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Review

Aging of mesenchymal stem cells

Sebastian Sethe [b], Andrew Scutt [a], Alexandra Stolzing [a,*]

[a] *Kroto Research Institute and Centre for Nanoscience and Technology, University of Sheffield, UK*
[b] *Sheffield Institute of Biotechnological Law and Ethics, University of Sheffield, UK*

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Abstract

The role of adult mesenchymal stem cells (MSC) in tissue maintenance and regeneration has received significant attention of late. Questions arise to what extent these cells are either subject to, or causes of aging; whether age-related changes in these cells are due to intrinsic factors or induced by the somatic environment.

This review collates and examines recent data in support of these different theories.

By means of introduction, a brief overview is given of current MSC definitions and their basic role in tissue regeneration followed by a comparative analysis of gerontological studies involving MSC. Evidence for extrinsic aging and various aging markers relating to morphology, proliferation, signalling, senescence markers, telomeres and telomerase, and other indicators is discussed.

We observe that while the literature might often appear to conflict, many apparent discrepancies are attributable to inconsistent methods of extracting and isolating MSC which in fact contains various subsets of adult stem cells, varying not only in their differentiation potential but also in their vulnerability to senescence—ranging from quasi-somatic lifespan to perennial vigour.

Thus, mesenchymal stem cells emerge as both subject to and key mediators of organismal aging.

Keywords: Aging; Mesenchymal stem cell; Senescence; Telomeres

* Corresponding author at: Kroto Research Institute and Centre for Nanoscience and Technology, Centre of Biomaterials and Tissue Engineering, University of Sheffield, Kroto Building, Broad Lane, Sheffield S3 7HQ, UK. Tel.: +44 114 222 5931; fax: +44 114 222 5943.

E-mail address: A.Stolzing@sheffield.ac.uk (A. Stolzing).

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1. Introduction

1.1. Defining mesenchymal stem cells

In recent years, multipotent stem cells in adult tissue have received considerable attention. The term mesenchymal stem cells (MSC) was popularized by Caplan (Gao et al., 2001), in reference to work by Friedenstein and Owen (Friedenstein et al., 1970) describing a plastic-adherent fibroblastic cell isolated by Percoll density centrifugation, reactive with monoclonal antibodies SH2 and SH3.

The adjective 'mesenchymal' is fraught with some ambiguity since 'mesenchyme' describes tissue of mesodermal origin, the middle embryological germ layer, giving rise to the musculoskeletal, blood, vascular and urinogenital systems, and to connective tissue (including dermis). Thus, developmentally speaking, the term 'mesenchymal' should include both blood and connective tissue cells.

In practice however, only the latter cells are usually described as being derived from MSC and considered distinct from haematopoietic stem cells (HSC), which are responsible for the development, maintenance, and regeneration of blood forming tissues [reviewed by Chen (2002)]. It is quite possible that MSC and HSC have a common precursor in the elusive ''haemangioblasts'' (Sabin, 1920), in the cells identified by the group of Verfaillie originally termed ''mesodermal progenitor'' (Reyes et al., 2001), later ''multipotent adult progenitor'' (MAPC) (Young and Black, 2004) cells, or in ''pluripotent stem cells'' (Howell et al., 2003), or 'tissue committed stem cells' (TCSC) (Ratajczak et al., 2004). However, this is contentious and the physiological relevance of these cells remains to be demonstrated.

Cells with non-haematopoietic multipotency can ultimately differentiate into multiple cell lineages including osteoblasts (Jaiswal et al., 1997), adipocytes (Purpura et al., 2004), chondrocytes (Johnstone et al., 1998), myoblasts (Wakitani et al., 1995), and early progenitors of neural cells (Deng et al., 2001). Such cells can be isolated from umbilical cord blood (Lee et al., 2004; Hou et al., 2003), connective tissue (Young et al., 1995), skin (Shih et al., 2005), synovium fluid (Jones et al., 2004), fat (De Ugarte et al., 2003), the placenta (Waller et al., 1995) and even teeth (Nakashima and de Crombrugghe, 2003), but most commonly they are taken from marrow of various bones.

