposed to cancerous tissue extracts showed that MSCs, but not HSCs, acquired expression of TFF2. The contribution of MSCs to tumor formation by cell fusion was ruled out by comparing the ploidy of stomach cells from infected and noninfected mice: the number of tetraploid cells in both groups did not differ significantly. Another recent study described the involvement of stem cells in human brain tumors (Singh et al. 2004). These results are consistent with an emerging view of cancer as a stem cell disorder, rather than a disease confined to fully or partially differentiated cells.

Besides their potential use for the study of basic cancer biology, modified MSCs may prove to be efficient antitumoral agents. Human MSCs have been shown to incorporate into tumor stroma (Studeny et al. 2002), a potentially useful tool for the delivery of gene products directly to tumors (see below).

8.2 Cell Therapy

Mesenchymal stem cells may participate in cell therapy protocols through two mechanisms. First, MSCs may contribute physically to injured sites when

administered locally or systemically. Second, MSCs may have a supportive role through means of secreted factors. Examples of these applications are given below.

8.2.1 Fibrosis

As mentioned earlier, murine MSCs administered systemically to mice subjected to lung injury show superior lung engraftment rates relative to uninjured animals. Furthermore, MSC treatments performed immediately after antibiotic challenge reduces the fibrotic and inflammatory effects of the lesion significantly more than that in animals receiving the cells 7 days after the challenge (Ortiz et al. 2003). An earlier work, using a poorly characterized murine MSC population, showed that infusion of the cells in mice subjected to lung injury by bleomycin showed enhanced reproducibility of engraftment (Kotton et al. 2001). The injected cells were found to engraft as type I pneumocytes, but not type II pneumocytes. Similar results were reported by Ortiz et al. (2003). In the case of bleomycin-induced lung injury, this indicated that the main cellular contribution from the plastic-adherent fraction of bone marrow can be attributed to MSCs. This information may be valuable for future therapies aiming to reduce lung fibrosis in humans by autologous bone marrow transplantation.

MSCs have also been used to treat liver fibrosis. Fang et al. (2004) depleted murine bone marrow from $CD45^+$, $GlyA^+$, and $CD34^+$ cells to obtain adherent Flk^+ cells that are expandable in vitro for more than 30 passages. Using a murine model of tetrachloride-induced liver injury, they showed in the animals receiving MSCs systemically immediately after the challenge, but not 1 week later, that the fibrotic effects caused by the lesion were reduced. The presence of albumin-producing cells that exhibit donor-derived markers was also detected, although at a low frequency.

8.2.2 Cardiovasculogenesis

In a study originally designed to assess the contribution of murine MSCs to the cardiac tissue, Gojo et al. (2003) showed that a 5-azacytidine-responsive, $CD34^{low/-}$c-$kit^+CD140a^+$Sca-1^{high} clone transduced with an EGFP construct contributed to several sites when implanted in vivo. After injection into the ventricular myocardium, $EGFP^+$ cardiomyocytes were detected, along with $EGFP^+CD31^+$ cells lining the vessels surrounding the site of injection. The number of endothelial cells and cardiomyocytes grafted in the ventricle was estimated to be, respectively, 1,625 and 75 1 week after injection, and 275 and 25 3 months later. When MSCs were infused systemically through the inferior vena cava, the cells engrafted predominantly in the lungs 1 week after

administration. Four weeks after the injection, the number of $EGFP^+$ cells in the lungs declined considerably. The grafted cells lacked CD31 expression, indicating that they had formed pericytes or smooth muscle cells. In addition to these results, $EGFP^+$ cells were found in the brain, thymus, uterus, and kidney. Engraftment in the stomach and small intestine was observed, and the number of donor-derived cells seemed to increase over time. When high cell numbers were implanted in the muscle, liver, or spleen, ectopic bone formation was observed, in contrast to the muscular and vascular fates adopted by cells delivered in low quantities. This finding cautions against experimental protocols involving the injection of large doses of MSCs directly in heart muscle, since they might differentiate into tissues other than those expected.

The use of purified MSCs to treat human heart diseases has not yet come into practice, despite the reported successes of blood or bone marrow-derived mononuclear cells (Assmus et al. 2002; Perin et al. 2003). Although studies demonstrating that bone marrow-derived c-kit^+lin^- cells, a cellular fraction putatively enriched in HSCs, regenerate mouse infarcted myocardium (Orlic et al. 2001) these findings been challenged by others (Murry et al. 2004; Baslam et al. 2004). The plastic-adherent fraction of bone marrow has also improved cardiac performance in a rat model of heart infarction (Olivares et al. 2004). The $CD34^-CD45^-$ cells collected for these latter experiments most likely comprised MSCs. Histological analyses indicated that the main contribution of the cells to the infarcted zone occurred through the formation of new myocardium and blood vessels.

Taken together, these studies indicate that the cell type most likely involved in cardiac regeneration is the MSC, possibly due more to its arteriogenic effects (see the next section) than to its cardiomyogenic properties.

8.2.3 Arteriogenic Effects

Kinnaird et al. (2004) induced unilateral hind limb ischemia in mice to demonstrate that murine bone marrow, devoid of $CD34^+CD45^+$ cells and delivered in situ 24 h after lesion, improved limb function despite little long-term cell engraftment. They found that medium conditioned by these cells contained several potent arteriogenic cytokines, such as basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), placental growth factor (PlGF), and monocyte chemoattractant protein-1 (MCP-1). Animals that received the cells after injury contained donor-marked cells surrounded by bFGF and VEGF positive cells, indicating that the transplanted MSCs expressed these cytokines in situ. These findings support the hypothesis that factors secreted by MSCs have a significant role in limb recovery. Interestingly, MCP-1, which is secreted by cells present in the vascular wall, has been shown to recruit circulating monocytes that can differentiate into endothelial cells (Fujiyama et al. 2003). The arteriogenic effects of MSCs may thus involve

the recruitment of circulating cells through the secretion of chemoattractant factors.

8.2.4 Immunosuppressive Effects

The subcutaneous co-injection of primary murine MSCs or of an embryonic mouse mesenchymal stem cell line (CH310T1/2) with a melanoma cell line (B16) was shown to favor tumor growth (Djouad et al. 2003). In vitro experiments in which activated murine splenocytes were co-cultured with CH310T1/2 cells in a transwell culture system indicated that soluble factors secreted by MSCs inhibit $CD8^+$ T cell proliferation. Even though the primary murine MSCs were not well characterized, the use of CH310T1/2 validated the hypothesis that MCSs favored tumor growth, since the results observed in vivo were similar for both cell types. These results indicated that the immunosuppressive effects should be considered whenever MSC transplantation takes place. Krampera et al. (2003) demonstrated even more clearly the immunosuppressive effects of MSCs by showing that culture-expanded murine MSCs are capable of inhibiting both naïve and memory antigen-specific T cell activation. The authors used a mixed lymphocyte reaction system in dose-dependent experiments, and determined that T cell inhibition was transient and independent of MHC antigen-presenting cells or $CD4^+CD25^+$ regulatory T cells. In contrast, the study by Djouad et al. (2003) showed that the MSC immunosuppressive effect required cell contact. Whether or not cell contact is required, it is clear that MSCs have immunomodulatory capabilities. Treatment of acute graft-versus-host disease in a human subject using third-party haploidentical MSCs can be taken as proof of this concept (Le Blanc et al. 2004).

The mechanisms involved in MSC-mediated immunosuppression are currently being investigated. Glennie et al. (2004) reported that MSCs suppress T cell effector function transiently, but the cells do not block activation. On the other hand, they induce an irreversible proliferation arrest not only in $CD4^+$ and $CD8^+$ T cells, but also in B cells, by downregulating cyclin-D2 expression. The interactions of MSCs and immune cells may have future implications not only for the knowledge of MSC biology, but also for the understanding of immune system homeostasis.

8.3 Mesenchymal Stem Cells and Tissue Engineering

In addition to systemic engraftment capabilities, MSCs show great potential for the replacement of damaged tissues such as bone, cartilage, tendon, and ligament. Although bone is capable of regeneration, the three other tissues often develop fibrous scar tissues when injured, which usually renders them unable to function properly. Large bone defects, however, do not heal spontaneously,

Table 4 Examples of tissues engineered with the use of MSCs

Engineered tissue	Methods	Reference
Respiratory mucosa	Human MSCs co-cultured with normal human bronchial epithelial cells, in vitro	Le Visage et al. 2004
Cartilage	Human MSCs cultured under chondrogenic conditions on 3D scaffold, in vitro	Chen et al. 2004a; Li et al. 2005
Bone	Critical-size cranial defect created in rabbit, repaired with BMP-2-expressing rabbit MSCs embedded in alginate	Chang et al. 2004
	Full-thickness mandibular defects created in pigs, and repaired with autologous MSCs previously seeded in poly-DL-lactic-coglycolic acid scaffolds and kept in osteo-inductive medium	Abukawa et al. 2004
Cardiac pacemakers	Human MSCs transfected with a cardiac pacemaker gene (*mHCN2*). Functional results observed in vitro by co-culture with neonatal rat ventricular myocytes, and in vivo by subepicardial injection into the canine left ventricular wall	Potapova et al. 2004

suggesting that MSC-based reconstitution may be feasible. The application of MSCs in the engineering of new tissue is dependent on the use of an appropriate scaffold to maintain an adequate three-dimensional distribution and on the use of specific molecules to drive their differentiation into cells that can restore the tissue-specific matrix (Huang et al. 2004).

The use of murine MSCs for tissue engineering is limited, due to the small size of the mouse. However, murine MSCs along with MSCs from other species are interesting candidates for the study of cell interactions with novel biomaterials, and the study of new molecules on differentiation. Examples of the use of MSCs for tissue engineering in larger animal models can be found in the literature, some of which are listed in Table 4.

8.4 Genetic Therapy

Genetic diseases can be generally classified into two categories: those caused by genetic alterations leading to loss of protein/gene function and those caused by a gain of function mutation. Other factors are involved such as the restriction of a disease phenotype to a specific organ as opposed to the whole body. When loss of function is the cause, introduction of genetic constructs expressing the missing product may be sufficient to revert or suppress the disease phenotype. When the disease involves gain of function, however, the insertion of vectors

expressing healthy transcripts is not enough to correct the disorder, and some sort of genome or transcriptome editing is necessary.

The molecular tools currently used to address these issues include vectors derived from plasmids, virus, and even transposons (reviewed by Selkirk 2004 and Nathwani et al. 2004). Anti-sense RNA and interference RNA can also be used. Plasmid-derived vectors do not integrate into the host genome, and so do not last for the lifetime of the individual. On the other hand, virus-derived vectors can integrate into the genome of proliferating or nonproliferating cells, depending on the type of viral-based expression system. Sustained expression following integration makes them the vector system of choice. The systemic in vivo administration of viral vectors represents the main hurdle to their direct utilization, since they may elicit a strong host immune response that could lead to death (Kaiser 2004) or cause insertional mutagenesis resulting in cancer as reported recently in the X-SCID clinical trial (Hacein-Bey-Abina et al. 2003). The use of stem cells to deliver genetic material represents the best way to circumvent the first obstacle. Stem cells can be manipulated ex vivo and receive the genetic modifications necessary for correction of the disease, avoiding the need to expose the patient directly to vectors. When proliferative stem cells such as MSCs are used, there is also the possibility of selecting successfully altered clones for reinfusion, which might suffice to minimize insertional mutagenesis risks. This is in contrast to the use of HSCs, the stem cell type altered in the X-SCID trial, which are largely nonproliferative in vitro.

Systemic delivery of genetic constructs mediated by stem cells is feasible: stem cells engraft in vivo, and particularly in the case of MSCs, they engraft to multiple sites. When autologous cells are genetically corrected, they are likely to acquire a proliferative advantage over the patient's cells, increasing the likelihood of engraftment and providing continued expression of the therapeutic construct. Moreover, if HLA-matched allogeneic stem cells are used there may be no need to use genetic manipulation tools, as the cells themselves may exert therapeutic effects through the expression of donor genes.

8.4.1
Correction of Genetic Disorders

The availability of homogeneous populations of murine MSCs has profound implications for the treatment of genetic diseases in mouse models, and by analogy to humans. The use of murine models is appropriate when trying to develop new therapeutic strategies. The cost of maintaining mice is not excessively high, and mouse genetics are well described. Moreover, knock-out mice can be generated by site-directed mutagenesis in embryonic stem cells, which means that many loss-of-function diseases, excluding those that lead to embryonic lethality, can be simulated in mice. The results obtained using a small animal model should, nevertheless, be validated using larger animals to avoid any unexpected effects when the therapy is applied to humans.

Table 5 Selected candidate mouse models of genetic disease for MSC-mediated therapy

Model	Expected role of transplanted MSCs	See also
Mucopolysaccharidosis type I	Production of α-L-iduronidase, particularly in the brain	Koç et al. 2002
Hemophilia A	Production and release into the blood of coagulation factor VIII	Van Damme et al. 2003
Niemann-Pick disease	Sphingomyelinase production, particularly in the brain	Jin et al. 2002
Osteogenesis imperfecta	Production of healthy collagen type I fibers	Pereira et al. 1995; Chamberlain et al. 2004

Reference: http://jaxmice.jax.org/jaxmice-cgi/jaxmicedb.cgi as of 03/28/2005 (mouse models database). See also complementary references for further information

In a search at The Jackson Laboratory mouse database (http://jaxmice.jax.org/jaxmice-cgi/jaxmicedb.cgi) using the term "genetic disease," 140 results were retrieved. Some of them were selected (Table 5) as examples of experimental models that could be used to test a MSC-based therapeutic approach, including the mouse model of mucopolysaccharidosis type I (Ohmi et al. 2003) that we currently study.

The application of MSC-mediated gene therapy in humans is still in its infancy, with no clinical trials reported so far. In vitro studies, however, show promise. Baxter et al. (2002) have successfully restored α-L-iduronidase expression by retroviral transduction of the human IDUA cDNA into MSCs obtained from patients affected by mucopolysaccharidosis type I. While this work demonstrates the possibility of reversing loss-of-function genetic disorders in humans, another study (Chamberlain et al. 2004) went even further. In this latter study, the correction of a gain-of-function genetic disease, osteogenesis imperfecta, was addressed. MSCs obtained from affected patients were genetically modified to disrupt the dominant-negative mutant allele of the COL1A1 gene. Because the site-directed mutagenesis method could affect both normal and mutant alleles and lead to inappropriate integration events in the genome, screening for correctly altered clones was performed. The investigators specifically screened several clones to identify those cells that had been appropriately modified. Once selected, these clones could generate cells that accumulated limited amounts of intracellular procollagen and could produce relatively normal collagen extracellular matrix.

8.4.2 Cancer Suppression

As mentioned earlier, the ability of MSCs to incorporate into tumor stroma could be used to design strategies to fight cancer. Studeny et al. (2002) trans-

duced human MSCs with a construct expressing human IFN-β, which has immunomodulatory properties and antiproliferative effects over melanoma cell lines. The transduced MSCs were administered subcutaneously to nude mice together with the A375SM human melanoma cell line. The results showed that the tumor area was strikingly reduced even when only 10% IFN-β-expressing MSCs were co-injected with the melanoma cells, as compared to control animals that received the melanoma cells alone. The same effect was not achieved when nontransduced MSCs were used, indicating that IFN-β was the mediator of tumor suppression. Moreover, the tumor area was not reduced by IFN-β injection alone, indicating that MSCs were required. The survival period of treated animals, compared to controls, also significantly increased (i.e., 41–110 days for animals receiving different proportions of IFN-β-expressing MSCs vs 21–27 days for animals that received melanoma cells alone). A study examining tumor metastasis in lungs yielded similar results (Studeny et al. 2004). The results obtained using xenograft tumor models in immunoincompetent mice also show promise.

Equivalent studies with syngeneic or allogeneic murine MSCs in immunocompetent mice are required to evaluate the efficacy of this treatment in individuals with normal immune activity, since the development of anti-cancer protocols without the need for immunosuppression are highly desirable for application in humans.

9
Pharmacologic Aspects of Mesenchymal Stem Cell Biology

The pharmacological relevance of MSCs can be divided into four categories. First, the molecules secreted by MSCs may be employed as therapeutic agents or adjuvants in animal models. A long list of biologically important molecules secreted by MSCs (Majumdar et al. 1998; Kinnaird et al. 2004) include interleukins 6, 7, 8, 11, 12, 14, and 15, M-CSF, Flt-3 ligand, SCF, LIF, bFGF, VEGF, PlGF, and MCP-1. Second, specific receptors expressed by MSCs (Table 6) may be used as targets for drugs aimed at MSCs in vivo. These studies may provide information on homing mechanisms when systemically infused. Third, genetic constructs can be made that are preferentially expressed in MSCs by the incorporation of cell-specific regulatory regions, similar to that described earlier. Fourth, natural or artificial molecules may be used modify the natural behavior of MSCs and alter the MSC compartment in vivo.

While a set of natural and synthetic compounds have been shown to exert many biological effects, such as differentiation induction (Table 7), many other compounds remain to be discovered and/or fully characterized. For instance, a small molecule termed reversine (a 2-(4-morpholinoanilino)-6-cyclohexylaminopurine analog) has the ability to reprogram myogenesis-committed precursor cells (the murine cell line C2C12) into a less differentiated

Table 6 Receptors expressed by MSCs render them responsive to specific molecules

Category	Expressed	Nonexpressed
Cytokine receptors	IL-1R (CD121a)	IL-2R (CD25)
	IL-3Ra (CD123)	
	IL-4R (CDw124)	
	IL-6R (CD126)	
	IL-7R (CD127)	
Chemokine receptors	CXCR4	
Factor receptors	EGFR	EGFR-3
	IGF1 R (CD221)	Fas ligand (CD178)
	NGFR	
	IFNγR (CDw119)	
	TNFIR (CD120a)	
	TNFIIR (CD120b)	
	TGFβIR	
	TGFβIIR	
	bFGFR	
	PDGFR (CD140a)	
	Transferrin (CD71)	
Matrix receptors	ICAM-1 (CD54)	ICAM-3 (CD50)
	ICAM-2 (CD102)	E-selectin (CD62E)
	VCAM-1 (CD106)	P-selectin (CD62P)
	L-Selectin (CD62L)	PECAM-1 (CD31)
	LFA-3 (CD58)	vW factor
	ALCAM (CD166)	Cadherin 5 (CD144)
	Hyaluronate (CD44)	Lewis^x (CD15)
	Endoglin (CD105)	

References: Pittenger et al. 1999; Gronthos et al. 1998; Wynn et al. 2004

state equivalent to that of MSCs (Chen et al. 2004b). The resulting MSCs were shown to differentiate into osteoblastic and adipocytic cells upon appropriate stimulation.

10 Conclusions

Mesenchymal stem cells are finally attracting the attention of the scientific community, some 30 years after the first insights on the existence of non-hematopoietic stem cells in bone marrow. Their ease of derivation and manip-

Table 7 Specific molecules can direct MSC differentiation or modulate their expansion capacity

Molecule	Main effect	Reference
5-azacytidine	Myogenesis	Wakitani et al. 1995
All-trans-retinoic acid	Neurogenesis	Sanchez-Ramos et al. 2000
Amphotericin B	Myogenesis	Phinney et al. 1999
Ascorbic acid	Osteogenesis; chondrogenesis	Pittenger et al. 1999
Beta-glycerophosphate	Osteogenesis; chondrogenesis	Pittenger et al. 1999
Beta-mercaptoethanol	Neurogenesis	Woodbury et al. 2000
bFGF	Proliferation	Kuznetsov et al. 1997; Bianchi et al. 2003
BHA	Neurogenesis	Woodbury et al. 2000
Dexamethasone	Adipogenesis; osteogenesis; chondrogenesis	Pittenger et al. 1999
EGF	proliferation	Kuznetsov et al. 1997
ETYA	Adipogenesis	Kopen et al. 1999
Hydrocortisone	Myogenesis	Zuk et al. 2001
IBMX	Adipogenesis	Pittenger et al. 1999
Indomethacin	Adipogenesis	Pittenger et al. 1999
Insulin	Adipogenesis	Pittenger et al. 1999; Zuk et al. 2001
PDGF	Proliferation	Kuznetsov et al. 1997
TGF-beta family members (incl. BMPs)	Proliferation; differentiation Roelen and Dijke 2003	Kuznetsov et al. 1997;

ulation ex vivo, together with the growing body of information provided by studies on their characterization and differentiation potential, have generated excitement in the field of stem cell based therapies. Clinical applications with MSCs are also relatively close to realization, when compared with most other stem cells.

Most of the knowledge generated so far, however, concerns their behavior in vitro, because little is known about their properties in vivo. Although this does not necessarily hinder their application for the treatment of severe diseases or for the replacement of damaged tissues, it is clear that a comprehensive understanding of their biology is required to achieve maximal benefits. The task will not be easy, because at present, tracking the fates of ex vivo manipulated MSCs after their systemic delivery in animal models may lead to skewed results. In vitro manipulation seems to alter the original properties of the cells. The lack of definitive markers also does not allow the direct observation of MSCs in situ, so that putative MSCs should be functionally characterized in vitro prior

to their use in the experiments. Ultimately, concrete data indicating that MSCs are present in multiple adult tissues together with findings suggesting the existence of a perivascular niche for mesenchymal precursors will help us to better understand the role of the mesenchymal stem cell in vivo and consequently will help in the development of more efficient strategies to treat a wide range of diseases.

Acknowledgements The authors are indebted to Conselho Nacional de Desenvolvimento Cientifico e Tecnologico (CNPq) and Fundação de Amparo a Pesquisa do Estado do Rio Grande do Sul (FAPERGS) for funding.