Accordingly, the nomenclature is not consistent. Designations for cells with non-haematopoietic multipotency have included ''colony-forming-unit-fibroblasts'', ''stromal (stem) cells'', ''bone marrow (stromal) cells'', ''skeletal stem cells'', ''mesodermal progenitor cells'', ''non-haematopoietic stem cells'', ''(bone marrow) stem cells'', ''mesenchymal progenitor cells'' and others (see Baksh et al., 2004; Young and Black, 2004 for enumeration). There is also an understandable tendency to designate such cells as ''pre-(lineage-under-investigation)'' cells (e.g. pre-osteoblast etc.). It has also been suggested that MSC are simply pericytes (Nakashima and de Crombrugghe, 2003).

Some of the inconsistencies surrounding the identification of MSC arise from the fact that specific markers have not yet been agreed on. In the absence of a universal antigenic indication (analogous to CD34+ for HSC) and an universal assay (analogous to the repopulation assays for HSC) MSC are often identified simply by testing a cultures' differentiation potential into colony forming units (CFU) indicative of proliferative

capacity (see below) and into several lineages of mesenchymal tissue as defined above (Pittenger et al., 1999). Also, the ability to adhere to tissue culture plastic and a fibroblast-like morphology are taken as characteristic markers for MSC (Prockop, 1997).

Recently, different surface markers have been associated with MSC including D7fib (Jones et al., 2002), Stro1 (Stenderup et al., 2001), CD45 and glycophorin A (Jones et al., 2004; Pittenger et al., 1999; Reyes et al., 2001), BMPR1a (Zvaifler et al., 2000) (for comparative analysis, see Young and Black, 2004).

Further complications for comparative review arise when different sources, extraction and cultivation methods are used. Even when narrowing sources to bone marrow (as in this review), the site of extraction is reported to influence cell behaviour: e.g. MSC from alveolar bone show less chondrogenic and adipogenic potential compared to iliac bone (Matsubara et al., 2004). Isolation is usually conducted by density centrifugation (sometimes enhanced by gradient solutions) to obtain the mononuclear fraction of marrow cells and by using the widely reported ability of MSC to adhere to tissue culture plastic (Sekiya et al., 2002). Newer methods employ magnetic beads (Stenderup et al., 2001) or FACS sorting (Fickert et al., 2003) in conjunction with antibodies to the proposed MSC markers above. Additionally, widely differing standards regarding serum composition, culture conditions, and growth factor application in MSC cultivation exist. Differing conditions can lead to enrichment of different subsets of MSC with differing clonogenic potential. All these potential deviation points in current methods are summarised in Table 1.

For the purposes of this review, MSC will be defined as *post-embryonic*, *bone-marrow derived cells*, *naturally capable of multipotent differentiation into connective tissue of non-haematopoietic lineage*; *in particular bone*, *ligaments*, *tendons*, *fibres*, *cartilage*, and *adipose tissue*. (Compare the cell type called PPIMSC by Young and Black (2004).)

1.2. Defining aging

If the definition of MSC is elusive, a definition of aging is even more daunting. The patchwork nature of different research foci, coupled with fundamental uncertainties about the nature and the evolutionary role of the aging process preclude a common definition. In cytological study, further challenges arise in distinguishing between aging in vivo and prolonged cultivation in vitro that might or might not simulate 'true' aging ('in vitro aging').

We adopt a definition of aging as "*the sum of primary restrictions in regenerative mechanisms of multicellular organisms*" (Sames and Stolzing, 2005). This definition highlights the involvement of MSC in cell replenishment and thus in influencing lifespan.

Aging can be conceptually distinguished from senescence, with the latter emphasising the cellular level. Here, we adopt the definition of Campisi (2000) that equates senescence with replicative senescence (Hayflick and Moorhead, 1961) by defining it as "*an essentially irreversible arrest of cell division*". Unlike apoptotic cells, senescent cells remain alive, despite a derangement of function (Itahana et al., 2001). Cellular senescence is a complex phenotype that entails changes in both function and replicative capacity. Different experimental protocols, culture conditions, and cell types yield different kinds of senescence. Generally, senescent cells display a characteristic enlarged, flattened