References

Abukawa H, Shin M, Williams WB, Vacanti JP, Kaban LB, Troulis MJ (2004) Reconstruction of mandibular defects with autologous tissue-engineered bone. J Oral Maxillofac Surg 62:601–606

Alfonso ZZ, Forneck ED, Allebrandt WF, Nardi NB (2000) Establishment of an adherent cell layer from human umbilical cord blood. Genet Mol Biol 23:519–522

Anjos-Afonso F, Siapati EK, Bonnet D (2004) In vivo contribution of murine mesenchymal stem cells into multiple cell-types under minimal damage conditions. J Cell Sci 117:5655–5664

Assmus B, Schachinger V, Teupe C, Britten M, Lehmann R, Dobert N, Grunwald F, Aicher A, Urbich C, Martin H, Hoelzer D, Dimmeler S, Zeiher AM (2002) Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOPCARE-AMI). Circulation 106:3009–3017

Azizi SA, Stokes D, Augelli BJ, DiGirolamo C, Prockop DJ (1998) Engraftment and migration of human bone marrow stromal cells implanted in the brains of albino rats—similarities to astrocyte grafts. Proc Natl Acad Sci U S A 95:3908–3913

Baddoo M, Hill K, Wilkinson R, Gaupp D, Hughes C, Kopen GC, Phinney DG (2003) Characterization of mesenchymal stem cells isolated from murine bone marrow by negative selection. J Cell Biochem 89:1235–1249

Baksh D, Song L, Tuan RS (2004) Adult mesenchymal stem cells: characterization, differentiation, and application in cell and gene therapy. J Cell Mol Med 8:301–316

Balsam LB, Wagers AJ, Christensen JL, Kofidis T, Weissman IL, Robbins RC (2004) Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. Nature 428:668–673

Barry FP, Murphy JM (2004) Mesenchymal stem cells: clinical applications and biological characterization. Int J Biochem Cell Biol 36:568–584

Baxter MA, Wynn RF, Deakin JA, Bellantuono I, Edington KG, Cooper A, Besley GT, Church HJ, Wraith JE, Carr TF, Fairbairn LJ (2002) Retrovirally mediated correction of bone marrow-derived mesenchymal stem cells from patients with mucopolysaccharidosis type I. Blood 99:1857–1859

Bianchi G, Banfi A, Mastrogiacomo M, Notaro R, Luzzatto L, Cancedda R, Quarto R (2003) Ex vivo enrichment of mesenchymal cell progenitors by fibroblast growth factor 2. Exp Cell Res 287:98–105

Bianco P, Cossu G (1999) Uno, nessuno e centomila: searching for the identity of mesodermal progenitors. Exp Cell Res 251:257–263

Bianco P, Riminucci M, Gronthos S, Robey PG (2001) Bone marrow stromal stem cells: nature, biology, and potential applications. Stem Cells 19:180–192

Breems DA, Blokland EAW, Siebel KE, Mayen AEM, Engels LJA, Ploemacher RE (1998) Stroma-contact prevents loss of hematopoietic stem cell quality during ex vivo expansion of CD34+ mobilized peripheral blood stem cells. Blood 91:111–117

Bruder SP, Jaiswal N, Haynesworth SE (1997) Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation. J Cell Biochem 64:278–294

Campagnoli C, Roberts IA, Kumar S, Bennett PR, Bellantuono I, Fisk NM (2001) Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow. Blood 98:2396–2402

Caplan AI (1994) The mesengenic process. Clin Plast Surg 21:429–435

Chamberlain JR, Schwarze U, Wang PR, Hirata RK, Hankenson KD, Pace JM, Underwood RA, Song KM, Sussman M, Byers PH, Russell DW (2004) Gene targeting in stem cells from individuals with osteogenesis imperfecta. Science 303:1198–1201

Chang SC, Chuang H, Chen YR, Yang LC, Chen JK, Mardini S, Chung HY, Lu YL, Ma WC, Lou J (2004) Cranial repair using BMP-2 gene engineered bone marrow stromal cells. J Surg Res 119:85–91

Chen G, Liu D, Tadokoro M, Hirochika R, Ohgushi H, Tanaka J, Tateishi T (2004a) Chondrogenic differentiation of human mesenchymal stem cells cultured in a cobweb-like biodegradable scaffold. Biochem Biophys Res Commun 322:50–55

Chen S, Zhang Q, Wu X, Schultz PG, Ding S (2004b) Dedifferentiation of lineage-committed cells by a small molecule. J Am Chem Soc 126:410–411

Colter DC, Class R, DiGirolamo CM, Prockop DJ (2000) Rapid expansion of recycling stem cells in cultures of plastic-adherent cells from human bone marrow. Proc Natl Acad Sci U S A 97:3213–3218

Cordero EAA, Silla LMR, Cañedo AD, Allebrandt WF, Fogliatto L, Nardi NB (2004) Interaction between normal and CML hematopoietic progenitors and stroma influences abnormal hematopoietic development. Stem Cells Devel 13:225–228

Davani S, Marandin A, Mersin N, Royer B, Kantelip B, Herve P, Etievent JP, Kantelip JP (2003) Mesenchymal progenitor cells differentiate into an endothelial phenotype, enhance vascular density, and improve heart function in a rat cellular cardiomyoplasty model. Circulation 108 Suppl 1:II253–II258

De Bari C, Dell'Accio F, Vandenabeele F, Vermeesch JR, Raymackers J-M, Luyten FP (2003) Skeletal muscle repair by adult human mesenchymal stem cells from synovial membrane. J Cell Biol 160:909–918

De Ugarte DA, Morizono K, Elbarbary A, Alfonso Z, Zuk PA, Zhu M, Dragoo JL, Ashjian P, Thomas B, Benhaim P, Chen I, Fraser J, Hedrick MH (2003) Comparison of multi-lineage cells from human adipose tissue and bone marrow. Cells Tissues Organs 174:101–109

Deans RJ, Moseley AB (2000) Mesenchymal stem cells: biology and potential clinical uses. Exp Hematol 28:875–884

Deng W, Obrocka M, Fischer I, Prockop DJ (2001) In vitro differentiation of human marrow stromal cells into early progenitors of neural cells by conditions that increase intracellular cyclic AMP. Biochem Biophys Res Commun 282:148–152

Dexter TM, Allen TD, Lajtha LG (1976) Conditions controlling the proliferation of haemopoietic stem cells in vitro. J Cell Physiol 91:335–344

Digirolamo CM, Stokes D, Colter D, Phinney DG, Class R, Prockop DJ (1999) Propagation and senescence of human marrow stromal cells in culture: a simple colony-forming assay identifies samples with the greatest potential to propagate and differentiate. Br J Haematol 107:275–281

D'Ippolito G, Diabira S, Howard GA, Menei P, Roos BA, Schiller PC (2004) Marrow-isolated adult multilineage inducible (MIAMI) cells, a unique population of postnatal young and old human cells with extensive expansion and differentiation potential. J Cell Sci 117:2971–2981

Djouad F, Plence P, Bony C, Tropel P, Apparailly F, Sany J, Noel D, Jorgensen C (2003) Immunosuppressive effect of mesenchymal stem cells favors tumor growth in allogeneic animals. Blood 102:3837–3844

Fang B, Shi M, Liao L, Yang S, Liu Y, Zhao RC (2004) Systemic infusion of FLK1(+) mesenchymal stem cells ameliorate carbon tetrachloride-induced liver fibrosis in mice. Transplantation 78:83–88

Friedenstein AJ, Gorskaja UF, Julagina NN (1976) Fibroblast precursors in normal and irradiated mouse hematopoietic organs. Exp Hematol 4:267–274

Fuchs E, Tumbar T, Guasch G (2004) Socializing with the neighbors: stem cells and their niche. Cell 116:769–778

Fujiyama S, Amano K, Uehira K, Yoshida M, Nishiwaki Y, Nozawa Y, Jin D, Takai S, Miyazaki M, Egashira K, Imada T, Iwasaka T, Matsubara H (2003) Bone marrow monocyte lineage cells adhere on injured endothelium in a monocyte chemoattractant protein-1-dependent manner and accelerate reendothelialization as endothelial progenitor cells. Circ Res 93:980–989

Gao J, Dennis JE, Muzic RF, Lundberg M, Caplan AI (2001) The dynamic in vivo distribution of bone marrow-derived mesenchymal stem cells after infusion. Cells Tissues Organs 169:12–20

Glennie S, Soeiro I, Dyson PJ, Lam EW, Dazzi F (2004) Bone marrow mesenchymal stem cells induce division arrest anergy of activated T cells. Blood 105:2821–2827

Gojo S, Gojo N, Takeda Y, Mori T, Abe H, Kyo S, Hata J, Umezawa A (2003) In vivo cardiovasculogenesis by direct injection of isolated adult mesenchymal stem cells. Exp Cell Res 288:51–59

Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC (1996) Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med 183:1797–1806

Gronthos S, Simmons PJ (1995) The growth factor requirements of STRO-1-positive human bone marrow stromal precursors under serum-deprived conditions in vitro. Blood 85:929–940

Gronthos S, Graves SE, Simmons PJ (1998) Isolation, purification and in vitro manipulation of human bone marrow stromal precursor cells. In: Beresford JN, Owen ME (eds) Marrow stromal cell culture. Cambridge University Press, New York, pp 26–42

Gronthos S, Zannettino AC, Hay SJ, Shi S, Graves SE, Kortesidis A, Simmons PJ (2003) Molecular and cellular characterisation of highly purified stromal stem cells derived from human bone marrow. J Cell Sci 116:1827–1835

Hacein-Bey-Abina S, von Kalle C, Schmidt M, Le Deist F, Wulffraat N, McIntyre E, Radford I, Villeval JL, Fraser CC, Cavazzana-Calvo M, Fischer A (2003) A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. N Engl J Med 348:255–256

Houghton J, Stoicov C, Nomura S, Rogers AB, Carlson J, Li H, Cai X, Fox JG, Goldenring JR, Wang TC (2004) Gastric cancer originating from bone marrow-derived cells. Science 306:1568–1571

Hu Y, Liao L, Wang Q, Ma L, Ma G, Jiang X, Zhao RC (2003) Isolation and identification of mesenchymal stem cells from human fetal pancreas. J Lab Clin Med 141:342–349

Huang JI, Yoo JU, Goldberg VM (2004) Orthopaedic applications of stem cells. In: Blau H, Melton D, Moore M, Thomas ED, Verfaillie C, Weissman I, West M (eds) Handbook of stem cells. Vol. 2. Elsevier, New York, pp 773–784

Hung SC, Chen NJ, Hsieh SL, Li H, Ma HL, Lo WH (2002) Isolation and characterization of size-sieved stem cells from human bone marrow. Stem Cells 20:249–258

Hung SC, Chang CF, Ma HL, Chen TH, Low-Tone Ho L (2004) Gene expression profiles of early adipogenesis in human mesenchymal stem cells. Gene 340:141–150

In 't Anker PS, Scherjon SA, Kleijburg-van der Keur C, de Groot-Swings GM, Claas FH, Fibbe WE, Kanhai HH (2004) Isolation of mesenchymal stem cells of fetal or maternal origin from human placenta. Stem Cells 22:1338–1345

Javazon EH, Beggs KJ, Flake AW (2004) Mesenchymal stem cells: paradoxes of passaging. Exp Hematol 32:414–425

Jeong JA, Hong SH, Gang EJ, Ahn C, Hwang SH, Yang IH, Han H, Kim H (2005) Differential gene expression profiling of human umbilical cord blood-derived mesenchymal stem cells by DNA microarray. Stem Cells 23:584–593

Jiang Y, Vaessen B, Lenvik T, Blackstad M, Reyes M, Verfaillie CM (2002) Multipotent progenitor cells can be isolated from postnatal murine bone marrow, muscle and brain. Exp Hematol 30:896–904

Kaiser J (2004) Gene therapy. Side effects sideline hemophilia trial. Science 304:1423–1425

Kassem M, Kristiansen M, Abdallah BM (2004) Mesenchymal stem cells: cell biology and potential use in therapy. Basic Clin Pharmacol Toxicol 95:209–214

Khosrotehrani K, Johnson KL, Cha DH, Salomon RN, Bianchi DW (2004) Transfer of fetal cells with multilineage potential to maternal tissue. JAMA 292:75–80

Kinnaird T, Stabile E, Burnett MS, Shou M, Lee CW, Barr S, Fuchs S, Epstein SE (2004) Local delivery of marrow-derived stromal cells augments collateral perfusion through paracrine mechanisms. Circulation 109:1543–1549

Kobayashi N, Yasu T, Ueba H, Sata M, Hashimoto S, Kuroki M, Saito M, Kawakami M (2004) Mechanical stress promotes the expression of smooth muscle-like properties in marrow stromal cells. Exp Hematol 32:1238–1245

Koç ON, Gerson SL, Cooper BW, Dyhouse SM, Haynesworth SE, Caplan AI, Lazarus HM (2000) Rapid hematopoietic recovery after coinfusion of autologous-blood stem cells and culture-expanded marrow mesenchymal stem cells in advanced breast cancer patients receiving high-dose chemotherapy. J Clin Oncol 18:307–316

Koç ON, Day J, Nieder M, Gerson SL, Lazarus HM, Krivit W (2002) Allogeneic mesenchymal stem cell infusion for treatment of metachromatic leukodystrophy (MLD) and Hurler syndrome (MPS-IH). Bone Marrow Transplant 30:215–222

Kojima S (1998) Hematopoietic growth factors and marrow stroma in aplastic anemia. Int J Hematol 68:19–28

Kopen GC, Prockop DJ, Phinney DG (1999) Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. Proc Natl Acad Sci U S A 96:10711–10716

Kotton DN, Ma BY, Cardoso WV, Sanderson EA, Summer RS, Williams MC, Fine A (2001) Bone marrow-derived cells as progenitors of lung alveolar epithelium. Development 128:5181–5188

Krampera M, Glennie S, Dyson J, Scott D, Laylor R, Simpson E, Dazzi F (2003) Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. Blood 101:3722–3729

Kuznetsov SA, Friedenstein AJ, Robey PG (1997) Factors required for bone marrow stromal fibroblast colony formation in vitro. Br J Haematol 97:561–570

Kuwana M, Okazaki Y, Kodama H, Izumi K, Yasuoka H, Ogawa Y, Kawakami Y, Ikeda Y (2003) Human circulating CD14+ monocytes as a source of progenitors that exhibit mesenchymal cell differentiation. J Leukoc Biol 74:833–845

Le Blanc K, Rasmusson I, Sundberg B, Gotherstrom C, Hassan M, Uzunel M, Ringden O (2004) Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. Lancet 363:1439–1441

Le Visage C, Dunham B, Flint P, Leong KW (2004) Coculture of mesenchymal stem cells and respiratory epithelial cells to engineer a human composite respiratory mucosa. Tissue Eng 10:1426–1435

Li WJ, Tuli R, Okafor C, Derfoul A, Danielson KG, Hall DJ, Tuan RS (2005) A three-dimensional nanofibrous scaffold for cartilage tissue engineering using human mesenchymal stem cells. Biomaterials 26:599–609

Lodie TA, Blickarz CE, Devarakonda TJ, He C, Dash AB, Clarke J, Gleneck K, Shihabuddin L, Tubo R (2002) Systematic analysis of reportedly distinct populations of multipotent bone marrow-derived stem cells reveals a lack of distinction. Tissue Eng 8:739–751

Mackay AM, Beck SC, Murphy JM, Barry FP, Chichester CO, Pittenger MF (1998) Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. Tissue Eng 4:415–428

Majumdar MK, Thiede MA, Mosca JD, Moorman M, Gerson SL (1998) Phenotypic and functional comparison of cultures of marrow-derived mesenchymal stem cells (MSCs) and stromal cells. J Cell Physiol 176:57–66

Makino S, Fukuda K, Miyoshi S, Konishi F, Kodama H, Pan J, Sano M, Takahashi T, Hori S, Abe H, Hata J, Umezawa A, Ogawa S (1999) Cardiomyocytes can be generated from marrow stromal cells in vitro. J Clin Invest 103:697–705

McBride C, Gaupp D, Phinney DG (2003) Quantifying levels of transplanted murine and human mesenchymal stem cells in vivo by real-time PCR. Cytotherapy 5:7–18

Meirelles Lda S, Nardi NB (2003) Murine marrow-derived mesenchymal stem cell: isolation, in vitro expansion, and characterization. Br J Haematol 123:702–711

Mihara K, Imai C, Coustan-Smith E, Dome JS, Dominici M, Vanin E, Campana D (2003) Development and functional characterization of human bone marrow mesenchymal cells immortalized by enforced expression of telomerase. Br J Haematol 120:846–849

Moscoso I, Centeno A, Lopez E, Rodriguez-Barbosa JI, Santamarina I, Filgueira P, Sanchez MJ, Dominguez-Perles R, Penuelas-Rivas G, Domenech N (2005) Differentiation "in vitro" of primary and immortalized porcine mesenchymal stem cells into cardiomyocytes for cell transplantation. Transplant Proc 37:481–482

Muraglia A, Cancedda R, Quarto R (2000) Clonal mesenchymal progenitors from human bone marrow differentiate in vitro according to a hierarchical model. J Cell Sci 113:1161–1166

Murry CE, Soonpaa MH, Reinecke H, Nakajima H, Nakajima HO, Rubart M, Pasumarthi KB, Virag JI, Bartelmez SH, Poppa V, Bradford G, Dowell JD, Williams DA, Field LJ (2004) Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. Nature 428:664–668

Nardi NB (2005) All the adult stem cells, where do they all come from? An external source for organ-specific stem cell pools. Med Hypoth 64:811–817

Nardi NB, Alfonso ZZC (1999) The hematopoietic stroma. Braz J Med Biol Res 32:601–609

Nathwani AC, Davidoff AM, Tuddenham EG (2004) Prospects for gene therapy of haemophilia. Haemophilia 10:309–318

O'Donoghue K, Choolani M, Chan J, de la Fuente J, Kumar S, Campagnoli C, Bennett PR, Roberts IA, Fisk NM (2003) Identification of fetal mesenchymal stem cells in maternal blood: implications for non-invasive prenatal diagnosis. Mol Hum Reprod 9:497–502

O'Donoghue K, Chan J, de la Fuente J, Kennea N, Sandison A, Anderson JR, Roberts IA, Fisk NM (2004) Microchimerism in female bone marrow and bone decades after fetal mesenchymal stem-cell trafficking in pregnancy. Lancet 364:179–182

Ogawa M (1993) Differentiation and proliferation of hematopoietic stem cells. Blood 81:2844–2853

Ohmi K, Greenberg DS, Rajavel KS, Ryazantsev S, Li HH, Neufeld EF (2003) Activated microglia in cortex of mouse models of mucopolysaccharidoses I and IIIB. Proc Natl Acad Sci U S A 100:1902–1907

Olivares EL, Ribeiro VP, Werneck de Castro JP, Ribeiro KC, Mattos EC, Goldenberg RC, Mill JG, Dohmann HF, dos Santos RR, de Carvalho AC, Masuda MO (2004) Bone marrow stromal cells improve cardiac performance in healed infarcted rat hearts. Am J Physiol Heart Circ Physiol 287:H464–H470

Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, Pickel J, McKay R, Nadal-Ginard B, Bodine DM, Leri A, Anversa P (2001) Bone marrow cells regenerate infarcted myocardium. Nature 410:701–705

Ortiz LA, Gambelli F, McBride C, Gaupp D, Baddoo M, Kaminski N, Phinney DG (2003) Mesenchymal stem cell engraftment in lung is enhanced in response to bleomycin exposure and ameliorates its fibrotic effects. Proc Natl Acad Sci U S A 100:8407–8411

Otto WR, Rao J (2004) Tomorrow's skeleton staff: mesenchymal stem cells and the repair of bone and cartilage. Cell Prolif 37:97–110

Owen M (1985) Lineage of osteogenic cells and their relationship to the stromal system. In: Peck WA (ed) Bone and mineral research. Vol. 3. Elsevier, New York, pp 1–25

Panepucci RA, Siufi JL, Silva WA Jr, Proto-Siquiera R, Neder L, Orellana M, Rocha V, Covas DT, Zago MA (2004) Comparison of gene expression of umbilical cord vein and bone marrow-derived mesenchymal stem cells. Stem Cells 22:1263–1278

Pereira RF, Halford KW, O'Hara MD, Leeper DB, Sokolov BP, Pollard MD, Bagasra O, Prockop DJ (1995) Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage, and lung in irradiated mice. Proc Natl Acad Sci U S A 92:4857–4861

Pereira RF, O'Hara MD, Laptev AV, Halford KW, Pollard MD, Class R, Simon D, Livezey K, Prockop DJ (1998) Marrow stromal cells as a source of progenitor cells for nonhematopoietic tissues in transgenic mice with a phenotype of osteogenesis imperfecta. Proc Natl Acad Sci U S A 95:1142–1147

Perin EC, Dohmann HF, Borojevic R, Silva SA, Sousa AL, Mesquita CT, Rossi MI, Carvalho AC, Dutra HS, Dohmann HJ, Silva GV, Belem L, Vivacqua R, Rangel FO, Esporcatte R, Geng YJ, Vaughn WK, Assad JA, Mesquita ET, Willerson JT (2003) Transendocardial, autologous bone marrow cell transplantation for severe, chronic ischemic heart failure. Circulation 107:2294–2302

Phinney DG (2002) Building a consensus regarding the nature and origin of mesenchymal stem cells. J Cell Biochem Suppl 38:7–12

Phinney DG, Kopen G, Isaacson RL, Prockop DJ (1999) Plastic adherent stromal cells from the bone marrow of commonly used strains of inbred mice: variations in yield, growth, and differentiation. J Cell Biochem 72:570–585

Pittenger MF, Martin BJ (2004) Mesenchymal stem cells and their potential as cardiac therapeutics. Circ Res 95:9–20

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147

Pochampally RR, Neville BT, Schwarz EJ, Li MM, Prockop DJ (2004) Rat adult stem cells (marrow stromal cells) engraft and differentiate in chick embryos without evidence of cell fusion. Proc Natl Acad Sci U S A 101:9282–9285

Potapova I, Plotnikov A, Lu Z, Danilo P Jr, Valiunas V, Qu J, Doronin S, Zuckerman J, Shlapakova IN, Gao J, Pan Z, Herron AJ, Robinson RB, Brink PR, Rosen MR, Cohen IS (2004) Human mesenchymal stem cells as a gene delivery system to create cardiac pacemakers. Circ Res 94:952–959

Pranke P, Failace RR, Allebrandt WF, Steibel G, Schmidt F, Nardi N (2001) Hematologic and immunophenotypic characterization of human umbilical cord blood. Acta Haematol 105:71–76

Prockop DJ (1997) Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 276:71–74

Reyes M, Verfaillie CM (2001) Characterization of multipotent adult progenitor cells, a subpopulation of mesenchymal stem cells. Ann N Y Acad Sci 938:231–233

Reyes M, Lund T, Lenvik T, Aguiar D, Koodie L, Verfaillie CM (2001) Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells. Blood 98:2615–2625

Roelen BA, Dijke P (2003) Controlling mesenchymal stem cell differentiation by TGFbeta family members. J Orthop Sci 8:740–748

Rombouts WJ, Ploemacher RE (2003) Primary murine MSC show highly efficient homing to the bone marrow but lose homing ability following culture. Leukemia 17:160–170

Safford KM, Hicok KC, Safford SD, Halvorsen YD, Wilkison WO, Gimble JM, Rice HE (2002) Neurogenic differentiation of murine and human adipose-derived stromal cells. Biochem Biophys Res Commun 294:371–379

Salingcarnboriboon R, Yoshitake H, Tsuji K, Obinata M, Amagasa T, Nifuji A, Noda M (2003) Establishment of tendon-derived cell lines exhibiting pluripotent mesenchymal stem cell-like property. Exp Cell Res 287:289–300

Sanchez-Ramos J, Song S, Cardozo-Pelaez F, Hazzi C, Stedeford T, Willing A, Freeman TB, Saporta S, Janssen W, Patel N, Cooper DR, Sanberg PR (2000) Adult bone marrow stromal cells differentiate into neural cells in vitro. Exp Neurol 164:247–256

Sato Y, Araki H, Kato J, Nakamura K, Kawano Y, Kobune M, Sato T, Miyanishi K, Takayama T, Takahashi M, Takimoto R, Iyama S, Matsunaga T, Ohtani S, Matsuura A, Hamada H, Niitsu Y (2005) Human mesenchymal stem cells xenografted directly to rat liver differentiated into human hepatocytes without fusion. Blood Apr 7; [Epub ahead of print]

Sekiya I, Larson BL, Smith JR, Pochampally R, Cui JG, Prockop DJ (2002) Expansion of human adult stem cells from bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality. Stem Cells 20:530–541

Selkirk SM (2004) Gene therapy in clinical medicine. Postgrad Med J 80:560–570

Seshi B, Kumar S, Sellers D (2000) Human bone marrow stromal cell: coexpression of markers specific for multiple mesenchymal cell lineages. Blood Cells Mol Dis 26:234–246

Short B, Brouard N, Occhiodoro-Scott T, Ramakrishnan A, Simmons PJ (2003) Mesenchymal stem cells. Arch Med Res 34:565–571

Silva WA Jr, Covas DT, Panepucci RA, Proto-Siqueira R, Siufi JL, Zanette DL, Santos AR, Zago MA (2003) The profile of gene expression of human marrow mesenchymal stem cells. Stem Cells 21:661–669

Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, Henkelman RM, Cusimano MD, Dirks PB (2004) Identification of human brain tumour initiating cells. Nature 432:396–401

Spees JL, Olson SD, Ylostalo J, Lynch PJ, Smith J, Perry A, Peister A, Wang MY, Prockop DJ (2003) Differentiation, cell fusion, and nuclear fusion during ex vivo repair of epithelium by human adult stem cells from bone marrow stroma. Proc Natl Acad Sci U S A 100:2397–2402

Studeny M, Marini FC, Champlin RE, Zompetta C, Fidler IJ, Andreeff M (2002) Bone marrow-derived mesenchymal stem cells as vehicles for interferon-beta delivery into tumors. Cancer Res 62:3603–3608

Studeny M, Marini FC, Dembinski JL, Zompetta C, Cabreira-Hansen M, Bekele BN, Champlin RE, Andreeff M (2004) Mesenchymal stem cells: potential precursors for tumor stroma and targeted-delivery vehicles for anticancer agents. J Natl Cancer Inst 96:1593–1603

Stute N, Holtz K, Bubenheim M, Lange C, Blake F, Zander AR (2004) Autologous serum for isolation and expansion of human mesenchymal stem cells for clinical use. Exp Hematol 32:1212–1225

Tang DG, Tokumoto YM, Apperly JA, Lloyd AC, Raff MC (2001) Lack of replicative senescence in cultured rat oligodendrocyte precursor cells. Science 291:868–871

Tavassoli M (1984). Marrow adipose cells and hemopoiesis: an interpretative review. Exp Hematol 12:139–146

Terada N, Hamazaki T, Oka M, Hoki M, Mastalerz DM, Nakano Y, Meyer EM, Morel L, Petersen BE, Scott EW (2002) Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. Nature 416:542–545

Tremain N, Korkko J, Ibberson D, Kopen GC, DiGirolamo C, Phinney DG (2001) MicroSAGE analysis of 2,353 expressed genes in a single cell-derived colony of undifferentiated human mesenchymal stem cells reveals mRNAs of multiple cell lineages. Stem Cells 19:408–418

Tuli R, Seghatoleslami MR, Tuli S, Wang ML, Hozack WJ, Manner PA, Danielson KG, Tuan RS (2003) A simple, high-yield method for obtaining multipotential mesenchymal progenitor cells from trabecular bone. Mol Biotechnol 23:37–49

Verfaillie CM (1992) Direct contact between human primitive hematopoietic progenitors and bone marrow stroma is not required for long-term in vitro hematopoiesis. Blood 79:2821–2826

Verfaillie CM (2002) Adult stem cells: assessing the case for pluripotency. Trends Cell Biol 12:502–508

Wakitani S, Saito T, Caplan AI (1995) Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine. Muscle Nerve 18:1417–1426

Wang Q-R, Yan Z-J, Wolf NS (1990) Dissecting the hematopoietic microenvironment. VI. The effects of several growth factors on the growth of murine bone marrow CFU-F. Exp Hematol 18:341–347

Watt FM, Hogan BL (2000) Out of Eden: stem cells and their niches. Science 287:1427–1430

Wexler SA, Donaldson C, Denning-Kendall P, Rice C, Bradley B, Hows JM (2003) Adult bone marrow is a rich source of human mesenchymal 'stem' cells but umbilical cord and mobilized adult blood are not. Br J Haematol 121:368–374

Whetton AD, Grahan GJ (1999) Homing and mobilization in the stem cell niche. Trends Cell Biol 9:233–238

Woodbury D, Schwarz EJ, Prockop DJ, Black IB (2000) Adult rat and human bone marrow stromal cells differentiate into neurons. J Neurosci Res 61:364–370

Wynn RF, Hart CA, Corradi-Perini C, O'Neill L, Evans CA, Wraith JE, Fairbairn LJ, Bellantuono I (2004) A small proportion of mesenchymal stem cells strongly expresses functionally active CXCR4 receptor capable of promoting migration to bone marrow. Blood 104:2643–2645

Ying QL, Nichols J, Evans EP, Smith AG (2002) Changing potency by spontaneous fusion. Nature 416:545–548

Young HE, Steele TA, Bray RA, Hudson J, Floyd JA, Hawkins K, Thomas K, Austin T, Edwards C, Cuzzourt J, Duenzl M, Lucas PA, Black AC Jr (2001) Human reserve pluripotent mesenchymal stem cells are present in the connective tissues of skeletal muscle and dermis derived from fetal, adult, and geriatric donors. Anat Rec 264:51–62

Zipori D (2004) Mesenchymal stem cells: harnessing cell plasticity to tissue and organ repair. Blood Cells Mol Dis 33:211–215

Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH (2001) Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 7:211–228

Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH (2002) Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 13:4279–4295

Functional characterization of cell hybrids generated by induced fusion of primary porcine mesenchymal stem cells with an immortal murine cell line.