Table 1
Difficulties in comparing MSC data: idiosyncrasies and ambiguities

Extraction sites (Prinz et al., 1999)
Bone marrow (live donors—partial samples only): hip, sternum, broken bones (rare)
Bone marrow (cadaver donors): all sites (rare)
Other tissue: teeth, fat, muscle, cartilage, synovial fluid, skin
Developmental tissue: foetal, umbilical, placenta
Dissociation method
Trypsination
Scraping
Suspension culture
Marker combinations
CD10+, CD13+, CD34+, CD56+, CD90+, MHC-1+ (Young et al., 1999)
CD10−, CD13+, CD31−, CD34−, CD44+, CD45−, CD90+, CD105+, CD133−
Wnt2+, Wnt4+, Wnt5a+, Wnt11+, Wnt16+, Fz2+, Fz3+, Fz4+, Fz5+, Fz6+ (Etheridge et al., 2004)
VCAM+, STRO-1+, CD73+, CD105+ (Tuli et al., 2003)
GlyA−, CD45− (Reyes et al., 2001)
D7-FIB+, CD13+; CD45−, GPA−, LNGFR+ (Jones et al., 2002)
SH2+; SH3+; CD14−; CD29+, CD34−, CD44+; CD45−, CD71+, CD90+, CD106+, CD120a+, CD124+ (Pittenger et al., 1999)
Identification
Adherence to plastic
Magnetic bead
FACS cell sorting
Other factors
Cell line or ex vivo
Donor age
Donor sex
Donor disease status
Point of first analysis
Seeding density
Medium composition
Feeder cells used
Culture conditions (temperature, motion, etc.)
Differentiation agent
Medium composition
First medium change
Frequency of medium change

Listed are some variables in MSC description where differing standards reportedly or likely result in influencing cell behaviour or otherwise lead to variant data.

morphology and are characterized by an irreversible G1 growth arrest involving the repression of genes that drive cell cycle progression and the upregulation of cell cycle inhibitors like p53/p21 and p16/RB. It was suggested that there are notable distinctions between senescent states induced by the p53 and p16/RB pathways; there is an emerging consensus that senescence occurs via one pathway or the other, with p53 mediating senescence due primarily to telomere dysfunction and DNA damage and the p16/RB

pathway mediating senescence due primarily to oncogenes, chromatin disruption, and various stresses (Campisi, 2005).

It has been speculated that senescence may lead to arrested regeneration in tissues and thus to organ failure and death (Knapowski et al., 2002). One degenerative factor in senescence is the accumulation of damage in the cell (Gao et al., 2001; von Zglinicki et al., 2001). Furthermore, senescent cells secrete factors including degradative enzymes, inflammatory cytokines, and growth factors that stimulate tissue aging and tumorigenesis (Krtolica and Campisi, 2003).

Nonetheless, there are persistent doubts about the phenomenon's relevance for in vivo aging (Hornsby, 2002). Critical tissues such as cardiac or neural tissue divides little if at all. Furthermore, senescence in metazoans composed entirely of postmitotic cells is just as predictable and robust as that in metazoans containing mitotic cells (Effros et al., 2005). The immediate relevance of senescence is likely closer to cancer than to other aging-related developments.

1.3. MSC in regeneration

The capacity of MSC to effect tissue and organ regeneration is still not well understood. MSC will populate a wide variety of tissues after systemic infusion (Gao et al., 2001).

Recent evidence suggests that the process of tissue repair is driven by tissue-specific progenitor cells which are replenished by MSC from bone marrow by migration–differentiation (Shake et al., 2002), fusion (Kotton et al., 2001; Prockop et al., 2003b) or the provision of a stromal support network (Alexanian and Kurpad, 2005; Sheng et al., 1998). Involvement of bone marrow-derived stem cells has been demonstrated in the regeneration of a number of organs/tissues including bone (Shirley et al., 2005), skin (Mori et al., 2005), liver (Grompe, 2005), kidney (Okada, 2005) and muscle (Natsu et al., 2004). Consequently, any loss in numbers or functionality with age would have profound consequences for the maintenance of tissue viability (Pelicci, 2004)

2. Age markers in MSC

2.1. Morphology

In vitro aged MSC are reportedly bigger (Baxter et al., 2004; Mauney et al., 2004; Stenderup et al., 2003) than their young counterparts; they exhibit more podia and spread further (Mauney et al., 2004) and contain more actin stress fibers (Stenderup et al., 2003). Increase in cell size is often associated with senescence (Dimri et al., 1995; Hayflick and Moorhead, 1961).

MSC from older patients show no spindle-formed (young) MSC morphology in culture, whereas MSC from young donors exhibit the spindle-type morphology in very early cultivation and a gradual loss of these features over cultivation time (Baxter et al., 2004).