Islam MQ, Ringe J, Reichmann E, Migotti R, Sittinger M, da S Meirelles L, Nardi NB, Magnusson P, Islam K.

Cell Tissue Res. 2006 Oct;326(1):123-37.

Cell Tissue Res
DOI 10.1007/s00441-006-0224-2

REGULAR ARTICLE

Functional characterization of cell hybrids generated by induced fusion of primary porcine mesenchymal stem cells with an immortal murine cell line

M. Q. Islam · J. Ringe · E. Reichmann · R. Migotti · M. Sittinger · L. da S. Meirelles · N. B. Nardi · P. Magnusson · K. Islam

Received: 17 January 2006 / Accepted: 11 April 2006
© Springer-Verlag 2006

Abstract Bone marrow mesenchymal stem cells (MSC) integrate into various organs and contribute to the regeneration of diverse tissues. However, the mechanistic basis of the plasticity of MSC is not fully understood. The change of cell fate has been suggested to occur through cell fusion. We have generated hybrid cell lines by polyethylene-glycol-mediated cell fusion of primary porcine MSC with the immortal murine fibroblast cell line F7, a derivative of the GM05267 cell line. The hybrid cell lines display fibroblastic morphology and proliferate like immortal cells. They contain tetraploid to hexaploid porcine chromosomes accompanied by hypo-diploid murine chromosomes. Interestingly, many hybrid cell lines also express high levels of tissue-nonspecific alkaline phosphatase, which is considered to be a marker of undifferentiated embryonic stem cells. All tested hybrid cell lines retain osteogenic differentiation, a few of them also retain adipogenic potential, but none retain chondrogenic differentiation. Conditioned media from hybrid cells enhance the proliferation of both early-passage and late-passage porcine MSC, indicating that the hybrid cells secrete diffusible growth stimulatory factors. Murine F7 cells thus have the unique property of generating immortal cell hybrids containing unusually high numbers of chromosomes derived from normal cells. These hybrid cells can be employed in various studies to improve our understanding of regenerative biology. This is the first report, to our knowledge, describing the generation of experimentally induced cell hybrids by using normal primary MSC.

Keywords Mesenchymal stem cell · Cell hybrid · Reprogramming · Immortality · Growth factor · Mouse · Pig

M. Q. Islam · K. Islam
Laboratory of Cancer Genetics, Laboratory Medicine Center (LMC), University Hospital Linkoping, SE-581 85 Linkoping, Sweden

M. Q. Islam · K. Islam
Department of Biomedicine and Surgery, Faculty of Health Sciences, Linkoping University, SE-581 85 Linkoping, Sweden

J. Ringe · E. Reichmann · R. Migotti · M. Sittinger
Tissue Engineering Laboratory, Department of Rheumatology, Charité University of Medicine Berlin, Tucholskystr 2, 10117 Berlin, Germany

L. da S. Meirelles · N. B. Nardi
Laboratorio de Imunogenetica, Departamento de Genetica, Universidade Federal do Rio Grande do Sul, Av. Bento Goncalves, Porto Alegre, RS, Brazil

P. Magnusson
Division of Clinical Chemistry, Department of Biomedicine and Surgery, Faculty of Health Sciences, Linkoping University, SE-581 85 Linkoping, Sweden

M. Q. Islam (✉)
Laboratory of Cancer Genetics, Main Building, Floor 11, Division of Clinical Chemistry, Department of Biomedicine and Surgery, Faculty of Health Sciences, Linkoping University, SE-581 85 Linkoping, Sweden
e-mail: quais@ibk.liu.se

Introduction

Mammalian somatic cells in vitro divide for a limited number of times before entering a non-dividing state called replicative senescence (Hayflick 1965). Cellular senescence is a complex phenotype characterized by irreversible G1 growth arrest. There are at least three major types of senescence: (1) replicative senescence, thought to be caused

by shortening of telomeres with successive cell divisions; (2) induced senescence, caused by forced expression of activated oncogenes in normal cells; (3) stress-induced senescence, resulting from cells cultured under sub-optimal conditions (Ben-Porath and Weinberg 2005).

In contrast to embryonic stem cells, somatic stem cells have a limited capacity for in vitro proliferation (Czyz et al. 2003). Somatic stem cells have been found to reside in a variety of tissues, e.g., bone marrow, muscle, heart, skin, intestine, liver, lung, prostate, central nervous system, and mammary gland (Poulsom et al. 2002; Young and Black 2004). Bone marrow is a major source of mesenchymal stem cells (MSC), which represent a population of plastic adherent non-hematopoietic cells that develop into mesenchymal tissues such as bone, cartilage, muscle, ligament, tendon, adipose, and stroma in vitro and in vivo (Baksh et al. 2004; Barry and Murphy 2004). Transplantation of MSC in humans and in animal models has demonstrated that they integrate into various organs and contribute to the regeneration of many cell lineages. Numerous studies have recently been undertaken to understand the mechanistic basis of somatic stem cell plasticity, i.e., the capacity of cells derived from one tissue type to form cells of other tissue types, but no clear result has emerged yet. Recently, cell fusion has received much attention as one explanation of stem cell plasticity in vivo (Vassilopoulos and Russell 2003; Camargo et al. 2004). However, convincing results are still required before accepting this hypothesis (Eisenberg and Eisenberg 2003; Dahlke et al. 2004). More research in this field is therefore desirable to uncover the role of cell fusion in stem cell plasticity. In this respect, new findings might be useful for developing cell-based therapies (Barry and Murphy 2004; Camargo et al. 2004).

By fusing cells of embryonic origins (embryonic stem cells, ES; embryonic germ cells, EG; embryonal carcinoma cells, EC) with unlimited proliferation and somatic cells of limited proliferation, many investigators have demonstrated that hybrid cells can be generated with indefinite proliferation through the reprogramming of somatic cell nuclei (Takagi 1997; Pells et al. 2002; Flasza et al. 2003; Ambrosi and Rasmussen 2005; Cowan et al. 2005). To ascertain the reprogramming of the somatic cell genome, various transcription factors and other traits of pluripotent embryonic cells have been used as markers (Tada et al. 2003; Do and Scholer 2004, 2005; Ambrosi and Rasmussen 2005). These studies have in essence demonstrated that the inactive X chromosome derived from female somatic cell becomes activated (Takagi 1997; Tada and Tada 2001; Tada et al. 2003), and a range of epigenetic changes takes place during the reprogramming of the two genomes of the hybrid cells through various modifications of histone proteins and alterations of the status of DNA methylation (Forejt et al. 1999; Tada and Tada 2001; Flasza et al. 2003; Kimura et al. 2004). One major difference between the reprogramming potentials of ES and EG cells is that the fusion of EG cells with somatic cells often erases the epigenetic marks of both imprinted and non-imprinted genes of the two genomes, resulting in a switching of monoallelically expressed imprinted genes into biallelic forms, unlike the ES-derived hybrid cells where epigenetic markers of both genomes remain intact (Tada et al. 1997; Surani 2001). However, hybrid cells derived from both ES and EG cells retain limited development potentials (Tada et al. 1997, 2001; Matveeva et al. 1998).

Cell fusion studies involving the generation of somatic cell hybrids between immortal and normal cells have suggested that the phenotype of limited proliferation of the normal cell is dominant over the unlimited proliferation of the immortal cell (Tominaga et al. 2002). In contrast, we have recently found that the fusion of the immortal murine cell line F7 with normal porcine fibroblasts produces immortal cell hybrids containing near-tetraploid (4N) chromosomes derived from porcine fibroblasts (M.Q. Islam, V. Panduri, K. Islam, in preparation). Normal MSC divide in vitro only for 30–40 population doublings before they become senescent (Sethe et al. 2005). This study has been designed to investigate (1) whether the fusion of the murine F7 cell line with porcine MSC generates hybrid cells capable of dividing indefinitely, (2) whether the generated hybrid cells retain the 4N genome from the porcine MSC, (3) whether the hybrid cells express distinct characteristics of porcine MSC, and (4) whether the conditioned media of hybrid cells can stimulate the proliferation of parental porcine MSC.

Materials and methods

Isolation of porcine MSC

Porcine MSC were isolated as described previously (Ringe et al. 2002). Briefly, femur and tibia bones from 6–month-old to 8-month-old porcine donors were sawed open, and bone marrow was extracted under sterile conditions. Gelatinous bone marrow was suspended in phosphate-buffered saline (PBS) and dispersed mechanically by passing through syringes fitted with a series of 16-, 18-, and 20-gauge needles. Cells were centrifuged and plated in complete Dulbecco’s Modified Eagle’s Medium (DMEM, Biochrom) containing 10% fetal bovine serum (FBS, Biochrom), at a density of 300,000 cells/cm^2. The medium was changed after 72 h and every 2–3 days thereafter. At 90% confluency, monolayer cells were detached by the addition of a solution containing 0.5% trypsin-EDTA (Biochrom) and replated at a density of 6,000 cells/cm^2.

Murine parental cell line F7

The immortal murine cell line GM05267, obtained from the National Institutes of General Medical Sciences (NIGMS), is deficient for the enzyme hypoxanthine-phosphoribosyl-transferase (HPRT-) and consequently is sensitive to hypoxanthine-aminopterin-thymidine (HAT). This fibroblast cell line was originally isolated from a postnatal/adult kidney of a male mouse heterozygous for the tfm (testicular feminization locus) mutation (Migeon et al. 1981; Jabs et al. 1984). Subsequently, these cells were transformed with simian virus 40 and sub-clones were isolated that were resistant to the toxic effects of 6-thioguanine (6-TG^R) because of the lack of HPRT. Although the original 6-TG^R cells had the modal number of 64 chromosomes, the derivative cell line available at the NIGMS, designated as GM05267 (http://www.locus.umdnj.edu/nigms/nigms_cgi/display.cgi?GM05267), contains the modal number of 38 chromosomes.

The GM05267 cell line was maintained in DMEM, supplemented with 15% FBS, 1% DMEM nonessential amino acids, and 1% penicillin-streptomycin. Tissue culture reagents were obtained from PAA Laboratories, if not stated otherwise. In order to selectively isolate hybrid cells and to eliminate the two parental cell types, we introduced the G418-resistant gene to the GM05267 cell line by a modified calcium-phosphate co-precipitation method (Islam and Islam 2000). Briefly, exponentially growing cells were seeded into 60-mm Petri dishes. The following day, calcium-phosphate-DNA solution was added and incubated with the cells overnight. Selective medium (complete DMEM plus 1% G418, Invitrogen) was added, and cells were fed with fresh selective medium twice a week. After 3 weeks, a single colony of G418-resistant cells was transferred to a culture flask, and the resulting cell line was designated as F7 (M.Q. Islam, V. Panduri, K. Islam, in preparation).

Cell fusion and isolation of hybrid cell lines

Cell fusion was induced in co-cultured porcine MSC and F7 cells briefly treated with polyethylene glycol (PEG) as follows. The F7 cells (HAT-sensitive and G418-resistant) and the porcine MSC (HAT-resistant and G418-sensitive) were detached from culture flasks by trypsin-EDTA treatment. Approximately equal numbers of cells from each parent were mixed in a centrifuge tube and plated in 60-mm Petri dishes to achieve nearly confluent cultures after 3–4 h. At this stage, growth medium was discarded, and fresh serum-free medium containing phytohemagglutinin-P (100 μg/ml, Sigma) was added for 30 min at 37°C to increase cell-cell contact. After the medium had been discarded, 2 ml 45% PEG (Sigma, MW 1 500, prepared in serum-free DMEM, w/v) was added for 1 min at room temperature and then aspirated off. The cell layer was washed four times with serum-free medium and then incubated in 5 ml complete DMEM (containing 15% FBS and 1% penicillin/streptomycin) at 37°C for 30 min. Medium was then aspirated away and replaced with complete DMEM, followed by incubation overnight at 37°C. The cell layer was then dissociated by trypsinzation; half of the cells were cryopreseved and the other half was mixed with complete DMEM supplemented with 2% HAT (Invitrogen) and 1% G418. The cells were plated into four 100-mm Petri dishes and two T75 flasks. HAT medium eliminated the F7 cells, and the G418 eliminated the porcine MSC. After 7 days, macroscopic double-drug-resistant colonies of hybrid cells were visible. Independently derived HAT and G418 double-resistant colonies (*n*=17) of hybrid cells were isolated individually by using cloning rings and were expanded in separate culture flasks as clonal cell lines. The expanded 17 cell lines were derived from two independent cell fusion experiments, 11 from one fusion and six from the other. Two cell lines (Pool A and Pool B), from each of the two fusion experiments, were also established by mixing all hybrid cell colonies of T75 flasks. All cell lines were cryopreserved after multiplication.

Chromosome analysis

Chromosome preparation and the chromosome banding procedure were as described previously (Islam and Levan 1987). Metaphase cells (20–35) were captured from each cell line by a charge-coupled device camera by using CytoVision software program (Applied Imaging). The chromosomes of the parental cell lines/strains were identified and counted from the metaphase images by using CytoVison. In the hybrid cells, chromosomes belonging to the same species were counted together, and then the total number of chromosomes was determined by adding the chromosomes of two species.

Induction of cellular differentiation

Osteogenic differentiation was induced by culturing cells of a selected hybrid cell line in DMEM (15% FBS) supplemented with 100 nM dexamethasone (Dex; Fortecortin Mono 40, Merck), 0.05 mM L-ascorbicacid-2-phosphate (AsAP; Sigma), and 10 mM β-glycerophosphate (Sigma; Bellows et al. 1990; Jaiswal et al. 1997). Cells were fixed with ice-cold methanol at specific time points and used for further histochemical analysis.

For chondrogenic differentiation, cells of a selected hybrid cell line were centrifuged to form a pelleted micromass (Johnstone et al. 1998). Pelleted cells were cultured in a defined medium consisting of DMEM, ITS+1 (Sigma:

Table 1 Oligonucleotide sequences

Gene	Accession number	Oligonucleotides (5′→3′; up/down)	Product size in base pair
GAPDH	AF017079	AGG GGC TCT CCA GAA CAT CAT TTG GCA GTA GGG ACA CGG AAG G	117
aP2	AF102872	GGC ATG GCC AAA CCC AAC CT TGT ACC AGG GCG CCT CCA TCT A	179
PPAR-γ2	AF103946	TGG CGA TAT TTA TAG CTG TCA TTA TGT CCG TCT CTG TCT TCT TTA TTA	237

10 mg/l insulin, 5 mg/l transferrin, 5 μg/l selenium, 0.5 mg/ml bovine serum albumin, 4.7 μg/ml linoleic acid), 1 mM sodium-pyruvate (Sigma), 100 nM Dex (Sigma), 0.35 mM proline (Sigma), 0.17 mM AsAP, and 10 ng/ml transforming growth factor-β3 (R&D Systems). For histological staining, the pellets were cryopreserved in OCT (Sakura), and 6-μm-thick sections were used for further analysis.

To induce adipogenic differentiation, cells of a selected hybrid cell line were treated with adipogenic induction medium containing DMEM, 15% FBS, 1 μM Dex, 0.2 mM indomethacin (Sigma), 10 μg/ml insulin (Hoechst Marion Roussel), and 0.5 mM 3-isobutyl-1-methylxanthine (Sigma; Gimble et al. 1992; Pittenger et al. 1999). Cells were used for further histochemical analysis or were lyzed for total RNA isolation.

Histological methods and immunohistochemistry

Osteoblasts exhibit high levels of alkaline phosphatase (ALP), which can be visualized by staining with SIGMA FAST BCIP/NBT (Sigma). Von Kossa staining identifies the deposition of mineralized bone matrix.

Proteoglycan-secreting chondrocytes were stained with Alcian Blue 8GS (Roth) at pH 2.5. Adipocytes were identified morphologically and by staining with Oil Red O (Sigma). For immunohistochemistry of type II collagen, cryosections (6 μm) were incubated for 1 h with primary antibodies (rabbit anti-human type II collagen, DPC-Biermann). Subsequently, sections were incubated with biotinylated anti-rabbit antibody and peroxidase-conjugated streptavidine (Dako). The color reaction was developed by the AEC substrate kit (Dako), followed by counterstaining with hematoxylin (Merck).

Real-time reverse transcription/polymerase chain reaction

To demonstrate adipogenesis on the mRNA level, total RNA of porcine MSC and a selected set of hybrid cell lines was isolated as described previously (Chomczynski 1993). Subsequently, 5 μg total RNA was reverse-transcribed by using the iScript cDNA Synthesis Kit (BioRad) according to the manufacturer′s instructions. The housekeeping gene, D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used to normalize marker gene expression in each sample in various concentrations. Real-time polymerase chain reaction (PCR) with the i-Cycler system (BioRad) was performed with 1 μl cDNA sample by using the SYBR Green PCR Core Kit (Applied Biosystems). Relative quantitation of adipogenic marker gene expression (Table 1) was performed in an ABI Prism 7700 as detailed by the manufacturer and was recorded as the percentage of the GAPDH product.

Measurement of ALP activity and protein concentration

Confluent cultures of hybrid cell lines were rinsed with PBS and homogenized in 10 mM TRIS buffer (pH 8.3) containing 10 μM zinc acetate, 0.1 mM $MgCl_2$, and 0.1% Triton X-100. ALP activities of homogenized cells were determined in 96-well microtiter plates. In brief, a total volume of 300 μl solution containing 1.0 M diethanolamine buffer (pH 9.8), 10 mM p-nitrophenyl phosphate (Sigma), and 1.0 mM $MgCl_2$ was added per well. The time-dependent increase in absorbance at 405 nm (reflecting p-nitrophenol production) was determined on a kinetic microplate reader (Model VMax, Molecular Devices).

In humans, tissue-nonspecific ALP (TNALP) isoforms are expressed in many tissues including bone, liver, and kidney. Various heat inactivation and inhibition techniques can distinguish the ALP isoenzymes (Magnusson et al. 1992). Heating at 56°C for 15 min inactivates TNALP activity, particularly bone ALP activity. Heat inactivation at 65°C for 10 min inactivates 100% of TNALP and intestinal ALP activity, but placental and germ cell ALP are resistant at 65°C. L-Phenylalanine (10 mM) inhibits intestinal ALP activity, but other isoenzymes are resistant to this treatment.

Protein concentrations were determined with the bicinchoninic acid (BCA) method (Smith et al. 1985) by using the BCA Protein Assay Kit (Pierce, Rockford). Since ALP genes are highly conserved across species (Narisawa et al. 2005), we used the same method established for human cell systems to assay ALP activity in the hybrid cell lines.

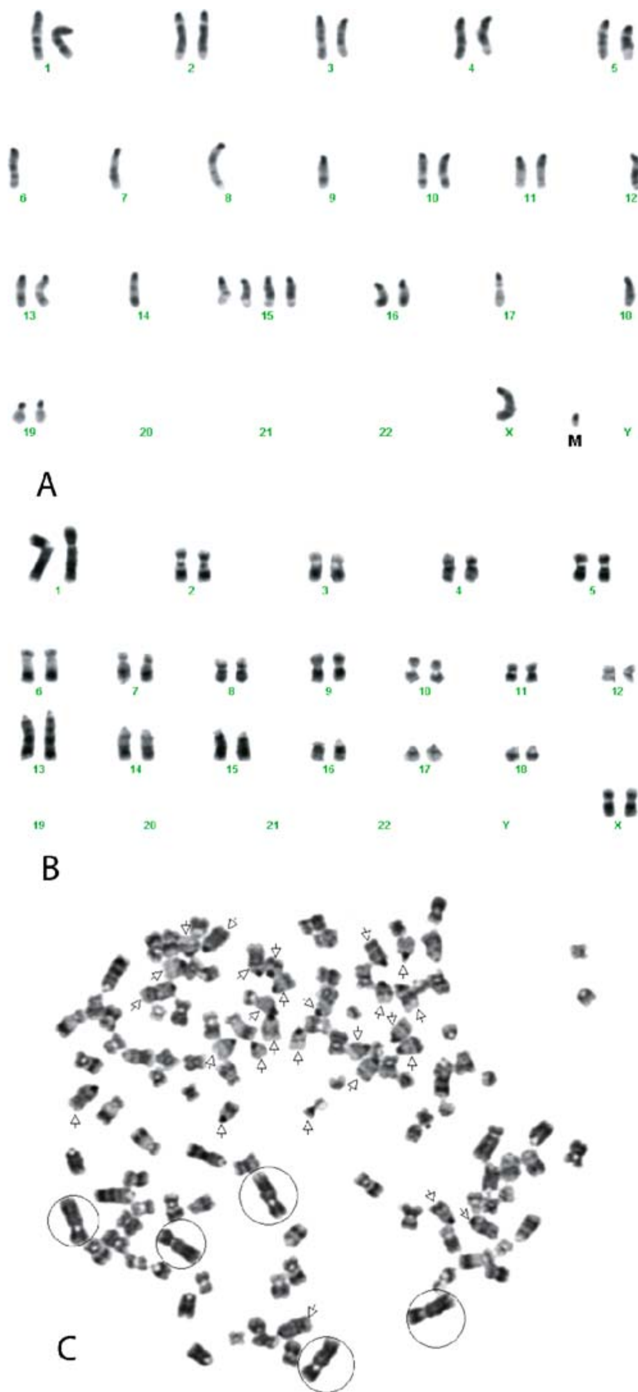

Fig. 1 Representative karyotypes of parental cells and derived hybrid cells. **a** Hypo-diploid karyotype of murine cell line F7 (*M* unidentified marker chromosome). Note that many chromosomes are represented by single copies including chromosome 9 and 17. **b** Diploid karyotype of porcine MSC. **c** A representative metaphase of hybrid cell line C4 containing a total of 124 chromosomes: 97 porcine (hyper-tetraploid, *circles* five copies of porcine chromosome 1) and 27 murine chromosomes (hypo-diploid, *arrows*)

Results

Inter-species porcine-murine hybrid (MSC-F7) cell lines were generated by fusing porcine MSC with the F7 murine cell line. Cell fusion was induced in co-cultured monolayers of two parental cell types followed by PEG treatment. To amplify the recombinant hybrid cells, the mixture of fused cells was plated in growth medium containing HAT (to eliminate F7 cells) and G418 (to eliminate porcine MSC). From these cells, 17 independently derived HAT and G418 double-resistant colonies of hybrids were isolated and expanded as clonal cell lines. Two cell lines (Pool A and Pool B), from each of the two fusion experiments, were also established by mixing all hybrid cell colonies that had developed in T75 flasks.

All MSC-F7 hybrid cell lines and two parental cell lines/strains were subjected to cytogenetic analysis by high-quality trypsin G-banding. The murine F7 cell line contained about 35 chromosomes, including single copies of chromosomes 6, 7, 8, 9, 12, 17, and 18, three to four copies of chromosome 15, and a single copy of the small marker chromosome M (Fig. 1a). The parental porcine MSC contained 38 normal chromosomes including two X chromosomes (Fig. 1b). Cytogenetic analyses of the 17 cell lines showed that 13 retained approximately 4N, and three retained approximately 6N porcine chromosomes, together with hypo-diploid murine chromosomes (Table 2, Fig. 1c). The hybrid cell lines retained about 22 murine chromosomes (Table 2) indicating that, following fusion, about 13 chromosomes were lost from the murine F7 cell parent. Retention of porcine chromosomes in the hybrid cell lines was extremely high compared with the murine chromosomes, with an average of 72 chromosomes, ranging from 56 to 119. The total number of chromosomes in the hybrid cell lines was about 94, ranging from 85 to 128. Thus, the ratio of murine to porcine chromosomes in the hybrid cells was approximately 1:4. Importantly, the chromosome number of all hybrid cell lines was highly variable, and therefore both murine and porcine genomes were represented by high anuploidy.

The morphology of all hybrid cell lines was fibroblastic, and the cells were large (Fig. 2). Closer examination revealed that minor morphological variations existed among the hybrid cell lines. In general, hybrid cell lines proliferated quickly (Fig. 3), but a few were slowly growing (e.g., C6, Fig. 2i, Table 2).

One cell line (Pool A) has been growing continuously in culture for more than a year and has exceeded 200 population doublings. So far, this cell line has not exhibited any sign of growth retardation. Interestingly, all these cell lines lack contact inhibition of growth and consequently produce multiple cell layers on plastics.

Since the in vitro expanded parental porcine MSC were capable of differentiating into different lineages (Ringe et al. 2002), a selected set of hybrid cell lines were investigated by using various differentiation induction protocols. The results of these investigations demonstrated

Table 2 Cytogenetic characterization of cell hybrids generated by fusion of primary porcine MSC with immortal murine cell line F7. All chromosome numbers represent mean values

Hybrid line	Murine chromosomes	Range	Porcine chromosomes	Range	Total chromosomes	Range	No. of cells analyzed	Hybrid type containing M and P[a]	Relative cell growth[b]
MSC-F7-C1	27	14–31	70	63–83	97	88–112	21	1:2=M/P/P	++
MSC-F7-C2	23	9–29	71	59–77	94	77–104	25	1:2=M/P/P	++++
MSC-F7-C3	27	20–42	79	73–119	106	97–161	24	1:2=M/P/P	++++
MSC-F7-C4	29	18–32	93	67–111	122	88–142	25	1:3=M/P/P/P	+++++
MSC-F7-C5	21	8–30	71	33–91	91	48–114	29	1:2=M/P/P	++++
MSC-F7-C6	24	19–28	68	61–75	92	86–97	21	1:2=M/P/P	+
MSC-F7-C7	23	10–34	105	69–138	128	93–164	23	1:3=M/P/P/P	++++
MSC-F7-C8	28	22–32	68	63–73	96	90–102	21	1:2=M/P/P	++
MSC-F7-C9	27	18–34	71	66–78	98	83–106	24	1:2=M/P/P	++
MSC-F7-C10	29	24–39	61	47–68	91	76–106	23	1:2=M/P/P	++
MSC-F7-C11	17	10–23	70	66–75	87	83–97	28	1:2=M/P/P	++
MSC-F7-C12	29	19–33	74	66–84	103	95–112	22	1:2=M/P/P	++++
MSC-F7-C13	21	16–25	67	66–73	88	90–96	24	1:2=M/P/P	++
MSC-F7-C14	25	10–31	96	82–105	121	105–131	20	1:3=M/P/P/P	+++
MSC-F7-C15	13	8–19	72	65–76	85	81–92	27	1:2=M/P/P	+++
MSC-F7-C16	18	7–33	72	56–94	90	71–127	28	1:2=M/P/P	++
MSC-F7-C17	26	17–33	70	57–84	97	74–117	35	1:2=M/P/P	+++
Mean	22		72		94				
Murine F7	35	33–36					24		
Porcine MSC	38						30		

[a]Each M and P represents one diploid cell

[b]Qualitative measurement from slowly growing (+) to rapid growth (+++++)

that the tested cell lines retained osteogenic differentiation (Table 3, Figs. 4, 5), a few of them also retaining adipogenic potential (Figs. 6, 7); none of them retained chondrogenic potential (Table 3).

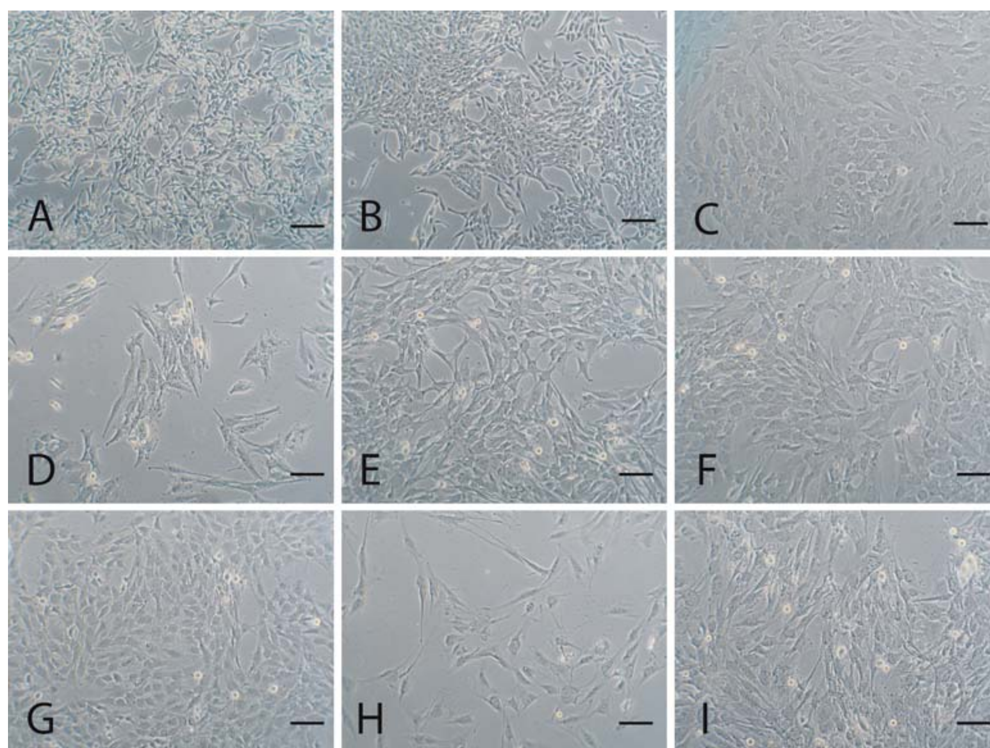

Fig. 2 Phase-contrast photomicrographs of murine F7 parental cells plated at low (**a**) and high (**b**) cell densities, porcine MSC (**c**), hybrid cell line C1 (**d**), hybrid cell line C2 (**e**), hybrid cell line C4 (**f**), hybrid cell line C5 (**g**), hybrid cell line C6 (**h**), and hybrid cell line C14 (**i**). The porcine MSC and all hybrid cell lines were photographed at passage 4. *Bars* 100 μm

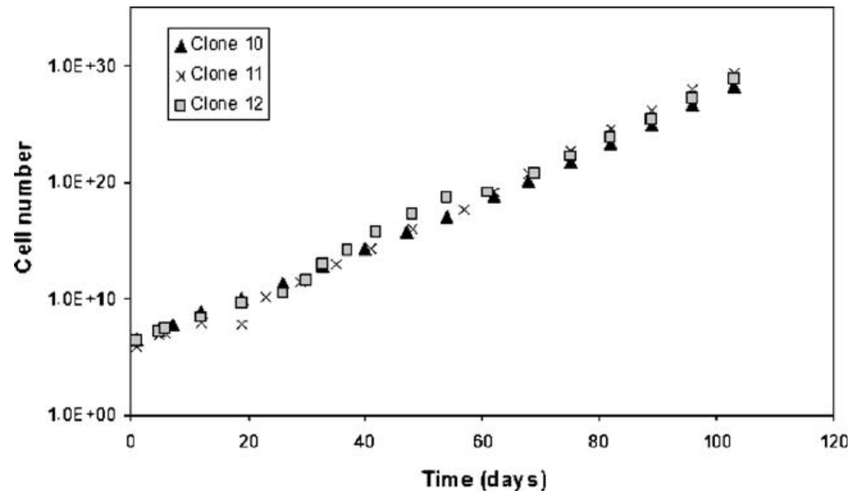

Fig. 3 Growth kinetics of the hybrid cell lines C10, C11, and C12 (*scale left*: *1,0E+10* 1.0×10^{10}, etc.). Hybrid cells were expanded for up to 103 days in monolayer culture. Cultures starting with fewer than 2×10^{6} cells expanded to more than 1×10^{29} cells after 103 days. The average specific growth rate (μ_{max}) of the cell lines was about $5.1\times10^{-1}\pm2.48\times10^{-2}$/day

We tested all hybrid cell lines for the expression of TNALP. The results showed that all hybrid cell lines expressed TNALP, although there was wide range variation for the amount of expressed enzyme among the cells lines (Table 4). Variable expression of TNALP enzyme by the hybrid cell lines was not unexpected because they contained variable numbers of the TNALP structural gene (ALPL), as the hybrid cell lines were highly aneuploid (Table 2).

Since the MSC-F7 hybrid cells were highly proliferative, we collected conditioned media from hybrid cell lines Pool A and Pool B at different time points to determine whether they secreted growth stimulatory factors into the media. The early-passage porcine MSC grown in continuous culture for 10 months in the presence of 20% conditioned medium showed the morphology of freshly isolated MSC with high mitotic activity (Fig. 8a), compared with the same cells that were grown without conditioned media and that exhibited signs of aging (Fig. 8b). Despite prolonged passaging in conditioned media, these cells maintained their diploid karyotype (Fig. 2b). The MSC grown without the addition of conditioned media showed mitotic activity within 5 days when sub-cultured and treated with hybrid-cell-derived conditioned media and, within 20 days, these cells acquired a morphology similar to that of freshly isolated porcine MSC (Fig. 8a,c). On the contrary, hybrid cells grown for more than 1 year as continuous cultures in standard DMEM containing 15% FBS showed high mitotic

Table 3 Differentiation of a selected set of hybrid cell lines generated by fusion of primary porcine MSC with immortal murine cell line F7

Hybrid cell line	Osteogenic differentiation[a]	Chondrogenic differentiation[b]	Adipogenic differentiation
MSC-F7-C3	+	ND	Cells contracted and detached before they could fully differentiate into adipocytes.
MSC-F7-C4	+++	ND	Cells contracted and detached before any sign of adipogenic differentiation could be detected
MSC-F7-C5	+++	ND	Cells contracted and detached before any sign of adipogenic differentiation could be detected
MSC-F7-C7	+++	ND	Cells contracted and detached before any sign of adipogenic differentiation could be detected
MSC-F7-C10	+++	–	Adipogenic differentiation of hybrid cells was weaker than the parental porcine MSC
MSC-F7-C11	+++	–	Adipogenic differentiation of hybrid cells was much weaker than the parental porcine MSC
MSC-F7-C12	+++	–	Adipogenic differentiation of hybrid cells was much weaker than the parental porcine MSC

[a]Qualitative measurement from low differentiation (+) to high differentiation (+++)
[b]*ND* not done, − no differentiation

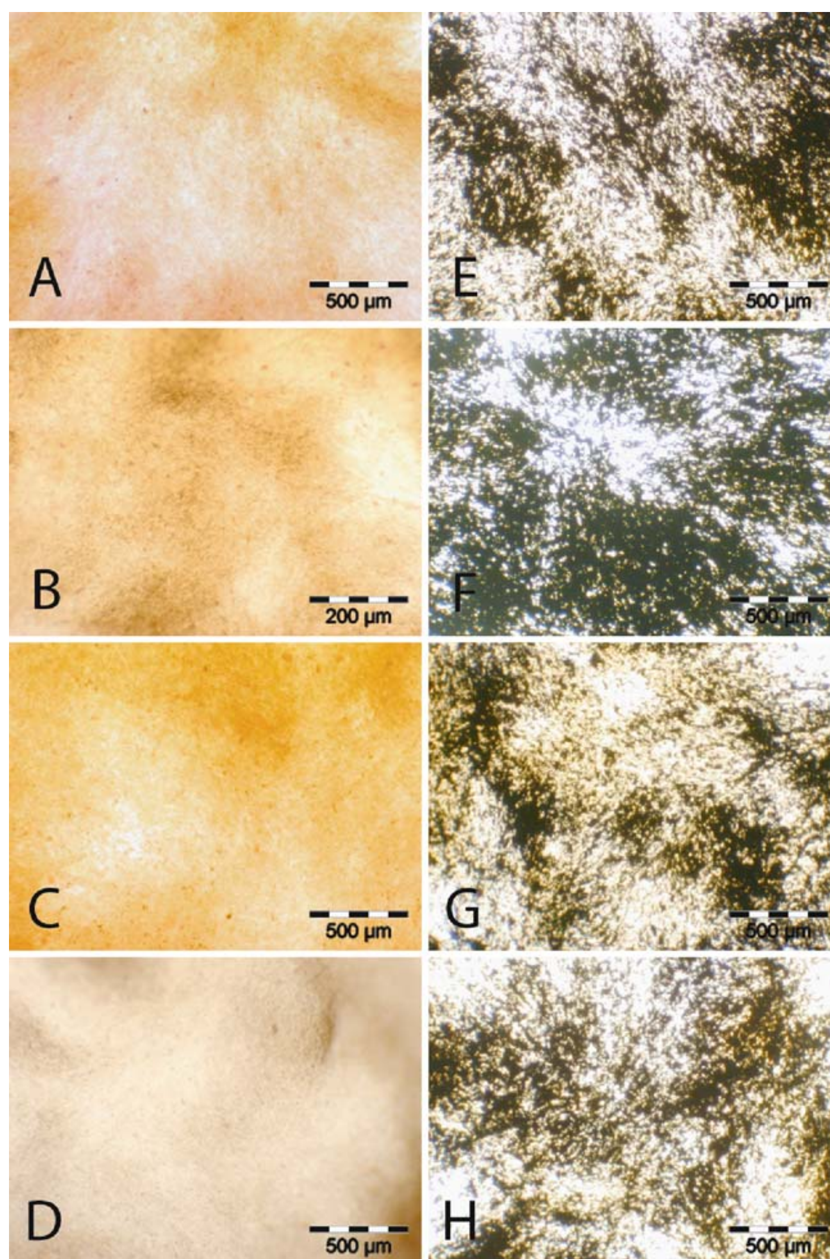

Fig. 4 Osteogenic differentiation of the hybrid cell line C10 documented by von Kossa staining of the mineralized bone matrix (**a**, **e** day 7, **b**, **f** day 14, **c**, **g** day 21, **d**, **h** day 28). **a–d** Untreated control cultures were negative for von Kossa staining. **e–h** Dexamethasone-stimulated hybrid cells started to form a calcified matrix and therefore were von-Kossa-positive. In contrast to untreated controls, induced hybrid cells were von-Kossa-positive on day 7

activity with no signs of aging (Fig. 8d). These results indicated that the MSC-F7 hybrid cells secreted diffusible factors into the culture media; these enhanced their own growth and that of other cells.

Discussion

We have demonstrated that the fusion of normal primary porcine MSC with the immortal murine fibroblast cell line F7 generates immortal hybrid cells containing 4N to 6N porcine chromosomes. This result indicates that whatever chromosome numbers may come from normal cell during fusion, the immortal F7 cell is capable of erasing the memory of the normal cell and resetting it into the immortal cell program. This unusual species-specific combination of genomes has not previously been described for any experimentally induced somatic cell hybrid. Although the spontaneous fusion of MSC, both in vitro and in vivo, with other cell types is known to occur (Vassilopoulos and

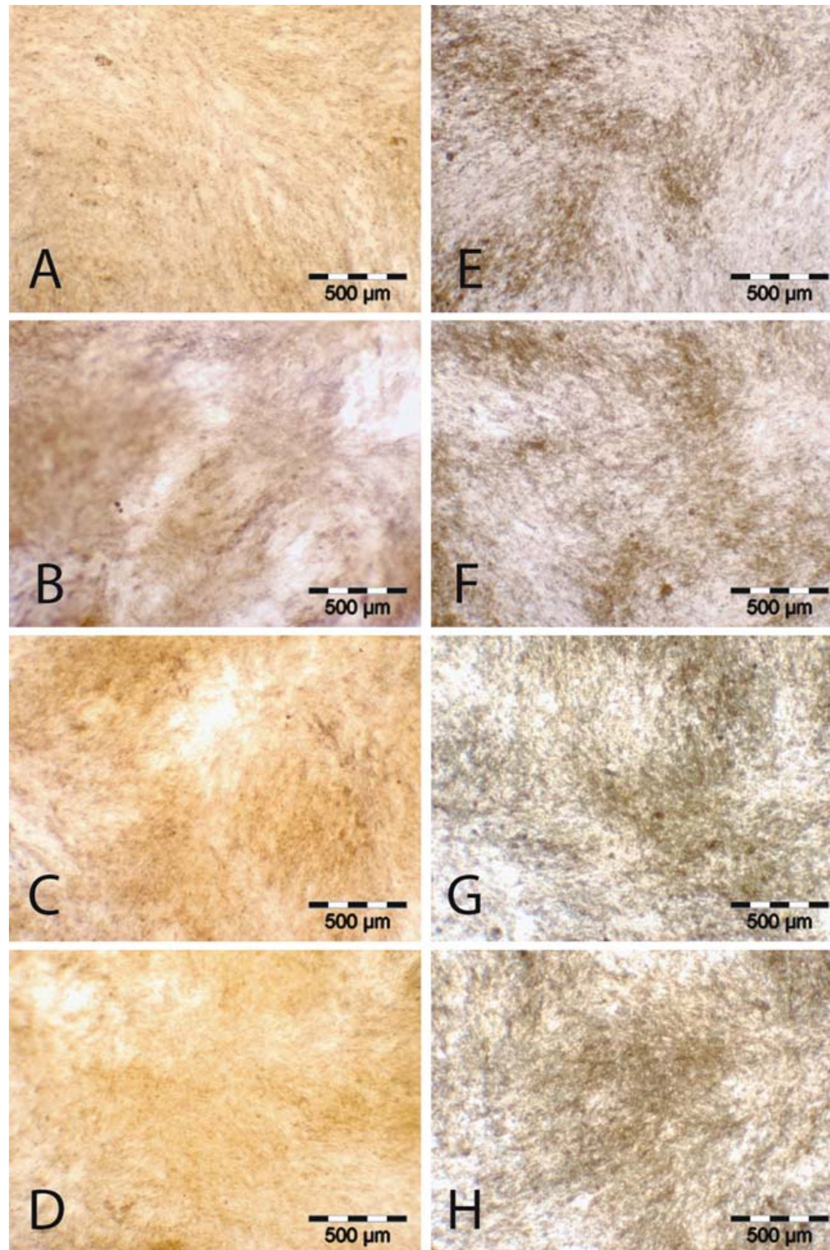

Fig. 5 Osteogenic differentiation potential of the hybrid cell line C10 documented by alkaline phosphatase (ALP) activity (**a**, **e** day 7, **b**, **f** day 14, **c**, **g** day 21, **d**, **h** day 28). **a–d** Controls showed low ALP activities during the whole culture period. **e–h** Osteogenic stimulation resulted in increased ALP activity that peaked at day 7

Russell 2003; Camargo et al. 2004; Que et al. 2004; Rodic et al. 2004), this is the first report, to our knowledge, describing the generation of experimentally induced cell hybrids by fusion of primary porcine MSC with an immortal cell line. The retention of an unusually high number of normal cell chromosomes is not limited to the MSC-F7 hybrid cell line. Similar hybrid cell lines have been generated by fusing normal porcine fibroblasts with F7 cells (M.Q. Islam, V. Panduri, K. Islam, in preparation). The fusion of F7 cells with normal porcine fibroblasts (both proliferating and senescent) containing the complete genome, but not with immortal cells containing a deleted genome, consistently produces hybrid cells with activated growth accompanied by a nearly 4N porcine genome. Introduction of the human telomerase gene (hTERT) into the immortal cell line and the subsequent fusion of these cells with F7 cells does not improve the growth of the resulting hybrid cells, indicating that a telomerase-independent mechanism is responsible for the activated growth phenotype.

We have previously demonstrated that porcine MSC can be induced to differentiate into various cell lineages

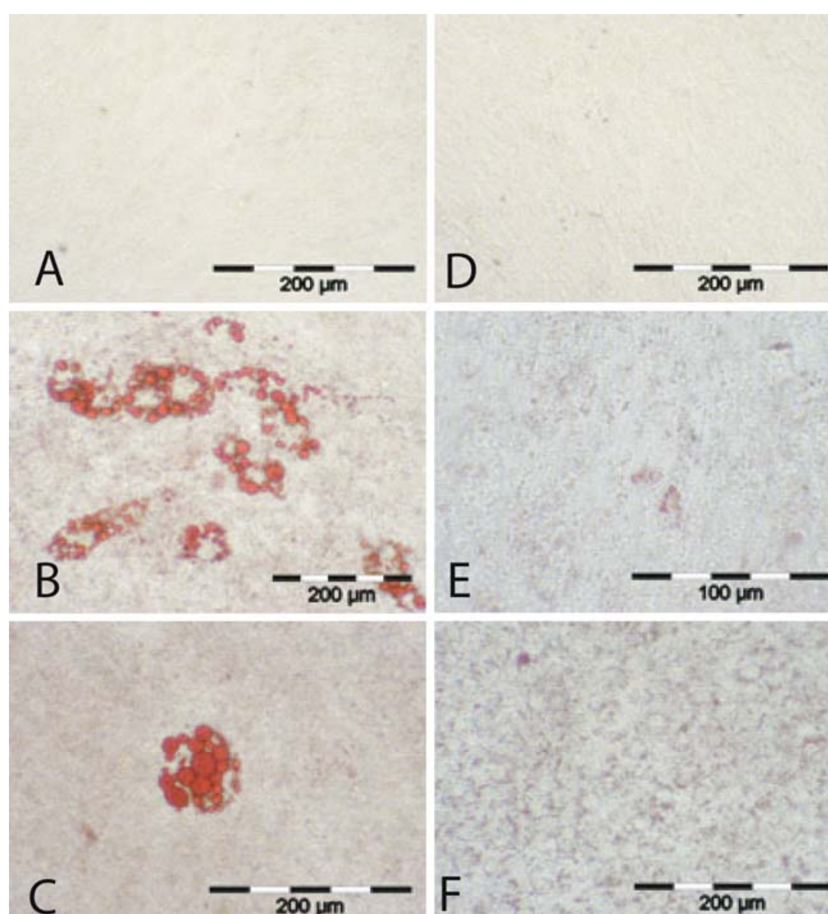

Fig. 6 Adipogenic differentiation potential of the hybrid cell line C10 documented by Oil red O staining of lipid droplets (**a**, **d** day 5, **b**, **e** day 15, **c**, **f** day 22). **a–c** Induced. **d–f** Untreated control

including bone, cartilage, and fat (Ringe et al. 2002). In the present study, we have shown that the hybrid cell lines retain full potential for osteogenic differentiation, partial potential for adipogenic differentiation, but no potential for chondrogenic differentiation. Several investigators have reported that chondrogenic differentiation is a sensitive phenotype that can be influenced by cell culture conditions and by genetic factors (Muraglia et al. 2000; Baddoo et al. 2003; Gregory et al. 2005; Magne et al. 2005; Sethe et al. 2005; Vacanti et al. 2005). The reprogramming of two genomes in hybrid cells is also known to be a general phenomenon during which the expression of previously silent genes and the extinction of previously expressed genes can occur, particularly if the hybrid cells are generated by fusing dissimilar cell types (Gourdeau and Fournier 1990; Takagi 1997; Surani 2001; Ambrosi and Rasmussen 2005). Whether the loss of chondrogenic potential in the hybrid cells is attributable to the silencing of a specific porcine gene(s) required for the differentiation pathway or because of interspecies genome incompatibility is currently unknown.

Although TNALP is one of the most consistent markers of embryonic stem cells (Carpenter et al. 2003), this enzyme is not expressed at a significant level in adult stem cells. A recent report, however, indicates that pluripotent epiblastic-like adult stem cells express high levels of TNALP. Interestingly, these pluripotent stem cells lack contact-inhibited cell growth and consequently form multiple cell layers on plastics (Young and Black 2004). Since the MSC-F7 hybrid cells also exhibit these features, we have tested all hybrid cell lines for the expression of TNALP. Interestingly, we have found that some of the MSC-F7 hybrid cell lines, particularly the C5, C7, C10, C11, C15, Pool A, and Pool B lines, produce high levels of TNALP in contrast to the immortal fibroblast cell line F7 (Table 4). Previous somatic cell genetic studies have revealed that the expression of this enzyme is extinguished upon cell fusion and re-expressed when substantial numbers of chromosomes are ejected from hybrid cells (Johnson-Pais and Leach 1995, 1996). Our limited data indicate that prolonged culture of hybrid cells in vitro has a positive effect on the expression of TNALP (Table 4). One

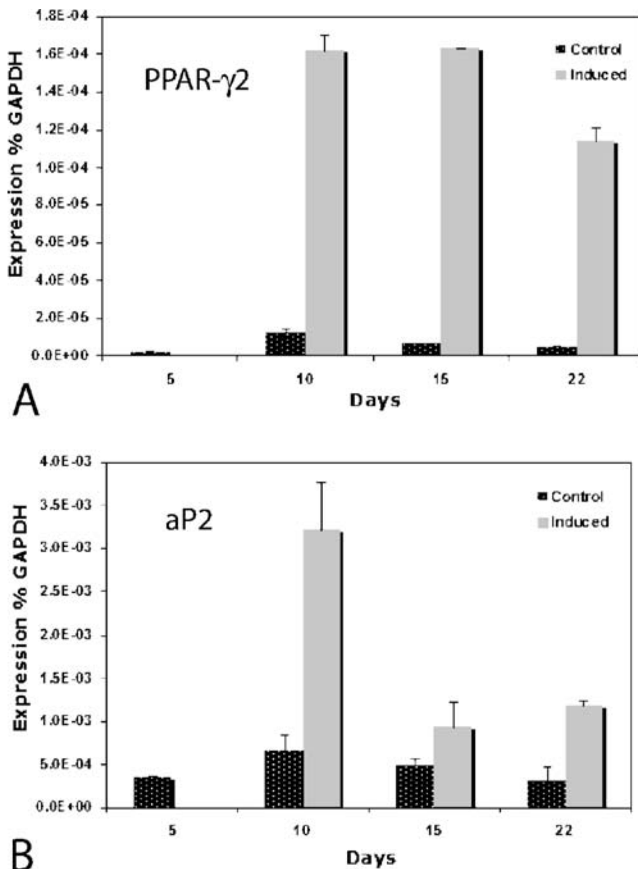

Fig. 7 Real-time RT-PCR performed on the hybrid cell line C12 for adipogenic marker genes (*scale left*: *4,0E-05* 4.0×10^{-5}, etc.). **a** PPAR-γ2, the early transcription factor. **b** aP2, the late marker. Each measure corresponds to the mean±SD of three independent experiments

possibility is that the extended culture selects cells of high TNALP content. Alternatively, chromosome carrying the repressor gene for ALPL is lost during cell culture resulting in the re-expression of this gene. Although the significance of high level expression of TNALP by embryonic stem cells is not fully understood, it may reflect the undifferentiated cell state, whereas reduced or loss of expression may reflect the differentiated state (Anneren et al. 2004). Our results indicate that there is ample scope for the selection of hybrid cell lines with high TNALP content.