Notably, MSC cell lines immortalised with sv40 (Negishi et al., 2000) or telomerase transfection (Kobune et al., 2003) are considerably smaller than the cells they were derived from.

2.2. Proliferation

2.2.1. CFU numbers

Colony forming unit (CFU) is a retrospective term used to describe a colony originated from a single cell. While some reports show that during aging, total CFU numbers from seeded MSC decrease, other groups find no significant change (Table 2). Given the remarkable sensitivity of totipotent cells, CFU quantification as a method suffers from idiosyncratic designations: e.g. where stimulants are employed to induce a differentiation response, questions about artificial reversing of age-related changes are relevant especially where these changes are due to extrinsic factors. These and other considerations are listed in Table 3.

2.2.2. Differentiation (see Table 2)

The capacity of MSC to differentiate into various types of tissue seems to change with age. Most work has been conducted on the osteogenic potential of aged MSC. One established method to ascertain the presence of osteoblast-MPC is counting the number of alkaline phosphatase-positive colonies. There is conflicting evidence with some groups reporting no change, while a majority finds an age-related decrease. It has been postulated early on (Meunier et al., 1971) that in advanced age MSC loose osteogenic potential and gain adipogenic potential—termed 'adipogenic switch' (Ross et al., 2000) leading to senile osteoporosis. While some recent reports do not find such changes, other groups corroborate the thesis by reporting elevated levels elevated levels of fat-progenitors.

Some interesting studies by Muraglia et al. (2000) investigated the occurrence of bi- and tripotential clones in young and aged bone marrow cells. Relative and absolute frequency of bipotential clones was found to decrease in aging; relative frequency of tripotential clones was higher in 'young' samples, but here absolute frequency did not change with in vitro aging. In contrast with the 'adipogenic switch' model, the authors find that the adipogenic lineage is lost earliest in differentiation.

In summary, in spite of some considerable research base, no definite statement regarding age-related effects on differentiation potential can be made. There is a notable tendency for CFU numbers (apart from adipose-CFU) to decrease during aging, but in humans, the decrease might be relatively minor. Interestingly, the efficiency of differentiating into local tissue ('homing') of transplanted MSC was found to be severely decreased following culture (Rombouts and Ploemacher, 2003), casting fundamental doubts on the applicability of in vitro data to differentiation studies.

2.2.3. Replicative lifespan—division potential

One sign of 'in vitro aging' is a diminishing division capacity. In contrast to embryonic stem cells that show no loss of proliferative potency (Rosenberger, 1995), the maximal population doublings MSC have achieved in vitro are reported as 30–40 (Banfi et al., 2000; Baxter et al., 2004; Bruder et al., 1997). A decline in replicative lifespan associated with donor age is found in somatic cells (Rubin, 2002), and this was also shown for MSC by Baxter et al. (2004) and Globerson (1997), but not by Liu et al. (2004).