Normal cells have been demonstrated to produce various growth factors and cytokines that are beneficial for cell proliferation and cell survival (Le Pillouer-Prost 2003; Li et al. 2005). Normal MSC are also known to produce multiple growth factors and cytokines (Majumdar et al. 1998, 2000; Deans and Moseley 2000; Dormady et al. 2001; Minguell et al. 2001). We have found that conditioned media from MSC-F7 hybrid cells can enhance the proliferation of both early-passage and late-passage MSC indicating that the hybrid cells secrete diffusible growth stimulatory factors into the culture media. We have previously reported that hybrid cells, generated by the fusion of normal porcine fibroblasts with F7 cells, also secrete growth stimulatory factors, which not only can enhance their own proliferation, but also can reinitiate cell division in the late-passage porcine fibroblasts (M.Q. Islam, V. Panduri, K. Islam, in preparation).

Unlike the fusion between embryonic and somatic cells (Tada and Tada 2001; Ambrosi and Rasmussen 2005; Cowan et al. 2005), we have generated somatic cell hybrids by fusing two types of somatic cells, viz., the immortalized murine fibroblast cell line F7 and primary porcine MSC, to ascertain whether the somatic cell hybrids grow indefinitely, and whether they exhibit any features of MSC. Our results demonstrate that porcine MSC with limited proliferation can be reprogrammed to give unlimited proliferation, and that they express many characteristics of MSC, although phenotypic differences occur among the hybrid cell lines (Tables 2 and 3). Somatic cell genetic studies have revealed that the phenotype of hybrid cells is determined by the relative contribution of the two parental genomes. For example, hybrid cells generated by fusion of two differentiated cell types representing the 2N genome from both parent (1:1 hybrid) express only housekeeping genes, with tissue-specific genes being systematically silenced. On the contrary, hybrid cells representing the 4N genome from one parent and the 2N genome from the other (2:1 or 1:2) generally maintain the phenotype of the 4N parent (Gourdeau and Fournier 1990; Massa et al. 2000). In our MSC-F7 hybrid cells, the porcine MSC genome is represented at the 4N to 6N level, and unsurprisingly many hybrid cell lines express the phenotypes of the MSC parent (e.g., TNALP, osteogenic and adipogenic differentiation potentials, and production of growth promoting factors). Interestingly, the limited proliferation of MSC is not evident in the hybrid cells, although the murine genome is represented by fewer than 2N chromosomes. Of note, the F7 is a unique cell line containing many single copy chromosomes in the karyotype, including chromosomes 9 and 17 (Fig. 1a). Fusion of F7 cells with other types of normal primary cells derived from various species (porcine, bovine, equine, canine, murine, and primate) consistently produces hybrid cells containing the 4N normal cell genome with activated growth phenotype. On the contrary, fusion of GM05267-derived independent cell lines, carrying disomy of chromosomes 9 and 17, with normal cells produces hybrid cells of neither activated growth nor the 4N normal cell genome (M.Q. Islam and K. Islam, unpublished). The activated growth phenotype seems to be mediated by epigenetic reprogramming of the normal cell genome through a direct involvement of the F7 genome containing a specific karyotype (Fig. 1a). Further studies are needed to understand the mechanism of this unusual growth phenotype of the hybrid cells resulting from the fusion of two somatic cell types.

Table 4 Quantitative kinetic assay of ALP in the hybrid cell lines generated by fusion of porcine MSC with immortal murine cell line F7

Cell line	Total protein (mg/l)	Total ALP (U/l)	ALP/protein (mU/mg)	Remaining ALP activity after 56°C, 15 min (%)	Remaining ALP activity after 65°C, 10 min (%)	Remaining ALP activity after 10 mM L-phenylalanine (%)	Passage number at which ALP assayed
MSC-F7-C1	4837	25	5	35	0	78	4
MSC-F7-C2	2977	47	16	33	0	83	4
MSC-F7-C3	3587	338	94	19	0	76	4
MSC-F7-C4	3347	133	40	30	0	76	4
MSC-F7-C5	3017	543	180	23	0	71	4
MSC-F7-C6	1357	57	42	26	0	72	4
MSC-F7-C7	3027	506	167	35	0	76	4
MSC-F7-C8	4427	346	78	26	0	77	4
MSC-F7-C9	3217	362	113	24	0	76	4
MSC-F7-C10	3147	2185	694	24	0	76	20
MSC-F7-C11	2747	490	178	32	0	78	20
MSC-F7-C12	3077	101	33	19	0	77	4
MSC-F7-C13	3917	13	3	39	0	81	4
MSC-F7-C14	2747	24	9	34	0	79	4
MSC-F7-C15	3437	971	282	18	0	78	4
MSC-F7-C16	3167	61	19	28	0	71	4
MSC-F7-C17	3497	469	134	24	0	79	4
MSC-F7-Pool A	4052	1281	316	18	0	59	20
MSC-F7-Pool B	5422	515	95	34	0	83	20
Murine F7	11163	100	9	28	4	92	Not known
Porcine MSC	1031	272	264	20	0	77	4

One limitation of present study is that we have not employed the parental cell lines as controls in all of the experiments. We have previously demonstrated the multi-lineage potentials of bone-marrow-derived porcine MSC (Ringe et al. 2002). The porcine MSC in the present study have been isolated from bone marrow in the same laboratory by identical methods and under standard cell culture conditions as previously. Additionally, the differentiation studies of the hybrid cell lines have been carried out in the same laboratory with identical protocols to those of our previous study (Ringe et al. 2002). Since the multilinage potential of MSC is well documented (Baksh et al. 2004; Barry and Murphy 2004), we have reasonably assumed that the MSC used in the present study retain similar properties to those obtained previously. Contrary to the potentials of MSC, postnatal mature fibroblasts do not differentiate into other lineages (Pittenger et al. 1999). Similarly, other workers have also concluded that tissue-specific fibroblasts have limited plasticity, unlike embryonic fibroblasts and stromal cells (also termed MSC; Bayreuther et al. 1988; Ronnov-Jessen et al. 1996). Since the F7 fibroblast cell line is also derived from a postnatal kidney, we consider that they are tissue-specific mature fibroblasts.

Although mammalian somatic cells under standard cell culture conditions do not proliferate indefinitely, several independent reports indicate that normal cells can be grown for extended periods under improved cell culture conditions (Loo et al. 1987; Mathon et al. 2001; Ramirez et al. 2001; Romanov et al. 2001; Tang et al. 2001). However, these cells are not expected to fulfill the strict criteria for immortal cells. The emergence of true immortal cells with unlimited proliferative capacity seems sometimes to occur spontaneously; however, this requires both structural and numerical chromosomal aberrations and multiple gene mutations (Romanov et al. 2001; Yaswen and Stampfer 2002; M.Q. Islam, V. Panduri, K. Islam, in preparation). In the present study, we have demonstrated that the proliferation of both early-passage and late-passage porcine MSC can be improved by the addition of conditioned media derived from hybrid cells (Fig. 8). Of note, the improved proliferation of porcine MSC following this treatment is a conditional phenotype; these cells are not truly immortal because porcine MSC grown for extended periods maintain their diploid karyotype (Fig. 1b). However, our present results and the findings of other investigators mentioned above support the notion that cellular senescence may not

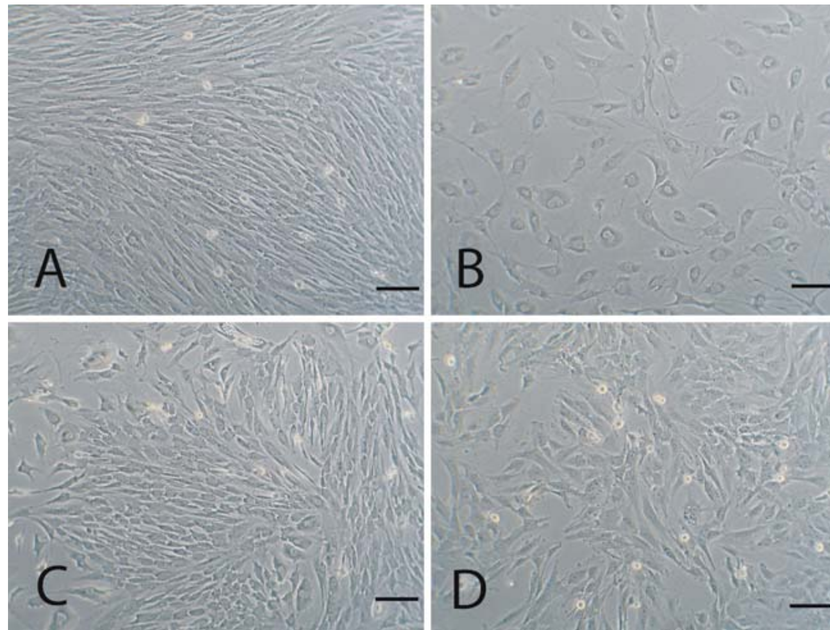

Fig. 8 Comparison of growth of porcine MSC in the presence or absence of conditioned media derived from MSC-F7 hybrid cell line Pool A. MSC at passage 30 grown continuously in DMEM containing 15% FBS and 20% conditioned media showing high mitotic activity even at confluent condition (**a**), MSC at passage 25 grown in the same medium but without conditioned media showing signs of aging (**b**), aged MSC previously grown in the absence of conditioned media (**b**) when cultured with conditioned media showed improved growth at day 20 (**c**), and hybrid cell line Pool A at passage 50 grown in DMEM plus 15% FBS showing high mitotic activity (**d**). *Bars* 100 μm

be a genetically programmed event, but rather the phenotype may be caused by the lack of availability of appropriate growth factors in the culture media.

With some exceptions, cell fusion is not a common phenomenon in vivo, particularly in mammals (Ogle et al. 2005). Interestingly, an increasing number of reports indicate that transplanted MSC frequently undergo cell fusion with native cells, and clonal expansion of the resulting hybrid cells is required for effective tissue regeneration. This is apparent in the fumarylacetoacetate-hydrolase-deficient mouse model in which regenerated livers contain mostly hybrid cells (Wang et al. 2003). The hybrid cells may have a selective growth advantage in vivo. Since the fusion of cell types with only limited proliferation produces hybrid cells of limited proliferation (Hoehn et al. 1978), methods must be developed in order to obtain proliferating hybrid cells in vitro before testing their ability in vivo.

Somatic stem cells have potentials for therapeutic applications, but their limited proliferation is a major barrier to achieving desirable therapeutic effects (Baxter et al. 2004; Javazon et al. 2004; Sethe et al. 2005). Although large numbers of cells are a requisite for in vivo use, cells with unlimited proliferation may not be essential for tissue regeneration. We have demonstrated here that our two-step cell culture protocol, viz., (1) immortalization of primary somatic stem cells of limited proliferation by fusion with F7 cells and (2) improvement of proliferation of primary cells through their treatment with hybrid-cell-derived conditioned media, may provide a means to produce large number of somatic stem cells of limited proliferation and of the hybrid cells derived from them. Thus, the application of our improved cell culture protocol should allow an evaluation of the regenerative capacity of hybrid and normal adult stem cells of limited proliferation.

Acknowledgement The authors thank Cecilia Linder for excellent technical assistance.

References

Ambrosi DJ, Rasmussen TP (2005) Reprogramming mediated by stem cell fusion. J Cell Mol Med 9:320–330

Anneren C, Cowan CA, Melton DA (2004) The Src family of tyrosine kinases is important for embryonic stem cell self-renewal. J Biol Chem 279:31590–31598

Baddoo M, Hill K, Wilkinson R, Gaupp D, Hughes C, Kopen GC, Phinney DG (2003) Characterization of mesenchymal stem cells isolated from murine bone marrow by negative selection. J Cell Biochem 89:1235–1249

Baksh D, Song L, Tuan RS (2004) Adult mesenchymal stem cells: characterization, differentiation, and application in cell and gene therapy. J Cell Mol Med 8:301–316

Barry FP, Murphy JM (2004) Mesenchymal stem cells: clinical applications and biological characterization. Int J Biochem Cell Biol 36:568–584
Baxter MA, Wynn RF, Jowitt SN, Wraith JE, Fairbairn LJ, Bellantuono I (2004) Study of telomere length reveals rapid aging of human marrow stromal cells following in vitro expansion. Stem Cells 22:675–682
Bayreuther K, Rodemann HP, Hommel R, Dittmann K, Albiez M, Francz PI (1988) Human skin fibroblasts in vitro differentiate along a terminal cell lineage. Proc Natl Acad Sci USA 85:5112–5116
Bellows CG, Heersche JN, Aubin JE (1990) Determination of the capacity for proliferation and differentiation of osteoprogenitor cells in the presence and absence of dexamethasone. Dev Biol 140:132–138
Ben-Porath I, Weinberg RA (2005) The signals and pathways activating cellular senescence. Int J Biochem Cell Biol 37:961–976
Camargo FD, Chambers SM, Goodell MA (2004) Stem cell plasticity: from transdifferentiation to macrophage fusion. Cell Prolif 37:55–65
Carpenter MK, Rosler E, Rao MS (2003) Characterization and differentiation of human embryonic stem cells. Cloning Stem Cells 5:79–88
Chomczynski P (1993) A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. Biotechniques 15:532–537
Cowan CA, Atienza J, Melton DA, Eggan K (2005) Nuclear reprogramming of somatic cells after fusion with human embryonic stem cells. Science 309:1369–1373
Czyz J, Wiese C, Rolletschek A, Blyszczuk P, Cross M, Wobus AM (2003) Potential of embryonic and adult stem cells in vitro. Biol Chem 384:1391–1409
Dahlke MH, Popp FC, Larsen S, Schlitt HJ, Rasko JE (2004) Stem cell therapy of the liver-fusion or fiction? Liver Transpl 10:471–479
Deans RJ, Moseley AB (2000) Mesenchymal stem cells: biology and potential clinical uses. Exp Hematol 28:875–884
Do JT, Scholer HR (2004) Nuclei of embryonic stem cells reprogram somatic cells. Stem Cells 22:941–949
Do JT, Scholer HR (2005) Comparison of neurosphere cells with cumulus cells after fusion with embryonic stem cells: reprogramming potential. Reprod Fertil Dev 17:143–149
Dormady SP, Bashayan O, Dougherty R, Zhang XM, Basch RS (2001) Immortalized multipotential mesenchymal cells and the hematopoietic microenvironment. J Hematother Stem Cell Res 10:125–140
Eisenberg LM, Eisenberg CA (2003) Stem cell plasticity, cell fusion, and transdifferentiation. Birth Defects Res C Embryo Today 69:209–218
Flasza M, Shering AF, Smith K, Andrews PW, Talley P, Johnson PA (2003) Reprogramming in inter-species embryonal carcinoma-somatic cell hybrids induces expression of pluripotency and differentiation markers. Cloning Stem Cells 5:339–354
Forejt J, Saam JR, Gregorova S, Tilghman SM (1999) Monoallelic expression of reactivated imprinted genes in embryonal carcinoma cell hybrids. Exp Cell Res 252:416–422
Gimble JM, Youkhana K, Hua X, Bass H, Medina K, Sullivan M, Greenberger J, Wang CS (1992) Adipogenesis in a myeloid supporting bone marrow stromal cell line. J Cell Biochem 50:73–82
Gourdeau H, Fournier RE (1990) Genetic analysis of mammalian cell differentiation. Annu Rev Cell Biol 6:69–94
Gregory CA, Gunn WG, Reyes E, Smolarz AJ, Munoz J, Spees JL, Prockop DJ (2005) How wnt signaling affects bone repair by mesenchymal stem cells from the bone marrow. Ann N Y Acad Sci 1049:97–106
Hayflick L (1965) The limited in vitro lifetime of human diploid cell strains. Exp Cell Res 37:614–636
Hoehn H, Bryant EM, Martin GM (1978) The replicative life spans of euploid hybrids derived from short-lived and long-lived human skin fibroblast cultures. Cytogenet Cell Genet 21:282–295
Islam MQ, Islam K (2000) Suppressor genes for malignant and anchorage-independent phenotypes located on human chromosome 9 have no dosage effects. Cytogenet Cell Genet 88:103–109
Islam MQ, Levan G (1987) A new fixation procedure for improved quality G-bands in routine cytogenetic work. Hereditas 107:127–130
Jabs EW, Wolf SF, Migeon BR (1984) Characterization of a cloned DNA sequence that is present at centromeres of all human autosomes and the X chromosome and shows polymorphic variation. Proc Natl Acad Sci USA 81:4884–4888
Jaiswal N, Haynesworth SE, Caplan AI, Bruder SP (1997) Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. J Cell Biochem 64:295–312
Javazon EH, Beggs KJ, Flake AW (2004) Mesenchymal stem cells: paradoxes of passaging. Exp Hematol 32:414–425
Johnson-Pais TL, Leach RJ (1995) Regulation of osteoblast gene expression in intratypic osteosarcoma hybrid cells. Exp Cell Res 221:370–376
Johnson-Pais TL, Leach RJ (1996) 1,25-Dihydroxyvitamin D3 and transforming growth factor-beta act synergistically to override extinction of liver/bone/kidney alkaline phosphatase in osteosarcoma hybrid cells. Exp Cell Res 226:67–74
Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU (1998) In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. Exp Cell Res 238:265–272
Kimura H, Tada M, Nakatsuji N, Tada T (2004) Histone code modifications on pluripotential nuclei of reprogrammed somatic cells. Mol Cell Biol 24:5710–5720
Le Pillouer-Prost A (2003) Fibroblasts: what's new in cellular biology? J Cosmet Laser Ther 5:232–238
Li H, Zou X, Baatrup A, Lind M, Bunger C (2005) Cytokine profiles in conditioned media from cultured human intervertebral disc tissue. Implications of their effect on bone marrow stem cell metabolism. Acta Orthop 76:115–121
Loo DT, Fuquay JI, Rawson CL, Barnes DW (1987) Extended culture of mouse embryo cells without senescence: inhibition by serum. Science 236:200–202
Magne D, Vinatier C, Julien M, Weiss P, Guicheux J (2005) Mesenchymal stem cell therapy to rebuild cartilage. Trends Mol Med 11:519–526
Magnusson P, Löfman O, Larsson L (1992) Determination of alkaline phosphatase isoenzymes in serum by high-performance liquid chromatography with post-column reaction detection. J Chromatogr 576:79–86
Majumdar MK, Thiede MA, Mosca JD, Moorman M, Gerson SL (1998) Phenotypic and functional comparison of cultures of marrow-derived mesenchymal stem cells (MSCs) and stromal cells. J Cell Physiol 176:57–66
Majumdar MK, Thiede MA, Haynesworth SE, Bruder SP, Gerson SL (2000) Human marrow-derived mesenchymal stem cells (MSCs) express hematopoietic cytokines and support long-term hematopoiesis when differentiated toward stromal and osteogenic lineages. J Hematother Stem Cell Res 9:841–848
Massa S, Junker S, Matthias P (2000) Molecular mechanisms of extinction: old findings and new ideas. Int J Biochem Cell Biol 32:23–40
Mathon NF, Malcolm DS, Harrisingh MC, Cheng L, Lloyd AC (2001) Lack of replicative senescence in normal rodent glia. Science 291:872–875

Matveeva NM, Shilov AG, Kaftanovskaya EM, Maximovsky LP, Zhelezova AI, Golubitsa AN, Bayborodin SI, Fokina MM, Serov OL (1998) In vitro and in vivo study of pluripotency in intraspecific hybrid cells obtained by fusion of murine embryonic stem cells with splenocytes. Mol Reprod Dev 50:128–138

Migeon BR, Brown TR, Axelman J, Migeon CJ (1981) Studies of the locus for androgen receptor: localization on the human X chromosome and evidence for homology with the Tfm locus in the mouse. Proc Natl Acad Sci USA 78:6339–6343

Minguell JJ, Erices A, Conget P (2001) Mesenchymal stem cells. Exp Biol Med (Maywood) 226:507–520

Muraglia A, Cancedda R, Quarto R (2000) Clonal mesenchymal progenitors from human bone marrow differentiate in vitro according to a hierarchical model. J Cell Sci 113:1161–1166

Narisawa S, Harmey D, Magnusson P, Millan JL (2005) Conserved epitopes in human and mouse tissue-nonspecific alkaline phosphatase. Second report of the ISOBM TD-9 workshop. Tumour Biol 26:113–120

Ogle BM, Cascalho M, Platt JL (2005) Biological implications of cell fusion. Nat Rev Mol Cell Biol 6:567–575

Pells S, Di Domenico AI, Gallagher EJ, McWhir J (2002) Multipotentiality of neuronal cells after spontaneous fusion with embryonic stem cells and nuclear reprogramming in vitro. Cloning Stem Cells 4:331–338

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147

Poulsom R, Alison MR, Forbes SJ, Wright NA (2002) Adult stem cell plasticity. J Pathol 197:441–456

Que J, El Oakley RM, Salto-Tellez M, Wong N, Kleijn DP de, Teh M, Retnam L, Lim SK (2004) Generation of hybrid cell lines with endothelial potential from spontaneous fusion of adult bone marrow cells with embryonic fibroblast feeder. In Vitro Cell Dev Biol Anim 40:143–149

Ramirez RD, Morales CP, Herbert BS, Rohde JM, Passons C, Shay JW, Wright WE (2001) Putative telomere-independent mechanisms of replicative aging reflect inadequate growth conditions. Genes Dev 15:398–403

Ringe J, Kaps C, Schmitt B, Buscher K, Bartel J, Smolian H, Schultz O, Burmester GR, Haupl T, Sittinger M (2002) Porcine mesenchymal stem cells. Induction of distinct mesenchymal cell lineages. Cell Tissue Res 307:321–327

Rodic N, Rutenberg MS, Terada N (2004) Cell fusion and reprogramming: resolving our transdifferences. Trends Mol Med 10:93–96

Romanov SR, Kozakiewicz BK, Holst CR, Stampfer MR, Haupt LM, Tlsty TD (2001) Normal human mammary epithelial cells spontaneously escape senescence and acquire genomic changes. Nature 409:633–637

Ronnov-Jessen L, Petersen OW, Bissell MJ (1996) Cellular changes involved in conversion of normal to malignant breast: importance of the stromal reaction. Physiol Rev 76:69–125

Sethe S, Scutt A, Stolzing A (2005) Aging of mesenchymal stem cells. Ageing Res Rev doi:10.1016/j.arr.2005.10.001

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC (1985) Measurement of protein using bicinchoninic acid. Anal Biochem 150:76–85

Surani MA (2001) Reprogramming of genome function through epigenetic inheritance. Nature 414:122–128

Tada T, Tada M (2001) Toti-/pluripotential stem cells and epigenetic modifications. Cell Struct Funct 26:149–160

Tada M, Tada T, Lefebvre L, Barton SC, Surani MA (1997) Embryonic germ cells induce epigenetic reprogramming of somatic nucleus in hybrid cells. EMBO J 16:6510–6520

Tada M, Takahama Y, Abe K, Nakatsuji N, Tada T (2001) Nuclear reprogramming of somatic cells by in vitro hybridization with ES cells. Curr Biol 11:1553–1558

Tada M, Morizane A, Kimura H, Kawasaki H, Ainscough JF, Sasai Y, Nakatsuji N, Tada T (2003) Pluripotency of reprogrammed somatic genomes in embryonic stem hybrid cells. Dev Dyn 227:504–510

Takagi N (1997) Mouse embryonal carcinoma cell-somatic cell hybrids as experimental tools for the study of cell differentiation and X chromosome activity. Cancer Genet Cytogenet 93:48–55

Tang DG, Tokumoto YM, Apperly JA, Lloyd AC, Raff MC (2001) Lack of replicative senescence in cultured rat oligodendrocyte precursor cells. Science 291:868–871

Tominaga K, Olgun A, Smith JR, Pereira-Smith OM (2002) Genetics of cellular senescence. Mech Ageing Dev 123:927–936

Vacanti V, Kong E, Suzuki G, Sato K, Canty JM, Lee T (2005) Phenotypic changes of adult porcine mesenchymal stem cells induced by prolonged passaging in culture. J Cell Physiol 205:194–201

Vassilopoulos G, Russell DW (2003) Cell fusion: an alternative to stem cell plasticity and its therapeutic implications. Curr Opin Genet Dev 13:480–485

Wang X, Willenbring H, Akkari Y, Torimaru Y, Foster M, Al-Dhalimy M, Lagasse E, Finegold M, Olson S, Grompe M (2003) Cell fusion is the principal source of bone-marrow-derived hepatocytes. Nature 422:897–901

Yaswen P, Stampfer MR (2002) Molecular changes accompanying senescence and immortalization of cultured human mammary epithelial cells. Int J Biochem Cell Biol 34:1382–1394

Young HE, Black AC Jr (2004) Adult stem cells. Anat Rec [A] 276:75–102

Polyethylene glycol-mediated fusion between primary mouse mesenchymal stem cells and mouse fibroblasts generates hybrid cells with increased proliferation and altered differentiation.

Islam MQ, Meirelles Lda S, Nardi NB, Magnusson P, Islam K.

Stem Cells Dev. 2006 Dec;15(6):905-19.

STEM CELLS AND DEVELOPMENT 15:905–919 (2006)
© Mary Ann Liebert, Inc.

Biotechnology

Polyethylene Glycol-Mediated Fusion between Primary Mouse Mesenchymal Stem Cells and Mouse Fibroblasts Generates Hybrid Cells with Increased Proliferation and Altered Differentiation

M.Q. ISLAM,[1,2] L. DA S. MEIRELLES,[4] N.B. NARDI,[4] P. MAGNUSSON,[3] and K. ISLAM[1,2]

ABSTRACT

Bone marrow-derived mesenchymal stem cells (MSCs) can differentiate into different cell lineages with the appropriate stimulation in vitro. Transplantation of MSCs in human and other animal models was found to repair tissues through the fusion of transplanted MSCs with indigenous cells. We have generated mouse–mouse hybrid cell lines in vitro by polyethylene glycol-mediated fusion of primary mouse MSCs with mouse fibroblasts to investigate the characteristics of hybrid cells, including their potentials for proliferation and differentiation. Similar to the parental MSCs, hybrid cells are positive for the cell-surface markers CD29, CD44, CD49e, and Sca-1, and negative for Gr-1, CD11b, CD13, CD18, CD31, CD43, CD45, CD49d, CD90.2, CD445R/B220, and CD117 markers. The hybrid cells also produce a high level of tissue nonspecific alkaline phosphatase compared to the parental cells. Conditioned medium of hybrid cells contain biologically active factors that are capable of stimulating proliferation of other cells. Although the parental MSCs can differentiate into adipogenic and osteogenic lineages, hybrid cells held disparate differentiation capacity. Hybrid cell lines in general have increased proliferative capacity than the primary MSCs. Our study demonstrates that proliferative hybrid cell lines can be generated in vitro by induced fusion of both immortal and primary somatic cells with primary MSCs.

INTRODUCTION

Adult tissues contain stem cells that contribute to the repair and regeneration of organs (1–3). Stem cell populations derived from variety of tissues offer great promise for cell-based therapies (4,5). Mesenchymal stem cells (MSCs) isolated from bone marrow (BM) are multipotent and capable of differentiating into many cell lineages when cultured under defined in vitro conditions (6–9). The MSCs also contribute to the regeneration of tissues such as bone, cartilage, muscle, ligament, tendon, adipose, and stroma in vivo (10–14). Transplantation of MSCs in vivo so far have demonstrated very limited regenerative capacity for using this procedure for meaningful therapies (12,15,16). However, delivery of MSCs in injury models shows promise for cell-based therapies,

[1]Laboratory of Cancer Genetics, Laboratory Medicine Center (LMC), University Hospital Linkoping, Sweden.
[2]Department of Biomedicine and Surgery and [3]Divison of Clinical Chemistry, Faculty of Health Sciences, Linkoping University, SE-581 85 Linkoping, Sweden.
[4]Laboratorio de Imunogenetica, Departamento de Genetica, Universidade Federal do Rio Grande do Sul, Porto Alegre RS, Brazil.

particularly in the fumarylacetoacetate hydroxylase-deficient mice where cell fusion plays a major role in regenerating new liver tissues (17–20). This observation may indicate that hybrid cells have better proliferative capacity than the unmodified natural MSCs. Because the frequency of spontaneous hybrid cell formation in vivo was expected to be low due to random chances of spontaneous cell fusion, optimum regeneration of organs may not be possible through the delivery of natural MSCs and relying on their unplanned fusion. In this respect, in vitro generation of hybrid cells by induced fusion of MSCs with organ-specific somatic cells and their proper characterization would permit selection of hybrid cell lines with improved regenerative capacity suitable for in vivo transplantation. This hypothesis has prompted us to generate hybrid cells by fusing mouse MSCs with mouse fibroblasts to investigate the proliferative capacity of in vitro-generated hybrid cells and their potentials for differentiation.

MATERIALS AND METHODS

Mouse MSCs

The isolation of mouse (m) MSC line LTC-4D (hereafter 4D) was described previously (21). Briefly, the 4D cell line was established from the BM of the mouse strain C57BL/6 with a targeted deletion of the gene *Idua* (IDUA-KO) introduced through a neomycin-resistant gene cassette (22). Animals were killed by cervical dislocation, and BM was flushed out of tibias and femurs. To initiate the mMSC culture, BM cells were plated in tissue culture dishes and kept in a humidified incubator with 5% CO2 at 37°C for 72 h, when nonadherent cells were removed by changing the medium. Confluent primary culture was trypsinized and passaged to a new culture dish with a split ratio of 1:2. Subsequent passages were done when the culture approached confluence, and split ratios were increased as needed to permit two subcultures a week. Long-term growing 4D cells at passage 22 were used for the present investigation. They were grown in complete Dulbecco's modified Eagle medium (DMEM) supplemented with 15% fetal bovine serum (FBS), 1% minimal essential medium (MEM) nonessential amino acids, and 1% penicillin and streptomycin. Tissue culture reagents were purchased from PAA Laboratories GmbH (Pasching, Austria), if not stated otherwise.

Primary mouse fibroblast cells

Primary mouse fibroblast cells were isolated from an adult female mouse of the strain T37H (kindly provided by M.F. Lyon) carrying reciprocal translocations involving the X chromosome and chromosome 4. The T37H fibroblasts (hereafter T37) were cultured in complete DMEM supplemented with 15% FBS, 1% MEM nonessential amino acids, and 1% penicillin and streptomycin. The T37 cells at passage 8 were used for the present study.

Immortal mouse fibroblast cells

The neomycin-resistant mouse cell line F7, a derivative of GM05267, has been described recently (23). The original immortal mouse fibroblast cell line GM05267, obtained from the National Institutes of General Medical Sciences, was deficient for the enzyme hypoxanthine phosphoribosyl transferase ($HPRT^{-}$) and consequently sensitive to hypoxanthine aminopterin thymidine (HAT). This fibroblast cell line was isolated from a postnatal/adult kidney of a heterozygous male for the testicular feminization (*tfm*) locus mutation (24,25). The F7 cell line was maintained in DMEM, supplemented with 15% FBS, 1% MEM nonessential amino acids, and 1% penicillin-streptomycin.

Introduction of hygromycin-resistant gene into T37 fibroblasts

A hygromycin-resistant gene was introduced into the T37 fibroblasts through retroviral infection using a published protocol (26). Briefly, proliferating cells were plated in a T75 culture flask with DMEM containing 15% FBS supplemented with 0.03 μg/ml tunicamycin (Sigma) and incubated overnight. Conditioned medium containing the HyTk retrovirus (kindly provided by R.F. Newbold) was collected from confluent culture of the producer cells and filtered through a 0.45-μm filter to remove any intact cell. The filtered supernatant was placed in the T75 flask after adding 4 μg/ml Polybrene (Sigma) and allowed to stay 5 h. The medium was then changed several times with serum-free DMEM and finally replaced with complete DMEM. Next day, complete DMEM with hygromycin (0.1 mg/ml; Calbiochem) was added to the flask. The medium was renewed every 3–4 days for 2 weeks. A pool of hygromycin-resistant cells was used for the present study.

Cell fusion

Cell fusion was induced following a protocol reported elsewhere (23). Briefly, a monolayer of 4D cells (HAT-resistant) and F7 cells (HAT-sensitive) were detached by trypsin treatment. Trypsinized cells were suspended in complete DMEM and 1×10^6 cells were taken from each parent and mixed in a centrifuge tube. Supernatant was discarded after centrifugation, and the pelleted cells were suspended in 5 ml of complete DMEM. The mixed parental cells were then seeded on a 60-mm Petri dish and incubated at 37°C with 5% CO_2 for 3–4 h. After aspirating medium, serum-free DMEM containing phyto-

hemagglutinin-P (100 μg/ml; Sigma) was added to increase the cell–cell contact and incubated at 37°C with 5% CO_2 for 30 min. After aspirating the medium, 2 ml of 45% polyethylene glycol (PEG; Sigma, MW 1,500), prepared by mixing with serum-free DMEM (wt/vol) and filter sterilized (MediaKap-2, 0.2 μm, Microgon, Inc., Laguna Hills, CA), was added at room temperature and allowed to cover the entire cell layer. One minute later, the PEG was aspirated and washed four times with serum-free medium. Complete DMEM was added to the dish after the final washing and then incubated at 37°C with 5% CO_2. The next day, the fused cell layer was trypsinized, and the dissociated cells were suspended in DMEM. The cells were divided equally into two centrifuge tubes. Cells from one tube were cryopreserved and the cells of the other tube were resuspended in complete DMEM supplemented with 2% HAT and plated into a 75-cm^2 tissue culture flask. Cells were grown in HAT medium for 7 days to eliminate F7 cells leaving the proliferating parental 4D cells and hybrid cells between 4D and F7 (half-selection). After propagating the mixed populations of HAT-resistant cells in DMEM for additional 14 days, 20 cells were plated per 100-mm Petri dish and allowed to form macroscopic colonies. Larger-size cell colonies were circled under the Petri dishes with a marker pen, and morphologically distinct colonies were isolated using cloning rings for establishing cell lines.

To generate the second series of hybrid cell lines, 4D cells (G418-resistant and hydromycin-sensitive) were fused with T37 cells (hygromycin-resistant and G418-sensitive) in monolayer culture similar to the first fusion experiment as described above. Hybrid cells were selected in DMEM containing hygromycin (0.1 mg/ml; Calbiochem) and G418 (1.5%, Invitrogen) to eliminate both 4D and T37 parental cells (complete selection). They were grown for 14 days in the presence of selective medium, and then 20 cells were plated per 100-mm cell culture dish to allow the formation of macroscopic colonies. After 7 days, hybrid cell colonies were isolated and then cryopreserved after multiplication.

Chromosome analysis

Chromosome preparation and the chromosome banding procedure were described previously (27). Metaphase cells (20–43) were captured from each cell line by a charge-coupled device camera by using CytoVision software program (Applied Imaging). Chromosomes of the parental and hybrid cell lines were identified and counted from the metaphase images using the CytoVision program.

Flow cytometry

Immunophenotyping of a selected set of hybrid cell lines and the MSC parental cell line 4D was performed by flow cytometric analysis following a published protocol (21,28). Briefly, cells were collected after trypsinization and incubated for 30 min at 4°C with phycoerythrin (PE)- or fluorescein isothiocyanate (FITC)-conjugated antibodies against murine Sca-1, Gr-1, CD11b, CD13, CD18, CD29, CD31, CD34, CD43, CD44, CD45, CD49d, CD49e, CD90.2, CD445R/B220, and CD117 (Pharmingen). Excess antibodies were removed by washing. Detection of PE and FITC labeling was accomplished on a FACScalibur cytometer equipped with 488-nm argon laser (Becton Dickinson) using CellQuest software. At least 10,000 events were collected. WinMDI 2.8 software was used to create histograms.

Induction of osteogenic differentiation

Osteogenic differentiation was induced by culturing cells of selected hybrid cell lines and the MSC parental cell line 4D for up to 4 weeks in DMEM containing 10% FBS and 15 mM HEPES supplemented with 10^{-8} M dexamethasone, 5 μg/ml ascorbic acid 2-phosphate, and 10 mM β-glycerophosphate (9,21,28). To observe calcium deposition, cultured cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 1 min at room temperature (RT), washed once with PBS, and stained for 5 min at RT with Alizarin Red S stain, pH 4.2. Excess stain was removed by several washes with distilled water.

Induction of adipogenic differentation

To induce adipogenic differentiation, selected hybrid cell lines and the MSC parental cell line 4D were kept in culture for up to 4 weeks in DMEM containing 10% FBS and 15 mM HEPES supplemented with 10^{-8} M dexamethasone and 5 μg/ml insulin. Adipocytes were easily distinguished from the undifferentiated cells by phase-contrast microscopy. To further confirm their adipogenic differentiation, cells were fixed with 4% paraformaldehyde in PBS for 1 h at RT, and stained with Oil Red O (Sigma) solution (three volumes of 3.75% Oil Red O in isopropanol plus two volumes of distilled water). In some experiments, $CoCl_2$ was added to the differentiation medium to a final concentration of 100 μM to mimic hypoxic conditions, because hypoxia has been previously shown to induce lipid vacuole formation in human MSCs (29).

Measurement of ALP activity and protein concentration

Parental and hybrid cells were rinsed with PBS and homogenized in 10 mM Tris buffer, pH 8.3, containing 10 μM zinc acetate, 0.1 mM $MgCl_2$, and 0.1% Triton X-100. Alkaline phosphatase (ALP) activities of homogenized cells were determined in 96-well microtiter plates.

In brief, a total volume of 300 μl of solution was added per well, containing 1.0 M diethanolamine buffer, pH 9.8, 10 mM *p*-nitrophenyl phosphate (Sigma), and 1.0 mM $MgCl_2$. The time-dependent increase in absorbance at 405 nm (reflecting *p*-nitrophenol production) was determined on a kinetic microplate reader (Model VMax, Molecular Devices Corp.).

Tissue-nonspecific ALP (TNALP) isoforms are expressed in many tissues. Different heat inactivation and inhibition techniques can distinguish the ALP isoenzymes (23,30). Heating at 56°C for 15 min inactivates the TNALP activity, particularly bone ALP activity. Heat inactivation at 65°C for 10 min inactivates 100% of TNALP and intestinal ALP activity, but placental and germ cell ALP are resistant at 65°C. L-Phenylalanine (10 mM) inhibits the intestinal ALP activity, but other isoenzymes are resistant to this treatment. Protein concentrations were determined by the bicinchoninic acid (BCA) method (31), using the BCA Protein Assay Kit (Pierce, Rockford). Because ALP genes are highly conserved across species (32), we used the same method established for human cell systems to assay ALP activity in the mouse cells.

RESULTS

Generation of hybrid cells

As the fusion of cells of limited proliferation usually generates hybrid cells of limited proliferation (33), we fused the long-term growing mouse MSC line 4D with the immortal mouse fibroblast cell line F7 to overcome the potential proliferative limitations in the resulting hybrid cells. Cell fusion was induced by PEG in co-cultured monolayer of F7 (HAT-sensitive) and 4D cells (HAT-resistant). Subsequently, HAT selection was applied for the initial enrichment of hybrid cells. The HAT medium prevented the proliferation of parental F7 cells but not the parental 4D cells and hybrid cells between 4D and F7 (half-selection, see Materials and Methods). We isolated clonal cell lines by dilution plating of mixed populations of the parental 4D cells and 4D–F7 hybrid cells at passage three. On the basis of distinct morphologies, we isolated five independently derived cell colonies using cloning rings and expanded separately as cell lines for further characterization.

Karyological evidence of generation of hybrid cells

Previous cytogenetic analyses revealed that the parental F7 fibroblast cell line contained approximately 35 chromosomes with monosomy of at least seven chromosomes. This cell line contained many copies of chromosome 15, one copy of X, one copy of a small marker chromosome, and no Y chromosome (23). Cytogenetic analyses of the parental cell line 4D showed nearly 80 chromosomes (Table 1). Initially, it appeared that this cell line was euploid and contained tetraploid chromosome complements. However, detailed karyotype analyses revealed that this was an aneuploid cell line containing variable numbers of copies of individual chromosome. This cell line also contained two copies of X, one to two copies of Y, and four marker chromosomes (Fig. 1A). Theoretically, fusion of one F7 cell with one 4D cell expected to produce a hybrid cell of 115 chromosomes. Cytogenetic analysis of HAT-resistant cell lines derived from the fusion of F7 and 4D cells revealed that four cell lines

TABLE 1. CYTOGENETIC ANALYSIS OF CELL HYBRIDS GENERATED BY FUSING MOUSE MSC CELL LINE LTC-4D WITH THE IMMORTAL FIBROBLAST CELL LINE F7

Cell line	*Total chromosome*[a]	*Normal X chromosome*[a]	*4D-derived Y chromosome*[a]	*F7-derived small marker*[a]	*Number of cells analyzed*	*Presumable fusion type 4D/F7*[b]
Hybrid						
4D-F7-C1	106.96 (99–110)	2.81 (1–3)	2.50 (1–4)	1.04 (1–2)	26	2:1
4D-F7-C2	107.70 (84–140)	3.10 (2–4)	1.40 (0–2)	1.20 (1–3)	20	2:1
4D-F7-C3	116.64 (63–139)	3.24 (2–4)	1.48 (0–2)	1.68 (1–2)	25	2:1
4D-F7-C4	107.91 (74–126)	2.78 (2–4)	0.56 (0–2)	1.66 (0–2)	32	2:1
4D-F7-C5	87.96 (67–116)	2.23 (1–3)	1.33 (0–3)	0.89 (0–1)	27	2:1
Parent						
F7	34.70 (33–36)	1.00 (1–1)	NA	1.04 (1–2)	24	NA
4D	79.12 (74–82)	1.98 (1–1)	1.07 (0–2)	NA	43	NA

NA, Not applicable.
[a]All numbers represent the mean value, and the number in parentheses represents the range.
[b]The number "1" represents a diploid cell and "2" represents a tetraploid cell.

contained chromosome numbers exceeding 100 ranged from 88 and 117 chromosomes (Table 1), and this number is close to the expected chromosome number of a hybrid cell. However, one cell line (4D–F7–C5) contained less than 90 chromosomes, which may resulted from the loss of several chromosomes. To confirm whether the HAT-selected cell lines were actually hybrid cells and carrying marker chromosomes of both 4D and F7 parental cells, we performed cytogenetic analysis of all cell lines. The results of these analyses revealed that they contained two copies of each of X and Y chromosomes, presumably derived from the 4D cell line, and one copy each of X and a small marker chromosome, derived from the F7 cell line (Table 1, Fig. 1B). The presence of marker chromosomes of both 4D and F7 parents in the metaphases of all cell lines confirmed that they were truly hybrid cells. Absence of any pure parental karyotype of 4D cells in the isolated cell line indicates that the hybrid cells had a proliferative advantage compared to the parental 4D cells.

Next, we generated a second series of cell hybrids by fusing the 4D cell line with primary T37 fibroblasts. The 4D cell line was constitutively carrying a neomycin-resistant gene (G418-resistant and hygromycin-sensitive), and a hygromycin-resistant gene was introduced into the T37 fibroblasts (hygromycin-resistant and G418-sensitive) before inducing cell fusion. After fusion, hybrid cells were selected in DMEM containing G418 (to kill T37 cells) and hygromycin (to kill 4D cells). Recombinant hybrid cells between 4D and T37 cells were proliferated in the medium containing two drugs because of genetic complementation. Cytogenetics is a powerful tool to identify hybrid cells generated by fusion of two cell types belonging to different species. However, it is dif-

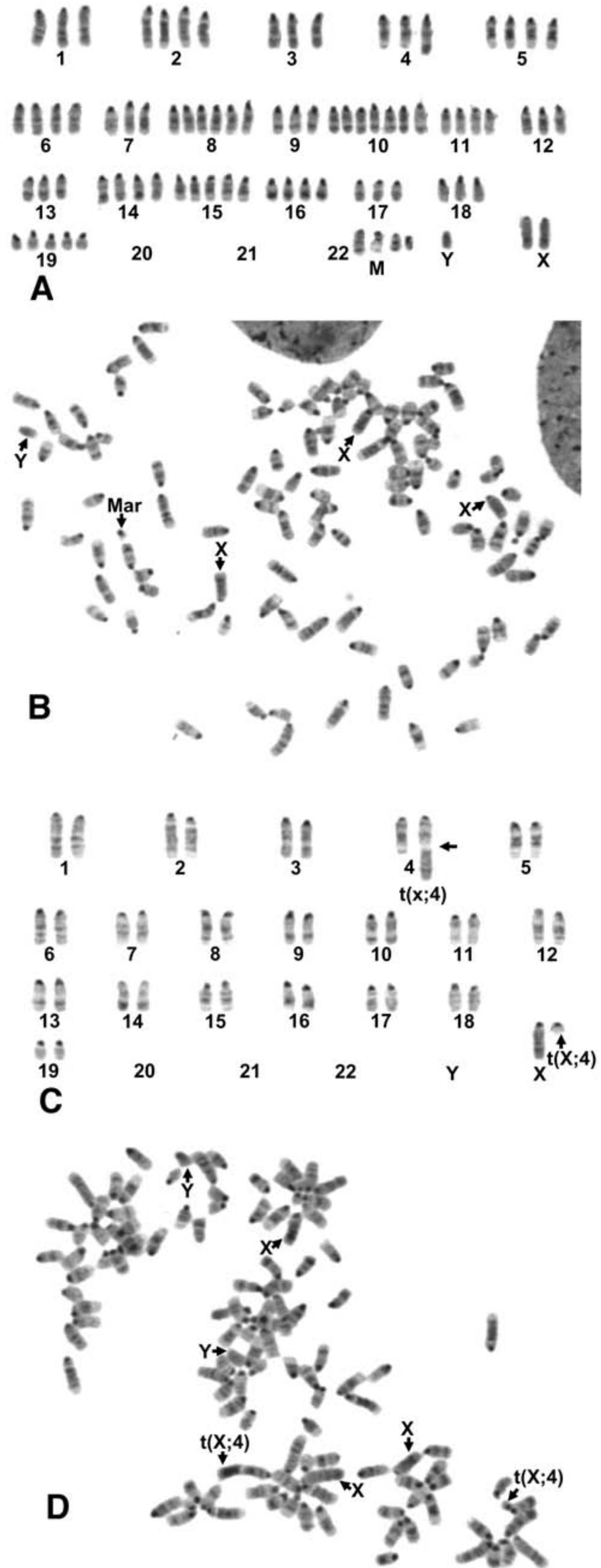

FIG. 1. Cytogenetic evidence of generation of hybrid cells between mouse 4D cells and mouse fibroblast cells. (**A**) Representative karyotype of the 4D cell line containing a total of 81 chromosomes. Besides the presence of four marker chromosomes (M), the karyotype showed variable numbers of copies of autosomes represented by three to seven copies, two copies of X chromosomes, and a single-copy Y chromosome. (**B**) Representative metaphase cell of the hybrid cell line 4D–F7–C1, containing a total of 105 chromosomes including two copies of X and one copy of Y, presumably derived from the 4D cell, and one copy each of X and a small marker chromosome derived from the F7 cell. (**C**) Representative karyotype of a diploid cell of primary mouse fibroblast strain T37 showing 40 chromosomes, including two translocated X;4 chromosomes, generated by reciprocal translocations of 4 and X, and one normal X chromosome. (**D**) Represenetative metaphase of the hybrid cell line 4D–T37–H containing a total of 114 chromosomes including two normal X chromosomes and two Y chromosomes, presumably derived from a 4D cell, and one normal X and two translocated X;4 chromosomes, presumably derived from a T37 cell.

ficult to determine unambiguously the parental origins of chromosomes of hybrid cells generated by fusion of cells of same species. To discriminate the parental origin of chromosomes in the mouse–mouse hybrid cells, we used mouse fibroblast cells T37 carrying reciprocal translocations between chromosome 4 and X chromosome (Fig. 1C). Cytogenetic analysis of the T37 parental cells showed that 56% of the metaphase cells contained diploid, 12% tetraploid, and 32% *heteroploid chromosomes* (Table 2). The fusion of 4D cells with 80 chromosomes with T37 cells having 40 chromosomes was expected to produce a hybrid cell of 120 chromosomes. Double drug-resistant eight hybrid cell lines (4D–T37 hybrid) of independent origins were subjected to cytogenetic analysis using the G-banding technique. These analyses showed that they contained chromosome numbers ranging from 111 to 145 (Table 2). It should be noted that two hybrid cell lines contained chromosome numbers slightly less than 120 (hybrid A and hybrid H), two contained slightly more than 120, and four contained between 134 and 145 chromosomes. These results suggest that hybrid cells were generated by the fusion of near-tetraploid 4D cells with three possible types of T37 cells (diploid, tetraploid, and heteroploid) (Table 2). Detailed karyotype analyses of 4D–T37 hybrid cells confirmed that that they contained marker chromosomes of both 4D and T37 parental cells (Table 2, Fig. 1D). The use of the T37 cells in the fusion experiments allowed us to determine indirectly the genomic contribution of each parent (2:1 or 2:2 combination) in the generation of hybrid cells by counting the copy numbers of t(4;X) markers, normal X, and Y chromosomes derived from two parents (Table 2).