Table 2
CFU potential of aged MSC

Indicator	Age	Medium	Species	Reference
MSC				
CFU	▼	?	Mouse	Mets and Verdonk (1981)
CFU	▲	?	Mouse	Xu et al. (1983)
CFU (+ Spleen?)	=	?	Mouse	Brockbank and Ploemacher (1983)
CFU	▼	?	Rat	Egrise et al. (1992)
CFU	▼	–	Rat	Quarto et al. (1995)
CFU	▼	?	Mouse	Kahn et al. (1995)
CFU	▼	Dex	Mouse	Bergman et al. (1996)
CFU (1 mm)	▼	–	Rat	Dobson et al. (1999)
CFU	▼	?	Mouse	Globerson (1997)
CFU	=	–	Human	Oreffo et al. (1998a)
CFU	=	–	Human	Oreffo et al. (1998b)
Stro-1 antib., CFU (16+)	=	–	Human	Stenderup et al. (2001)
CFU (16+/3+)	=[a]	–	Human	Justesen et al. (2002)
Growth rate	▼	VitC, bFGF	Human	Mendes et al. (2002)
CFU	▼	–	Human	Baxter et al. (2004)
CFU	▼	VitC, Gly, Dex	Mouse	Chen (2002)
CFU	▼	?	Various[b]	Lebedinskaia et al. (2004)
CFU-"Bioimage"	▼	–	Rat	Stolzing et al. (2005)
Osteoblasts				
ALP expression	=	?	Human	Evans et al. (1990)
ALP-CFU	▼	–	Rat	Muraglia et al. (2000)
ALP-CFU	=	Dex	Mouse	Bergman et al. (1996)
ALP	=	Dex, VitC	Rat	Dobson et al. (1999)
ALP-CFU	▼	VitD	Human	Majors et al. (1997)
ALP-CFU indep count	▼	-	Human	Oreffo et al. (1998a)
ALP-CFU indep count	=	-	Human	Oreffo et al. (1998b)
ALP-CFU (50+)	▼	Dex	Human	D'Ippolito et al. (1999)
ALP-CFU (50+)	▼	-	Human	Nishida et al. (1999)
ALP expression	=/▲	VitD	Human	Martinez et al. (1999)
ALP-CFU (8+)	▼	VitC	Human	Muschler et al. (2001)
ALP-CFU (16+)	▼	VitD	Human	Justesen et al. (2002)
Fuchsin, methylene blue	▼	VitC, Gly, (Dex)[c]	Human	Mendes et al. (2002)
ALP-CFU (5+)	=/▼[d]	Dex	Human	Leskela et al. (2003)
ALP histochemistry	▼	?	Rat	Chen (2002)
ALP expression	▼	VitD	Human	Baxter et al. (2004)
Von Cossa-CFU	▼	VitC, Gly	Mouse	Moerman et al. (2004)
Adipopcyte				
?	▲	?	?	Meunier et al. (1971)
Oil-Red O CFU (16+)	=	IBMX, insulin, Dex, horse serum	Human	Justesen et al. (2002)
Oil-Red O quantification	=	Horse serum, Dex,	Human	Stenderup et al. (2003)

Table 2 (*Continued*)

Indicator	Age	Medium	Species	Reference
Oil Red O quantification	▲	?	Rat	Chen (2002)
Oil-Red O CFU (10%)	▲	IBMX, indomethacin, hydrocortisone	Mouse	Moerman et al. (2004)
Chondrocyte				
Collagen histochemistry	▼	N/A	Chicken	Nakahara et al. (1991)
Safranin O	▼	Prolin, TGF-β, VitC	Rabbit	O'Driscoll et al. (2001)

Keys: indicator, which cell type and how measured; CFU: selected by colony forming capacity; CFU (*x*): *x* indicates criterion for qualifying as 'countable' CFU, e.g. minimum cell number; age: age-related effect on CFU number; (▼) decrease, (▲) increase, (=) no change (with age); medium: additions to typical culture medium (usually FCS); Dex: dexamethasone; Gly: glycerophospathe; ?: unknown.

[a] ▼ not significant.

[b] Mice, rat, guniea pig.

[c] Dex added in 50% of cases, no correlation found.

[d] No changes in males, decline in females.

2.2.4. Colony size

In addition to decreases in total CFU numbers, there is also evidence that the average colony size decreases in aged MSC (Chen, 2002; Globerson, 1997) (mice) (Oreffo et al., 1998a, 1998b; Prinz et al., 1999). Big colonies tend to be composed of

Table 3
Variables in the assessment of proliferative potential by CFU-count

Donor species and strain
Donor age
Donor sex
Donor disease
Extraction site
Extraction method
Markers used
Gradient used
Dissociation method
Seeding density
Medium composition
Feeder cells
Culture conditions
Differentiation agent
First medium change
Frequency of medium change
Measuring intervals
CFU minimum cell number for counting
% of marked cells in CFU
Counting accuracy (independence, double-blind, computer, etc.)

Listed are some protocol variables in CFU-based analysis of MSC differentiation potential where differing standards reportedly or likely result in influencing cell behaviour or otherwise lead to variant data.

spindle-shaped cells, small colonies often consist of broad, flattened (senescent) cells (Liu et al., 2004).

2.2.5. Growth rate

MSC divide with a donor-dependent average initial doubling time of 12–24 h, dependent on initial plating density (Prockop et al., 2003a). Some groups observe a significant decrease in the growth rate of MSC from aged donors (Baxter et al., 2004; Mendes et al., 2002; Park et al., 2005). In contrast, Bergman et al. (1996) report that the proliferative rate in cultures from older mice, measured by ^{3}H-thymidine uptake, was more than three times that observed in cultures from young animals. However, in response to stimulation, increase in growth rate was 10-fold in the 'young' cultures and insignificant ($p < 0.4$) in the 'older' cultures.

2.3. Telomeres

2.3.1. Telomere length

When telomeres reach a certain length, cells generally stop dividing and enter senescence (Harley et al., 1990). The gradual loss of telomeres is interpreted as a regulator for cell life span and is considered a mechanism to prevent cancer (Deiss et al., 1996). Age-related telomere shortening was observed in osteoblasts (Yudoh et al., 2001; Prinz et al., 1999), myocytes (Torella et al., 2004) (mice), and chondrocyte (Martin and Buckwalter, 2001) (unsupported by Parsch et al. (2002)).

In MSC, a similar trend has been observed (extrapolated by Baxter et al. (2004) to be 17 bp/year), in many cases, MSC in vitro seem to lose telomeric repeats at roughly the same rate as non-stem cells (30–120 bp/PD), but results vary and significance is disputed (Table 4). Remarkably, telomeres of chondrocytes (Parsch et al., 2004) and osteoblasts (Schieker et al., 2004) were found to be longer than the telomeres of MSC from which they were derived.

2.3.2. Telomerase activity

Telomerase is an enzyme counteracting the gradual loss of telomeres by de novo synthesizing telomere repeats (Greider and Blackburn, 1985). It was shown that telomerase activity and differentiation are linked (Armstrong et al., 2000). Apart from cancer cells, telomerase activity is observed in germ-line cells (Deiss et al., 1996), embryonic stem cells (Thomson et al., 1998), and to a certain degree also in adult stem cells such as HSC (Chiu et al., 1996), neuronal stem cells (Ostenfeld et al., 2000), skin stem cells and intestine crypt stem cells (Forsyth et al., 2002). This activity is repressed after stem cells start to differentiate (Armstrong et al., 2000; Forsyth et al., 2002; Ravindranath et al., 1997).

In myocytes, telomerase activity declines with age (Torella et al., 2004) (mice). Most adult cartilage is telomerase negative in vivo (Parsch et al., 2002), but proliferating adult chondrocytes express telomerase activity in vitro (Parsch et al., 2004). Human 'preadipocytes' (Darimont et al., 2003), 'osteoblast precursors' (Darimont et al., 2002), and fetal osteoblasts (Montjovent et al., 2004) apparently show telomerase activity when proliferating in vitro.

Table 4
Telomere and telomerase analysis in MSC

Reference	Age groups	MSC selection	Telomere loss/age	Telomere mean length	Telomere loss in vitro	Telomerase activity
Banfi et al. (2000)	2.5–45 (3)	Adherence Ficoll-g (culture FGF-2)	/	15.7 kb (early pas.), 14.1 kb (growth arrest)	50–85 bp/PD	Negative
Baxter et al. (2004)	0–18 (10)	Adherence	17 bp per year in vivo	11.5 kb (16 PD), 10.4 kb (growth arrest)	88 bp/PD (±10)	/
	59–75 (5)			10.4 kb (16 PD), 10.3 kb (growth arrest)	78 bp/PD (±34)	
Fu et al. (2001)	Fetal	?	?	?	?	Positive
Jiang et al. (2002)	Mouse: 8 weeks (2)	Bead: CD45, TER119 (culture: LIF, ITS, LA-BSA, dex, VitC, FCS2%, EGF, PDGF)	/	27 kb	No loss (40–142 PD)	/
	Rat: 8 weeks (3)		/	27 kb	No loss (42–100 PD)	Positive
Lee et al. (2003)	4–74 (18, control + 11)	Adherence, Ficoll-g	(∼7.8 in youngest vs. 6.1 oldest at pas. 3)	∼7.3 kb (pas. 3)	/	/
Reyes et al. (2001)	2–50 (5)	Bead: CD45, glycophorinA (culture: EGF, PDGF, IGF-1)	Yes but not significant	11–15 kb (35 PD)	Unchanged over 30 PD (one sample)	/
Schieker et al. (2004)	Cambrex, healthy donor	Adherence	/	7.5 kb (pas. 5) stable over 3 weeks in culture	40 bp/PD pas. 5 vs.14	Active only in one highly proliferative subset