Cellular morphology and growth properties

Morphologically, the F7 parental cells were smaller in size with high proliferation (Fig. 2A) and the 4D cells were flat with slow proliferation (Fig. 2B). The 4D–F7 hybrid cells had flattened morphology with high proliferation rates. These hybrid cells grew even at low cell densities and proliferated like immortal cells (Fig. 2C–F). The morphology of the parental T37 fibroblas was thin, elongated, and tightly packed (Fig. 3A). The morphology of 4D–T37 hybrid cells was large and flat (Fig. 3C–F). The 4D–T37 hybrid cells have been grown for at least 60 population doublings, and they showed superior proliferative capacity compared to the parental cells (Fig. 3A–H). In general, the growth of 4D–F7 hybrid cell lines (Fig. 2C–F) was better compared to the growth of 4D–T37 hybrid cell lines (Fig. 3C–F). It should be noted that the 4D–T37 hybrid cells required moderately high cell density for optimum proliferation.

Immunophenotyping

The MSC parental cell line 4D found to express a distinct set of cell-surface antigens (21). We randomly selected cell lines from two series of hybrids and performed immunophenotyping by flow cytometry. Similar to the cell-surface antigens of the parental 4D cell line, all tested hybrid cell lines were found to be positive for the mark-

TABLE 2. CYTOGENETIC ANALYSIS OF CELL HYBRIDS GENERATED BY FUSING MOUSE MSC CELL LINE LTC-4D WITH ADULT FIBROBLASTS DERIVED FROM THE MOUSE STRAIN T37H

Hybrid cell line	*Total chromosome*[a]	*Normal X chromosome*[a]	*4D-derived Y chromosome*[a]	*T37-derived long X;4 marker*[a]	*Number of cells analyzed*	*Presumable fusion type 4D/T37*[b]
Hybrids						
4D-T37-A	111.1 (104–142)	3.2 (3–4)	2.2 (1–4)	0.9 (0–1)	22	2:1
4D-T37-B	126.8 (120–131)	3.8 (3–4)	2.2 (2–3)	1.0 (1–1)	20	2:1
4D-T37-C	143.1 (118–146)	3.9 (3–4)	2.0 (1–3)	1.7 (1.2)	20	2:2
4D-T37-D	140.4 (108–150)	3.1 (3–4)	1.6 (1–2)	1.0 (1–1)	20	2:2
4D-T37-E	145.4 (133–158)	3.6 (3–4)	1.3 (1–2)	1.0 (1–1)	20	2:2
4D-T37-G	134.5 (106–146)	3.3 (2–4)	2.1 (1–3)	1.2 (1–2)	20	2:2
4D-T37-H	114.0 (110–146)	3.2 (3–4)	2.6 (2–4)	1.0 (1–1)	20	2:1
4D-T37-I	126.5 (107–131)	3.7 (3.4)	1.7 (1–2)	1.0 (1–1)	20	2:1
4D-T37-J	125.3 (113–130)	3.4 (2–4)	2.5 (2–3)	1.0 (1–1)	20	2:1
Parent						
4D	79.1 (74–82)	1.9 (1–2)	1.1 (0–2)	NA	43	NA
T37[c]	52.4 (60–84)	1.2 (1–2)	NA	1.2 (1–2)	25	

NA, Not applicable.
[a]All numbers represent mean value, and the number in parentheses represents the range.
[b]The number “1” represents a diploid cell, and “2” represents a tetraploid cell.
[c]A total of 56% of the cells were diploid, 12% cells tetraploid, and 32% cells aneuploid.

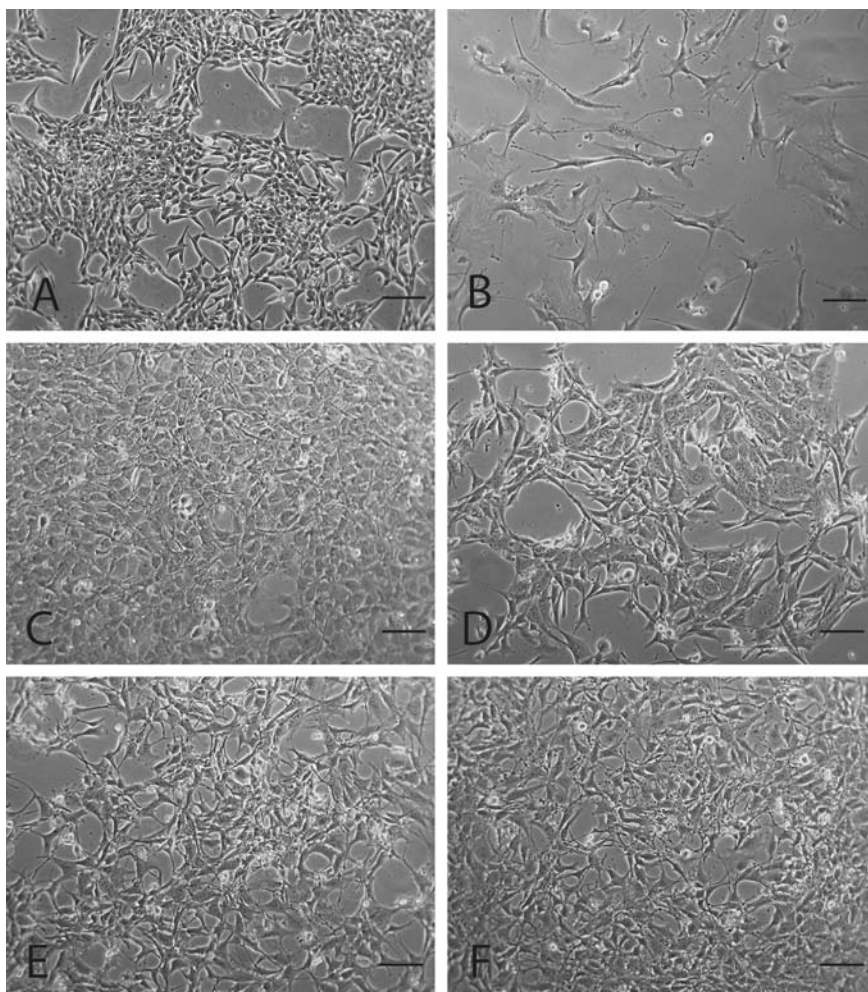

FIG. 2. Phase-contrast photomicrographs of the parental mouse immortal fibroblast F7 cells (**A**), mouse MSC 4D cells (**B**), hybrid cell lines 4D–F7–C1 (**C**), 4D–F7–C2 (**D**), 4D–F7–C3 (**E**), and 4D–F7–C4 (**F**). Note the increased proliferation of hybrid cells, as evident from the presence of numerous dividing rounded cells, compared to the parental 4D cells. Bars, 100 μm.

ers CD29 (integrin β1 chain), CD44 (hyaluronan receptor), CD49e (integrin α5 chain), and Sca-1 (stem cell antigen-1). There was no expression or barely detectable expression of following markers: CD49d (integrin α4 chain), CD11b and CD45 (hematopoetic markers), CD13 (a monocyte marker), CD18 (a leukocyte marker), CD31 (an endothelial marker), CD34 (a hematopoetic progenitor marker), CD117 (stem cell factor receptor), GR-1 (a granulocyte marker), CD41 (a megakaryocyte marker), CD90.2 (a thymus cell antigen), and CD445R/B220 (B lymphocyte marker) (Fig. 4). These results indicate that the fusion of mouse MSCs with mouse fibroblasts does not alter the cell-surface antigen expression of MSCs, irrespective of the mortality status of fibroblast parents.

TNALP analysis

In contrast to embryonic stem (ES) cells, adult stem cells express limited amounts of TNALP. Recently, we have demonstrated that fusion of pig MSCs with mouse F7 fibroblast cells produces hybrid cells where the expression of TNALP was elevated (23). To know whether the fusion of mouse MSCs with mouse fibroblasts produces hybrid cells of similar characteristics, we assayed the total TNALP content of all cell lines of 4D–T37 and 4D–F7 hybrids. The results of these analyses showed that the expression of TNALP was elevated in most of the hybrid cell lines compared to the parents (Table 3). It should be noted that the expression level of TNALP in the 4D–T37 hybrids was much higher than that of 4D–F7 hybrids.

Differentiation studies

The parental 4D cells were found to differentiate into osteoblasts and adipocytes under induced conditions. To find out whether the fusion of these cells with fibroblasts maintained similar differentiation as MSCs, we tested se-

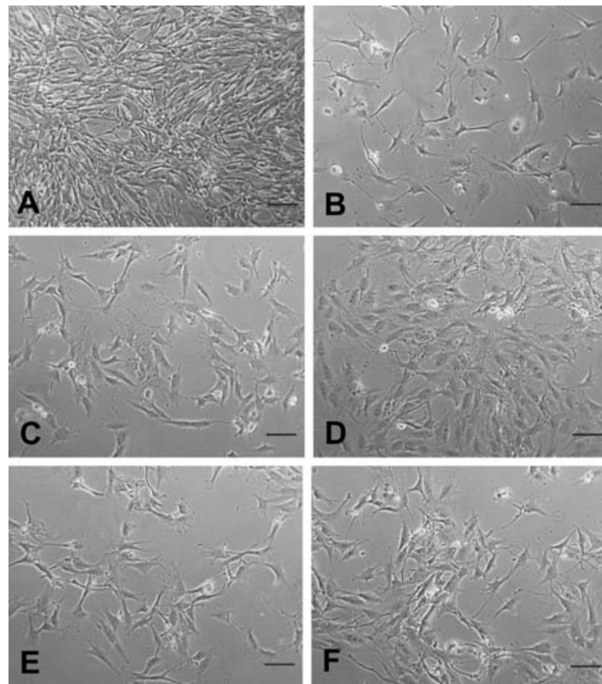

FIG. 3. Phase-contrast photomicrographs of the parental mouse normal fibroblast T37 cells (**A**), cells of mouse MSC 4D cell line (**B**), hybrid cell lines 4D–T37–A (**C**), 4D–F7–B (**D**), 4D–T37–C (**E**), and 4D–F7–E (**F**). Note the increased number of mitotic cells in hybrid cells compared to the parental 4D cells. Bars, 100 μm.

lected hybrid cell lines for osteogenic and adipogenic differentiation. In vitro differentiation assays revealed that hybrid cell lines generated by fusion of 4D cells with normal fibroblasts (4D–T37 hybrids) retained osteogenic differentiation, although with variable degrees in different lines (Table 4, Fig. 5). On the contrary, hybrid cell lines generated by the fusion of 4D cells with immortal fibroblasts (4D–F7 hybrids) failed to differentiate into osteogenic lineage except one cell line (4D–T37–C1). Most of the 4D–T37 hybrid cell lines retained adipogenic differentiation but the degree of fat accumulation was variable among the cell lines (Table 4, Fig. 5). Adipogenic studies of the 4D–F7 hybrid cell lines could not be completed because of detachment of cells from the plastic surface before cellular fat deposition could be observed.

Unusual cellular proliferation and bioactivity of conditioned medium

It should be noted that the confluent monolayer of 4D–F7 hybrid cells could be kept in culture for weeks with a regular change of medium. However, when confluent cells were maintained in culture for prolonged periods, the renewal of medium was difficult because of detachment of cells from the sides of culture flasks. This

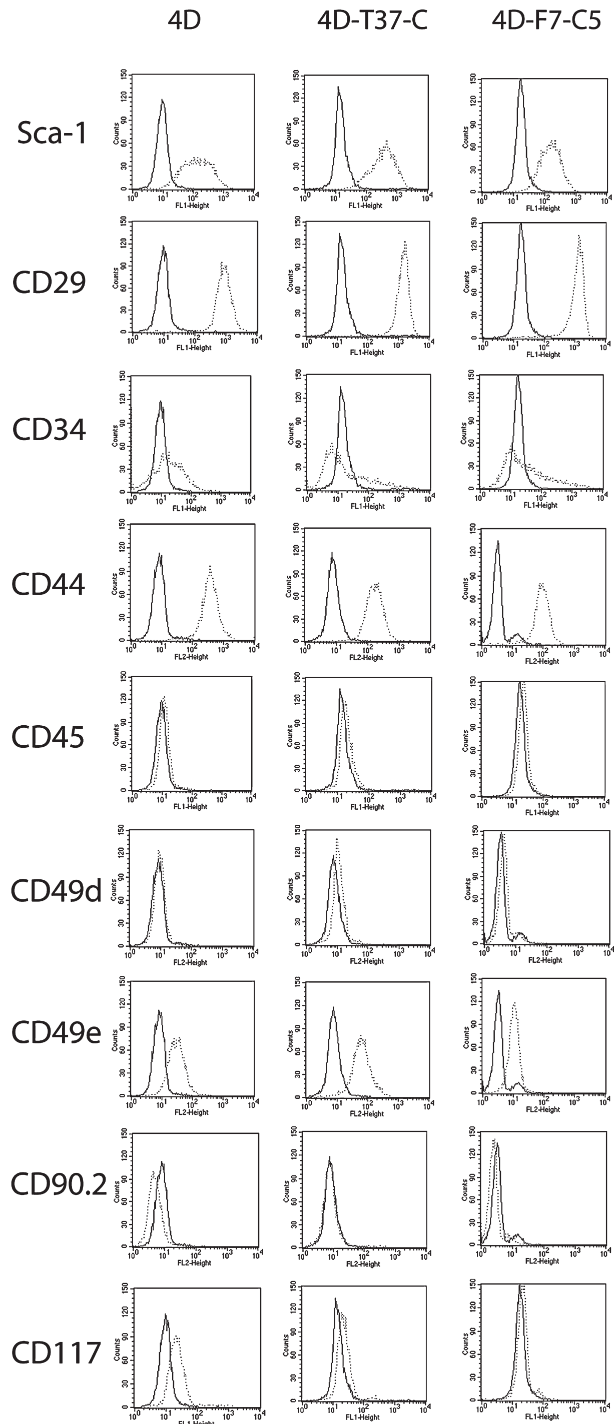

FIG. 4. Immunophenotypic profile of mouse MSC 4D cell line and 4D–T37 and 4D–F7 hybrid cells. (*Left panel*) 4D–hTERT (an hTERT introduced cell line); (*middle panel*) 4D–T37–C; and (*right panel*) 4D–F7–5. Flow cytometry histograms demonstrate the expression of Sca-1, CD29, CD44, and CD49e and lack of expression or weak expression of CD34, CD45, CD49d, CD90.2, and CD117 (see text for detail description of markers). Note the consistency of retention of cell-surface antigen expression of the 4D cells after fusion of these cells with both normal and immortal fibroblast cells.

TABLE 3. QUANTITATIVE KINETIC ASSAY OF ALP IN THE HYBRID CELL LINES GENERATED BY FUSION OF MOUSE MSCs WITH PRIMARY AND IMMORTAL MOUSE FIBROBLASTS

Cell strains/lines	*Total protein (mg/L)*	*Total ALP (U/L)*	*ALP/ protein (mU/mg)*	*Remaining ALP after 56°C, 15 min (%)*	*Remaining ALP after 65°C, 10 min (%)*	*Remaining ALP after 10 mM L-phenylalanine (%)*
Parents						
F7[a]	6431	78	12	18	2	80
LTC-4D[b]	611	5	8	28	0	76
T37[c]	3301	5	2	34	10	85
Hybrids (4DXF7)						
4D-F7-C1	3001	119	39	15	0	93
4D-F7-C2	6791	85	12	14	2	77
4D-F7-C3	5031	53	10	18	2	77
4D-F7-C4	4931	10	2	33	9	92
4D-F7-C5	3654	205	56	15	1	76
Hybrids (4DXT37)						
4D-T37-A	1171	441	377	11	0	80
4D-T37-B	2101	295	140	10	0	76
4D-T37-C	1371	4	3	27	1	76
4D-T37-D	791	327	413	11	0	77
4D-T37-E	1781	31	17	11	0	81
4D-T37-G	1321	6	4	27	1	79
4D-T37-H	1271	373	293	11	0	76
4D-T37-I	761	19	25	18	0	82
4D-T37-J	1557	56	36	14	0	76

[a]F7, Immortal mouse cell line derived from GM05267.
[b]LTC-4D, Long-term growing mouse MSC line.
[c]T37, Long-term growing adult fibroblasts derived from the mouse strain T37H.

happened particularly when the acidic old medium was replaced by alkaline new medium (Fig. 6A). The detached cell sheets frequently folded into multiple cell layers, showing brownish to blackish colors (Fig. 6B), and eventually the empty space created on the plastic surface because of cell detachment was covered by new cell division from the side of the folded cells (Fig. 6C). Occasionally, the detached cells never reattached on plastics and they eventually died. On the contrary, the confluent culture of 4D–T37 hybrid cells never detached, even if the medium was not changed regularly. On one occasion, we found a culture flask of the hybrid cell line 4D–T37–J where medium was not renewed at least for 115 days. Although the cells were in the nondividing state, they still

TABLE 4. DIFFERENTIATION POTENTIALS OF THE MOUSE MSC HYBRID CELL LINES

Cell line	*Quantitative score of adipogenesis*	*Quantitative score of osteogenesis*	*Remarks*
mMSC parent			
LTC-4D	+++	+/−	
4DXF7 hybrids			
4D-F7-C1	+	++	Partial adipogenic differentiation
4D-F7-C2			Cell detached during differentiation
4D-F7-C5			Cell detached during differentiation
4DXT37 Hybrids			
4D-T37-C	+++	+/−	
4D-T37-E	+	+/−	
4D-T37-H	−	+/−	
4D-T37-J	+++	+/−	

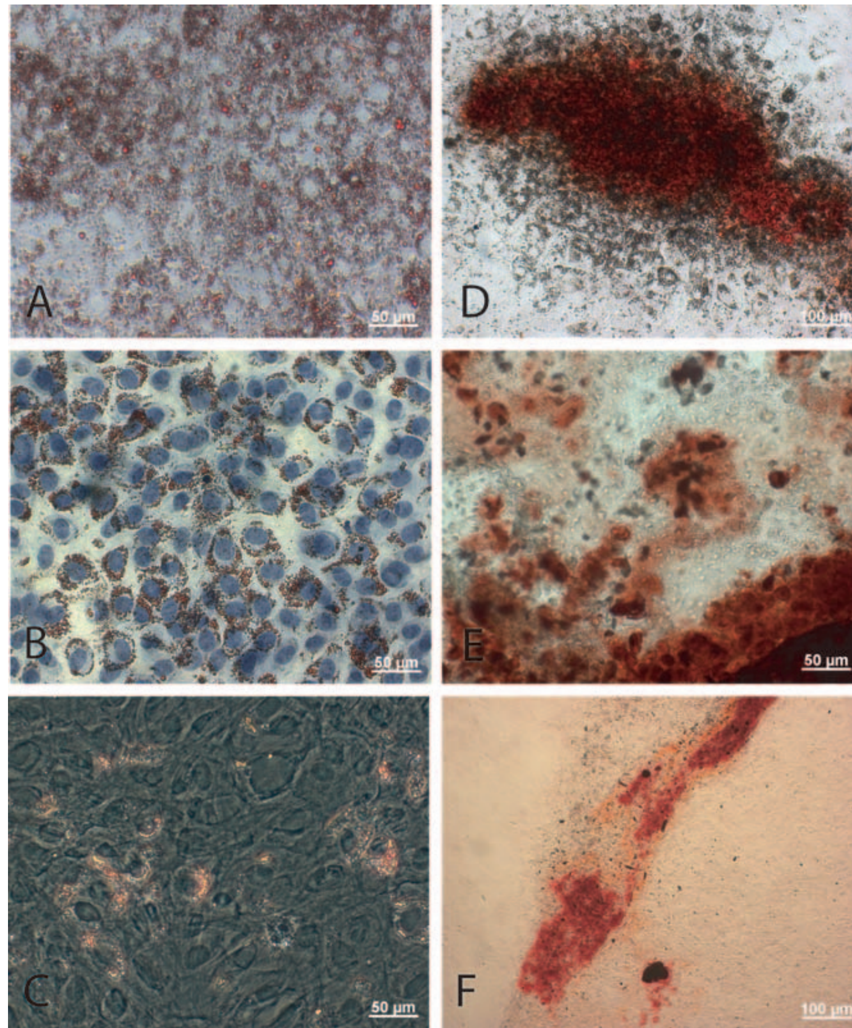

FIG. 5. Differentiation of mouse MSC 4D cells and derived hybrid cell lines. Induced adipogenic differentiation of parental 4D cell line (**A**), hybrid cell line 4D–T37–J (**B**), and hybrid cell line 4D–T37–E (**C**). Induced osteogenic differentiation of parental 4D–hTERT cell line (**D**), hybrid cell line 4D–T37–J (**E**), and hybrid cell line 4D–T37–E (**F**).

looked healthy (Fig. 7A). These post-mitotic hybrid cells when subcultured in standard DMEM containing 15% FBS did not show any mitotic activity (Fig. 7B). However, subculturing of these cells at low cell densities with DMEM containing 15% FBS supplemented with 20% conditioned medium derived from the hybrid cell line 4D–F7–C5 reinitiated mitotic activities (Fig. 7C,D). In contrast, the conditioned medium of 4D–T37 hybrid cells failed to show same effect (not shown).

DISCUSSION

We have generated two series of intraspecies somatic cell hybrids by fusing the mouse MSC cell line 4D with the immortal mouse fibroblast cell line F7 and primary fibroblast strain T37. Hybrid cell lines derived from the fusion of immortal F7 cell line were rapidly proliferating with immortal growth potential, and the T37-derived hybrid cell lines were moderately proliferating with an extended life span. Previously, we generated interspecies pig–mouse hybrid cell lines with indefinite growth by fusing the F7 cell line with primary pig MSCs (23) and primary pig fibroblasts (M.Q. Islam, V. Panduri, and K. Islam, in preparation). These results demonstrate that the fusion of the immortal F7 cell line with various primary cells consistently produces hybrid cells with immortal growth. Significantly, there are reports indicating that fusion of immortal cells of unlimited proliferation and normal cells of limited proliferation often produce hybrid cells of limited proliferation (34,35). In this respect, the F7 line is a unique immortal cell line that not only can produce immortal hybrid cells but also contains an excessive number of chromosomes derived from normal

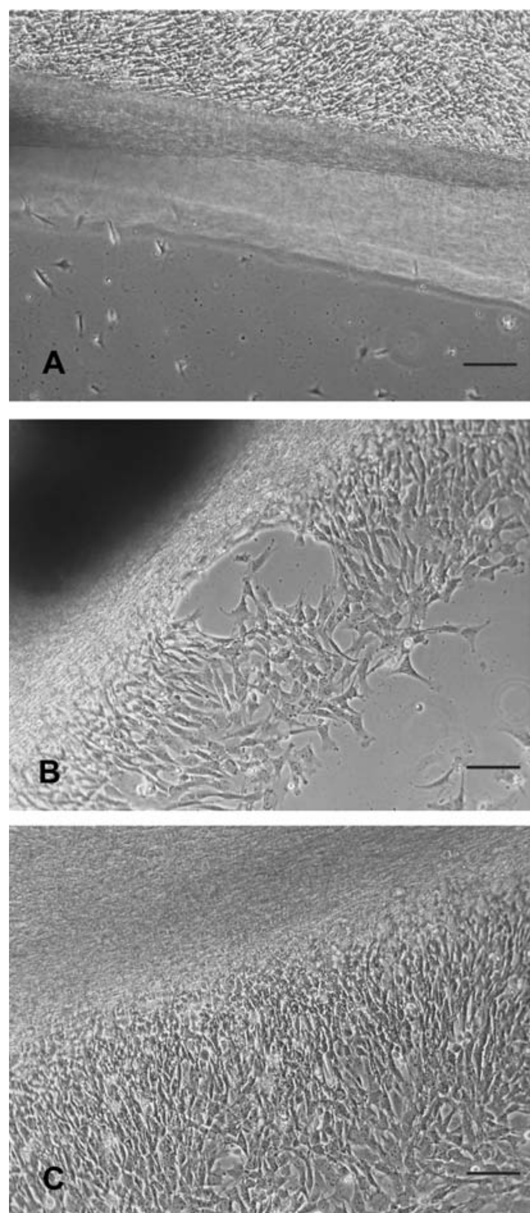

FIG. 6. Phase-contrast photomicrographs of abnormal growth phenotype of hybrid cell line 4D–F7–C5. Note that cells reaching the confluent state tended to detach from the plastic and eventually formed a multilayered cell sheet (**A**), reinitiating cell growth from the side of multilayered cells (**B**), and reaching confluency upon renewal of medium (**C**). Bars, 100 μm.

cell. It is interesting to note that the karyotype of the F7 cell line contains as many as seven single-copy autosomes (23) and at least six of these chromosomes are known to carry imprinted genes that are normally expressed from one of the two alleles (36). We suggested earlier that the F7 cells might have achieved the rapid proliferative capacity by accumulating imprinted gene(s) with growth-promoting effects, located on one or more single-copy chromosomes, through the loss of antagonistically acting growth suppressive imprinted gene(s), located on the missing chromosome(s) (23). Several reports indicate that fusion between two normal cell types of limited proliferation commonly produces hybrid cells of only limited proliferation (33,37,38). Although the 4D cell line has been growing in culture for an extended period and carrying polyploid chromosome complements, this cell line may not be truly immortal. This is probably why the fusion of 4D cells with primary T37 cells produces hybrid cells of extended proliferation instead of indefinite proliferation (33).