Seruya et al. (2004)	Rat	MSC	?	?	?	Positive
Simonsen et al. (2002)	22 + 46 (2)		/	9.2 kb (15 PD)	/	Negative
		+TERT	/	18.6 kb (75 PD)	/	(induced)
Stenderup et al. (2003)	18–29 (6)	Adherence, lymphoprep gradient	Yes but not significant	10.4 kb	100 bp/PD	Negative
	66–81 (5)			9.1 kb	?	
Parsch et al. (2004)	51–79 (12)	Adherence, Ficoll-g (culture: epidermal GF, platet-derived GF)	No correlation	11.4 kb (16 PD)	/	Low activity, no correlation with age
Pittenger et al. (1999)	19–57 (3)	Adherence Percoll-g	/	/	/	Positive (pas. 1 and 12)
Yoon et al. (2005)	Cambrex, healthy donor	MSC (HSC eliminated by NH_4Cl)	/	15.*x* kb	None (5 PD vs. 120 PD)	Positive throughout
Zimmermann et al. (2003)	(8)	Adherence	/	/	/	Detectable at PD 2.2–13.7 but extremely faint

Findings of telomere studies in human (exceptions: Jiang (2002) and Serjia (2004)) MSC. Key: age groups listed as age ranges (years) and sample numbers in parenthesis; adherence: cells selected by their ability to adhere to plastic; X-g commercial gradient used; kb: kilo base; PD: population doubling; pas.: cell culture passage; /: not investigate; ?: unknown.

In MSC, expression of telomerase is still disputed; some studies find no telomerase activity, but in others some activity is detected (Table 4). Such differences may arise from different sensitivity of measurement and differing standards regarding which amount of telomerase activity can be referred to as telomerase-negative. The disparities could also indicate – in particular considering the results of Zimmermann et al. (2003) and Schieker et al. (2004) – that telomerase-active mesenchymal stem cells might be a very rare subpopulation of MSC.

Overexpression of telomerase in HSC had no effect on cell lifespan in serial transplantation experiments (Allsopp and Weissman, 2002). In contrast, forced telomerase expression leads to an extended life span and enhanced differentiation potential in human MSC (Shi et al., 2002; Simonsen et al., 2002).

2.4. Senescence markers in vitro

At pH 6 beta-galactosidase (beta-GAL) activity is associated with senescence in vitro.

One theory suggests that beta-GAL activity is associated with the RAS pathway (Minamino et al., 2003) and with lysosomal dysfunction (Kurz et al., 2000).

Liu et al. (2004) find that MSC of telomerase-knockout mice show premature senescence. In pig MSC, Vacanti et al. (2005) find that in prolonged MSC cultivation beta-GAL activity increases. This is confirmed for humans by Park et al. (2005) who also find increases in p53, and p16/RB. Stenderup et al. (2003) confirms that beta-GAL activity increases in late-passage MSC, but report no differences between MSC from young and aged donors. This highlights the limitation of beta-GAL as a marker for MSC aging: while it is a reliable marker for senescence in low-density culture, and correlated to aging in vivo (Dimri et al., 1995) its application in vivo is limited (de Magalhaes, 2004; Severino et al., 2000). As long as both the self renewal capacity of MSC and the mechanisms of beta-Gal staining are relatively poorly understood, it can only be remarked that even comparatively few numbers of senescent cells – whether MSC or not – can have a marked impact on surrounding tissue (Campisi, 2005; Patil et al., 2005). Some of these interactions are explored in the next section.

2.5. Emissions/signalling

Senescence-associated functional changes include the emission of a variety of agents including proteases, cytokines, and growth factors that can act at a distance within tissues and thus alter the tissue microenvironment (Campisi, 1997, 2000). Among the molecules emitted by senescent cells are metalloproteinases, inflammatory cytokines, and growth factors (reviewed in Krtolica and Campisi (2003)). For MSC, the findings are less comprehensive.

2.5.1. TGF-β

TGF-β regulates osteoblast differentiation in a biphasic manner. It stimulates development and proliferation of early osteoblasts, but it inhibits their maturation (Alliston et al., 2001). TGF-β also inhibits epithelial growth (Han et al., 2005).

TGF-β was found to be decreased in somatic cells (e.g. Han et al., 2005; Wei and Messner, 1998) and also in MSC from aged (Moerman et al., 2004) and SAMP mice (Tsuboi et al., 2004).