Cytogenetic analysis of the hybrid cell lines revealed that they contained variable chromosome contents. Although the chromosome numbers of the F7-derived hybrid cell lines ranged from 88 to 117, the chromosome numbers of T37-derived hybrid cell lines ranged from 111 to 145. The presence of two sets of marker chromosomes from the 4D cell line and one set of marker chromosomes from the F7 cell line in the metaphases of hybrid cell lines indicates that they were produced by the fusion of tetraploid 4D cells with hypo-diploid F7 cells. Cytogenetic analyses of 4D–T37 hybrid cell lines revealed that most of them were generated either by the fusion of tetraploid 4D cells with diploid of T37 cells or by the fusion of tetraploid 4D and tetraploid T37 cells. To discriminate the parental origin of chromosomes in 4D–T37 hybrid cell lines, we fused primary mouse fibroblasts derived from a female mouse of strain T37, carrying reciprocal translocations involving chromosome 4 and X, with 4D cells carrying an XXYY sex chromosome composition. The use of the T37 fibroblast cells allowed us to determine indirectly the genomic contribution of parental cells in the resultant hybrids (2:1 or 2:2 hybrids) by counting the copy numbers of the t(4;X) marker chromosome and normal X and Y chromosomes (Table 2). Taken together, our cytogenetic data indicate that the 4D–T37-derived hybrid cell lines were chromosomally more diverse than the 4D–F7-derived hybrid cell lines. It should be noted that the fusion of mouse ES cells and MSCs with various somatic cell types frequently produces hybrid cells with near-diploid chromosome numbers (17,18,39–41). This observation has led to the suggestion that the hybrid cells undergo a reduction division resulting the loss of nearly a diploid set of chromosomes (18,42). Interestingly, hybrid cells containing tetraploid chromosomes, without the loss of chromosomes from any parent, have also been generated by fusing embryonic and adult stem cells with somatic cells (43–51).

Because the normal MSCs produce diffusible growth stimulatory factors in the culture medium (10,52–54), we saved the conditioned medium of mouse 4D-derived hybrid cells and tested their ability to promote the proliferation of other cells. Although the conditioned medium of 4D–F7 hybrids retained full capacity to promote proliferation of other cells, the conditioned medium derived

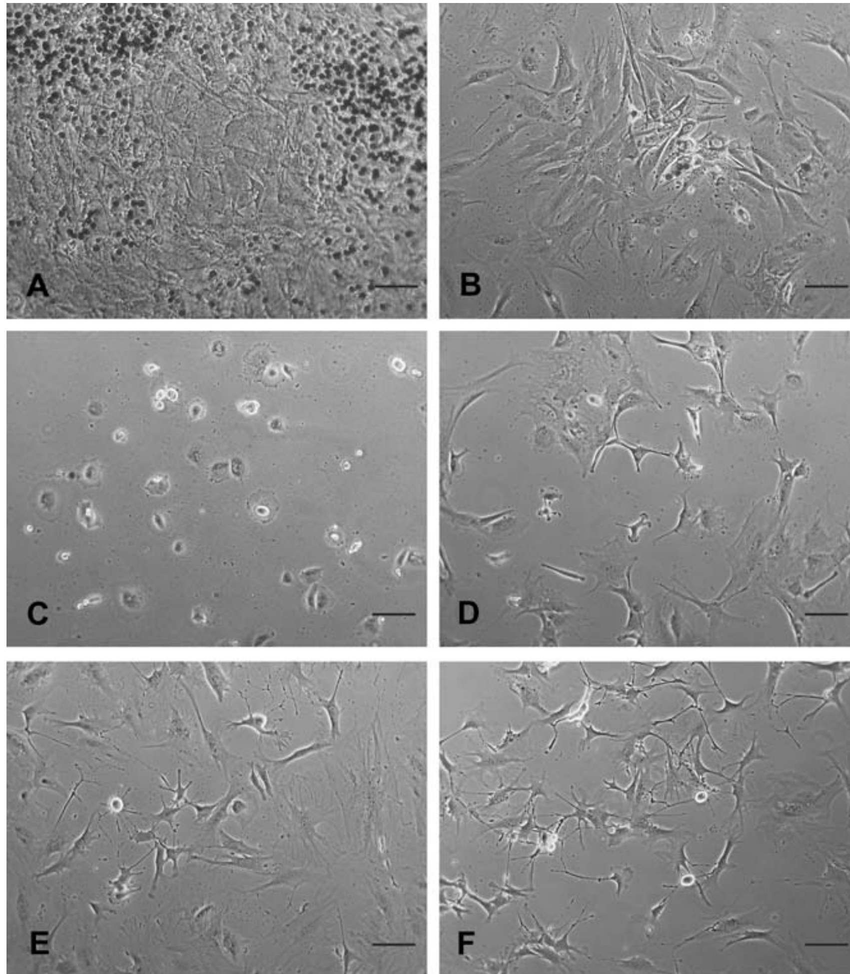

FIG. 7. Phase-contrast photomicrographs of hybrid cell line 4D–T37–J grown under nonpermissive conditions for normal cells and their subsequent recovery with addition of conditioned media derived from the hybrid cell line 4D–F7–C5. (**A**) 4D–T37–J cells kept in the same culture flask for 115 days without change of medium remained alive, but without proliferation. (**B**) Passaging of these cells into a new flask containing DMEM with 15% FBS show no sign of proliferation, at day 4. (**C**) Cells passaged in a new flask containing DMEM with 15% FBS (80%) and conditioned medium (20%) derived from the hybrid cell line 4D–F7–C5 show no mitotic cells at day 0. (**D**) The same cells show both mitotic and nonmitotic cells at day 4. (**E**) The same cells at day 8 show complete recovery and normal growth. (**F**) A culture of 4D–T37–J cells maintained in DMEM containing 15% FBS with regular change of medium served as control. Bars, 100 μm.

from the 4D–T37 hybrid cells was less effective for this capacity. This may indicate that the immortal F7 cell line has a special competence to convert the polyploid normal genome into an immortal state capable of producing growth-promoting factors in the conditioned medium. This conclusion is consistent with our recent demonstration that the fusion of F7 cells with both pig fibroblasts and pig MSCs generates hybrid cells capable of producing diffusible factors in the conditioned media that can enhance their own growth as well as growth of other cells (23) (M.Q. Islam, V. Panduri, and K. Islam, in preparation). This unique property of the F7 cell line can be applied for the generation of hybrid cells by fusing this cell line with various types of normal cells to produce cell type-specific growth-factor(s) to improve the proliferation of other cells.

By immunophenotyping the hybrid cell lines, we have demonstrated here that they express common cell-surface antigens as the parental 4D cells. For example, the parental 4D cell line and hybrid cell lines were positive for the makers CD29, CD44, CD49e, and Sca-1 and negative for the markers CD11b, CD13, CD18, CD31, CD41, CD45, CD90.2, CD117, CD445R/B220, and GR-1. These data indicate that the fusion of mouse MSCs with mouse fibroblast cells does not change the expression pattern of cell-surface antigens of MSCs in the resulting hybrid cells. One possible explanation of dominant expression of cell-surface markers of MSCs by the hybrid cells is that the parental 4D cells contained more chromosomes than the fibroblastic parents. Alternatively, hybrid cells between MSCs and fibroblasts maintained mesenchymal

cell-surface markers, because both cell types have a common mesenchymal origin. To our knowledge, this is the first report demonstrating the expression pattern of cell-surface antigens by intraspecies mouse hybrid cells using flow cytometry.

Although the parental mouse cell line 4D is capable of differentiating into adipogenic and osteogenic lineages (21), the differentiation capacity is largely altered following the fusion of 4D cells with fibroblasts. For example, most of the hybrid cell lines generated by fusion of 4D cells with immortal fibroblasts (4D–F7 hybrids) failed to differentiate into either adipogenic or osteogenic cell lineages. On the other hand, the osteo- and adipogenic differentiation potentials of hybrid cell lines generated by the fusion of 4D cells with primary fibroblasts (4D–T37 hybrids) were mostly maintained, but quantitative differences exist among the cell lines. Because most of the 4D–F7 hybrid cell lines can proliferate ceaselessly, the failure of differentiation in these hybrid cells may be caused by lack of cell cycle arrest. Alternatively, this failure may be due to resetting of the genetic program of parental genomes during cell fusion through the modification of gene expression pattern. The second possibility is unlikely in light of the observation that one of the hybrid cell lines maintained both adipo- and osteogenic differentiation, although all hybrid cell lines were derived from the same cell fusion experiment. These results demonstrate that the fusion between MSCs and fibroblasts generates hybrid cells with dissimilar differentiation potentials. The phenotypic diversity in our hybrid cell lines is not unexpected because they were genetically different as they contained disparate chromosome content.

Previously, we produced interspecies hybrid cells by fusing pig MSCs with mouse F7 fibroblast cells where osteogenic differentiation largely maintained, adipogenic differentiation diminished, and chondrogenic differentiation was completely lost (23). Collectively, these results clearly indicate that generation of hybrid cells by fusing MSCs with other somatic cells does not necessarily mean that the MSC phenotypes are maintained by the hybrid cells. In this respect, hybrid cells with contrasting characters could be useful to select cell lines with desirable traits by screening a large number of cell lines to use them as therapeutic agents.

Recently, it has been reported that systemic transplantation of various adult stem cells in human and other animal models resulted in spontaneous hybridization with endogenous cells generating hybrid cells in vivo (20,55–64). Spontaneously formed hybrid cells through the fusion of exogenous MSCs with endogenous somatic cells were found to repair experimentally damaged organs more effectively than the MSCs alone (17–20). In case of the fumarylacetoacetate hydroxylase-deficient mouse model, the regenerated livers contained mainly hybrid cells (17,18). This may indicate that the hybrid cells have potentials for improved proliferation compared to nonfused natural MSCs.

In the present study, we generated intraspecies hybrid cells through experimentally induced fusion of mouse MSCs with mouse fibroblasts to discover whether the resulting hybrid cells gain a superior capacity for proliferation and whether they retain the phenotypes of normal MSCs, including multilineage differentiation. Our results demonstrate that the hybrid cells in general maintain better proliferative capacity than the parental cells, and they also express many phenotypes of MSCs. Although the differentiation phenotypes of parental MSCs were drastically altered following the fusion of these cells with fibroblasts, some hybrid cell lines maintained phenotypes of parental cell differentiation. The nonuniform differentiation capacities of our experimentally induced hybrid cell lines indicate that the in vivo fusion of transplanted MSCs with indigenous cells may not produce hybrid cells with a consistent cell differentiation phenotype.

In the present study, we deliberately used at least one parental cell line with the capacity of either indefinite cell growth or extended cell growth to generate proliferating hybrid cells. In this context, in vitro generation of hybrid cells by fusing diploid MSCs with diploid primary somatic cells would be more similar with the spontaneously formed hybrid cells in vivo following the fusion of systemically transplanted MSCs with indigenous cells. Application of our two-step cell culture protocol, immortalization of adult stem cells by fusing with F7 cells, and improvement of proliferation of other cells by treatment of the conditioned medium of F7-derived hybrid cells would allow the production of a large number of diploid–diploid hybrid cells, derived from the fusion of normal somatic cells with normal adult stem cells to evaluate the differentiation potential of hybrid cells in vitro and their regenerative competence in vivo.

ACKNOWLEDGMENT

The authors thank Cecilia Linder for excellent technical assistance.

REFERENCES

1. Poulsom R, MR Alison, SJ Forbes and NA Wright. (2002). Adult stem cell plasticity. J Pathol 197:441–456.
2. Czyz J, C Wiese, A Rolletschek, P Blyszczuk, M Cross and AM Wobus. (2003). Potential of embryonic and adult stem cells in vitro. Biol Chem 384:1391–1409.
3. Young HE and AC Black Jr. (2004). Adult stem cells. Anat Rec A 276:75–102.
4. Blau HM, TR Brazelton and JM Weimann. (2001). The evolving concept of a stem cell: entity or function? Cell 105:829–841.

5. Wagers AJ and IL Weissman. (2004). Plasticity of adult stem cells. Cell 116:639–648.
6. Jaiswal N, SE Haynesworth, AI Caplan and SP Bruder. (1997). Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. J Cell Biochem 64:295–312.
7. Johnstone B, TM Hering, AI Caplan, VM Goldberg and JU Yoo. (1998). In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. Exp Cell Res 238:265–272.
8. Pittenger MF, AM Mackay, SC Beck, RK Jaiswal, R Douglas, JD Mosca, MA Moorman, DW Simonetti, S Craig and DR Marshak. (1999). Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147.
9. Phinney DG, G Kopen, RL Isaacson and DJ Prockop. (1999). Plastic adherent stromal cells from the bone marrow of commonly used strains of inbred mice: variations in yield, growth, and differentiation. J Cell Biochem 72:570–585.
10. Deans RJ and AB Moseley. (2000). Mesenchymal stem cells: biology and potential clinical uses. Exp Hematol 28:875–884.
11. Jiang Y, BN Jahagirdar, RL Reinhardt, RE Schwartz, CD Keene, XR Ortiz-Gonzalez, M Reyes, T Lenvik, T Lund, M Blackstad, J Du, S Aldrich, A Lisberg, WC Low, DA Largaespada and CM Verfaillie. (2002). Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 418:41–49.
12. Herzog EL, L Chai and DS Krause. (2003). Plasticity of marrow-derived stem cells. Blood 102:3483–3493.
13. Barry FP and JM Murphy. (2004). Mesenchymal stem cells: clinical applications and biological characterization. Int J Biochem Cell Biol 36:568–584.
14. Baksh D, L Song and RS Tuan. (2004). Adult mesenchymal stem cells: characterization, differentiation, and application in cell and gene therapy. J Cell Mol Med 8:301–316.
15. Javazon EH, KJ Beggs and AW Flake. (2004). Mesenchymal stem cells: paradoxes of passaging. Exp Hematol 32:414–425.
16. Kassem M. (2004). Mesenchymal stem cells: biological characteristics and potential clinical applications. Cloning Stem Cells 6:369–374.
17. Vassilopoulos G, PR Wang and DW Russell. (2003). Transplanted bone marrow regenerates liver by cell fusion. Nature 422:901–904.
18. Wang X, H Willenbring, Y Akkari, Y Torimaru, M Foster, M Al-Dhalimy, E Lagasse, M Finegold, S Olson and M Grompe. (2003). Cell fusion is the principal source of bone-marrow-derived hepatocytes. Nature 422:897–901.
19. Ogle BM, M Cascalho and JL Platt. (2005). Biological implications of cell fusion. Nat Rev Mol Cell Biol 6:567–575.
20. Thorgeirsson SS and JW Grisham. (2006). Hematopoietic cells as hepatocyte stem cells: a critical review of the evidence. Hepatology 43:2–8.
21. Meirelles Lda S and NB Nardi. (2003). Murine marrow-derived mesenchymal stem cell: isolation, in vitro expansion, and characterization. Br J Haematol 123:702–711.
22. Ohmi K, DS Greenberg, KS Rajavel, S Ryazantsev, HH Li and EF Neufeld. (2003). Activated microglia in cortex of mouse models of mucopolysaccharidoses I and IIIB. Proc Natl Acad Sci USA 100:1902–1907.
23. Islam MQ, J Ringe, E Reichmann, R Migotti, M Sittinger, L Da S Meirelles, NB Nardi, P Magnusson and K Islam. (2006). Functional characterization of cell hybrids generated by induced fusion of primary porcine mesenchymal stem cells with an immortal murine cell line. Cell Tissue Res 326:123–137.
24. Migeon BR, TR Brown, J Axelman and CJ Migeon. (1981). Studies of the locus for androgen receptor: localization on the human X chromosome and evidence for homology with the Tfm locus in the mouse. Proc Natl Acad Sci USA 78:6339–6343.
25. Jabs EW, SF Wolf and BR Migeon. (1984). Characterization of a cloned DNA sequence that is present at centromeres of all human autosomes and the X chromosome and shows polymorphic variation. Proc Natl Acad Sci USA 81:4884–4888.
26. Trott DA, AP Cuthbert, CM Todd, M Themis and RF Newbold. (1995). Novel use of a selectable fusion gene as an "in-out" marker for studying genetic loss in mammalian cells. Mol Carcinog 12:213–224.
27. Islam MQ and G Levan. (1987). A new fixation procedure for improved quality G-bands in routine cytogenetic work. Hereditas 107:127–130.
28. da Silva Meirelles L, PC Chagastelles and NB Nardi. (2006). Mesenchymal stem cells reside in virtually all post-natal organs and tissues. J Cell Sci 119:2204–2213.
29. Fink T, L Abildtrup, K Fogd, BM Abdallah, M Kassem, P Ebbesen and V Zachar. (2004). Induction of adipocyte-like phenotype in human mesenchymal stem cells by hypoxia. Stem Cells 22:1346–1355.
30. Magnusson P, O Löfman and L Larsson. (1992). Determination of alkaline phosphatase isoenzymes in serum by high-performance liquid chromatography with post-column reaction detection. J Chromatogr 576:79–86.
31. Smith PK, RI Krohn, GT Hermanson, AK Mallia, FH Gartner, MD Provenzano, EK Fujimoto, NM Goeke, BJ Olson and DC Klenk. (1985). Measurement of protein using bicinchoninic acid. Anal Biochem 150:76–85.
32. Narisawa S, D Harmey, P Magnusson and JL Millan. (2005). Conserved epitopes in human and mouse tissue-nonspecific alkaline phosphatase. Second report of the ISOBM TD-9 workshop. Tumour Biol 26:113–120.
33. Hoehn H, EM Bryant and GM Martin. (1978). The replicative life spans of euploid hybrids derived from short-lived and long-lived human skin fibroblast cultures. Cytogenet Cell Genet 21:282–295.
34. Ran Q and OM Pereira-Smith. (2000). Genetic approaches to the study of replicative senescence. Exp Gerontol 35:7–13.
35. Bunn CL and GM Tarrant. (1980). Limited lifespan in somatic cell hybrids and cybrids. Exp Cell Res 127:385–396.
36. Morison IM and AE Reeve. (1998). A catalogue of imprinted genes and parent-of-origin effects in humans and animals. Hum Mol Genet 7:1599–1609.
37. Migeon BR, RA Norum and CM Corsaro. (1974). Isolation and analysis of somatic hybrids derived from two human diploid cells. Proc Natl Acad Sci USA 71:937–941.
38. Norwood TH, WR Pendergrass, CA Sprague and GM Martin. (1974). Dominance of the senescent phenotype in heterokaryons between replicative and post-replicative human fibroblast-like cells. Proc Natl Acad Sci USA 71:2231–2235.
39. Matveeva NM, AG Shilov, EM Kaftanovskaya, LP Maximovsky, AI Zhelezova, AN Golubitsa, SI Bayborodin, MM

Fokina and OL Serov. (1998). In vitro and in vivo study of pluripotency in intraspecific hybrid cells obtained by fusion of murine embryonic stem cells with splenocytes. Mol Reprod Dev 50:128–138.
40. Matveeva NM, IE Pristyazhnyuk, SA Temirova, AG Menzorov, A Vasilkova, AG Shilov, A Smith and OL Serov. (2005). Unequal segregation of parental chromosomes in embryonic stem cell hybrids. Mol Reprod Dev 71:305–314.
41. Serov O, N Matveeva, S Kuznetsov, E Kaftanovskaya and J Mittmann. (2001). Embryonic hybrid cells: a powerful tool for studying pluripotency and reprogramming of the differentiated cell chromosomes. An Acad Bras Cienc 73:561–568.
42. Medvinsky A and A Smith. (2003). Stem cells: Fusion brings down barriers. Nature 422:823–825.
43. Cowan CA, J Atienza, DA Melton and K Eggan. (2005). Nuclear reprogramming of somatic cells after fusion with human embryonic stem cells. Science 309:1369–1373.
44. Tada M, T Tada, L Lefebvre, SC Barton and MA Surani. (1997). Embryonic germ cells induce epigenetic reprogramming of somatic nucleus in hybrid cells. EMBO J 16:6510–6520.
45. Tada M, A Morizane, H Kimura, H Kawasaki, JF Ainscough, Y Sasai, N Nakatsuji and T Tada. (2003). Pluripotency of reprogrammed somatic genomes in embryonic stem hybrid cells. Dev Dyn 227:504–510.
46. Kimura H, M Tada, S Hatano, M Yamazaki, N Nakatsuji and T Tada. (2002). Chromatin reprogramming of male somatic cell-derived XIST and TSIX in ES hybrid cells. Cytogenet Genome Res 99:106–114.
47. Tada M, Y Takahama, K Abe, N Nakatsuji and T Tada. (2001). Nuclear reprogramming of somatic cells by in vitro hybridization with ES cells. Curr Biol 11:1553–1558.
48. Mise N, T Sado, M Tada, S Takada and N Takagi. (1996). Activation of the inactive X chromosome induced by cell fusion between a murine EC and female somatic cell accompanies reproducible changes in the methylation pattern of the Xist gene. Exp Cell Res 223:193–202.
49. Terada N, T Hamazaki, M Oka, M Hoki, DM Mastalerz, Y Nakano, EM Meyer, L Morel, BE Petersen and EW Scott. (2002). Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. Nature 416:542–545.
50. Ying QL, J Nichols, EP Evans and AG Smith. (2002). Changing potency by spontaneous fusion. Nature 416: 545–548.
51. Que J, RM El Oakley, M Salto-Tellez, N Wong, DP de Kleijn, M Teh, L Retnam and SK Lim. (2004). Generation of hybrid cell lines with endothelial potential from spontaneous fusion of adult bone marrow cells with embryonic fibroblast feeder. In Vitro Cell Dev Biol Anim 40:143–149.
52. Majumdar MK, MA Thiede, SE Haynesworth, SP Bruder and SL Gerson. (2000). Human marrow-derived mesenchymal stem cells (MSCs) express hematopoietic cytokines and support long-term hematopoiesis when differentiated toward stromal and osteogenic lineages. J Hematother Stem Cell Res 9:841–848.
53. Dormady SP, O Bashayan, R Dougherty, XM Zhang and RS Basch. (2001). Immortalized multipotential mesenchymal cells and the hematopoietic microenvironment. J Hematother Stem Cell Res 10:125–140.
54. Minguell JJ, A Erices and P Conget. (2001). Mesenchymal stem cells. Exp Biol Med (Maywood) 226:507–520.
55. Rodic N, MS Rutenberg and N Terada. (2004). Cell fusion and reprogramming: resolving our transdifferences. Trends Mol Med 10:93–96.
56. Zhang S, D Wang, Z Estrov, S Raj, JT Willerson and ET Yeh. (2004). Both cell fusion and transdifferentiation account for the transformation of human peripheral blood CD34-positive cells into cardiomyocytes in vivo. Circulation 110:3803–3807.
57. Willenbring H, AS Bailey, M Foster, Y Akkari, C Dorrell, S Olson, M Finegold, WH Fleming and M Grompe. (2004). Myelomonocytic cells are sufficient for therapeutic cell fusion in liver. Nature Med 10:744–748.
58. Camargo FD, M Finegold and MA Goodell. (2004). Hematopoietic myelomonocytic cells are the major source of hepatocyte fusion partners. J Clin Invest 113:1266–1270.
59. Reinecke H, E Minami, V Poppa and CE Murry. (2004). Evidence for fusion between cardiac and skeletal muscle cells. Circ Res 94:e56–e60.
60. Oh H, SB Bradfute, TD Gallardo, T Nakamura, V Gaussin, Y Mishina, J Pocius, LH Michael, RR Behringer, DJ Garry, ML Entman and MD Schneider. (2003). Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. Proc Natl Acad Sci USA 100:12313–12318.
61. Nygren JM, S Jovinge, M Breitbach, P Sawen, W Roll, J Hescheler, J Taneera, BK Fleischmann and SE Jacobsen. (2004). Bone marrow-derived hematopoietic cells generate cardiomyocytes at a low frequency through cell fusion, but not transdifferentiation. Nature Med 10:494–501.
62. Alvarez-Dolado M, R Pardal, JM Garcia-Verdugo, JR Fike, HO Lee, K Pfeffer, C Lois, SJ Morrison and A Alvarez-Buylla. (2003). Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. Nature 425:968–973.
63. Camargo FD, R Green, Y Capetanaki, KA Jackson and MA Goodell. (2003). Single hematopoietic stem cells generate skeletal muscle through myeloid intermediates. Nature Med 9:1520–1527.
64. Camargo FD, SM Chambers and MA Goodell. (2004). Stem cell plasticity: from transdifferentiation to macrophage fusion. Cell Prolif 37:55–65.

Address reprint requests to:
Dr. M. Quamrul Islam
Laboratory of Cancer Genetics
Department of Biomedicine and Surgery
Division of Clinical Chemistry
Main Building, Floor 11
Faculty of Health Sciences
Linkoping University
S-581 85 Linkoping, Sweden

E-mail: quais@ibk.liu.se

Received September 7, 2006; accepted September 14, 2006.

Errata

As referências Jin et al., 2002, e Van Damme et al., 2003, citadas na Tabela 5, página 270 do artigo *Mesenchymal stem cells: isolation, in vitro expansion and characterization*, não aparecem listadas na bibliografia do mesmo. Estas são, em sua forma completa:

Jin HK, Carter JE, Huntley GW and Schuchman EH (2002) Intracerebral transplantation of mesenchymal stem cells into acid sphingomyelinase-deficient mice delays the onset of neurological abnormalities and extends their life span. J Clin Invest 109:1183-1191.

Van Damme A, Chuah MK, Dell'accio F, De Bari C, Luyten F, Collen D and VandenDriessche T (2003) Bone marrow mesenchymal cells for haemophilia A gene therapy using retroviral vectors with modified long-terminal repeats. Haemophilia. 2003 Jan;9(1):94-103.