2.5.2. BMP2/4

BMP2/4 cytokines are essential for osteoblasts to achieve their mature phenotype (Canalis et al., 2003). BMP promote development and regeneration in the brain (Ebendal et al., 1998). In aged rats, BMP2/4 levels are decreased in some brain areas (Chen, 2002). Expression of BMP2/4 is decreased in MSC of old mice (Moerman et al., 2004). Decreases expressions of TGF-β and BMP2/4 in aged MSC might contribute to the 'adipogenic switch' described above.

2.5.3. Interleukin-6 (IL-6)

Interleukin-6 (IL-6) is a cytokine capable of regulating proliferation, differentiation and activity of a variety of cell types (Heinrich et al., 2003). In particular, IL-6 plays a major role in acute phase response, in the balancing of the pro-inflammatory/anti-inflammatory pathways, and in the stress response. Population-based studies confirm IL-6 serum level as a predictor of disability and mortality among the elderly (Fagiolo et al., 1993; Ferrucci et al., 1999; Harris et al., 1999). IL-6 emission by human MSC increases with age (Cheleuitte et al., 1998).

2.5.4. Interleukin-7 (IL-7)

MSC-released interleukin-7 (IL-7) regulates pre-B-lymphocyte maturation. B-Lymphopoiesis decreases with age, but levels of IL-7 protein within MSC remained unchanged (Stephan et al., 1998). A mechanistic explanation suggests that aging affects secretion or receptor systems so that less IL-7 is available to pre-B-lymphocytes.

2.5.5. Interleukin-11 (IL-11)

Interleukin-11 (IL-11) is a cytokine with haematopoietic, osteotrophic, and mucosa protective properties, as well as anti-inflammatory functions (Zheng et al., 2001).

After stimulation, IL-11 activity and mRNA expression were found to be reduced in MSC from aged mice (Tohjima et al., 2003) and humans (Kuliwaba et al., 2000). In contrast, Cheleuitte et al. (1998) report an increase in IL-11 emission in human MSC (after 9 days of in vitro culturing, 13 donors, 20–80 years).

2.5.6. Reactive oxygen species (ROS)

Reactive oxygen species (ROS) and other free radical emissions by cells and tissue are often taken as an indicator and a cause of aging in vitro (Young et al., 2001) and in vivo (Droge, 2003). In the case of MSC however, ROS was also shown to be involved in signalling, down-regulating proliferation (Meagher et al., 1988) and stimulating differentiation processes (Carriere et al., 2003; Reykdal et al., 1999). During traumatic bone injury ROS emissions activate repair mechanisms (Symons, 1996). Superoxide radicals in (umbilical cord blood derived) MSC stimulate TGF-beta 1 which in turn stimulates CFU proliferation stimulation and osteogenic differentiation (Wang et al., 2004). On the other hand, ROS is clearly harmful to MSC-dependent regeneration as well. Iron overloading in young females resulted in reduced glutathione peroxidase and alkaline phosphatase activities similar to old females, and the authors conclude that this was due to increased ROS production (Isomura et al., 2004).

2.5.7. Nitric oxide (NO)

Nitric oxide (NO) promotes the ability of MSC to differentiate into osteoblasts (Xiao et al., 2001). On the other hand, apoptosis level in osteoblasts increases after NO release resulting in bone loss in wild-type but not in NO-synthase knockouts (Armour et al., 2001). Similarly, NO promotes MSC differentiation into adipocytes (Geula et al., 1998), but apparently has no role in progenitor proliferation of preadipocytes (Geula et al., 1998) (in contrast with HSC where a reduction is reported (Reykdal et al., 1999)).

2.5.8. Antioxidants

Antioxidants are usually beneficial in MSC aging: Antioxidant levels were observed to be abnormally low in osteoporotic humans (Maggio et al., 2003), and in bone marrow cells from aged rats (Umegaki et al., 2001). A prolonged lifespan and an enhanced growth rate is observed in human MSC cultures supplemented with antioxidants (Zou et al., 2005).

In summary, while oxidation processes and oxidation end products clearly play an important part in MSC aging, their usefulness as an indicative age marker is limited by their still poorly understood propensity to regulate MSC differentiation and proliferation.

3. Extrinsic and intrinsic MSC aging

When considering the link between aging and MSC, we are faced with two interrelated components: The effect of aging on MSC themselves, and the contribution of MSC to the aging of the organism. In this, a primary concern is to determine whether MSC are aging internally (intrinsic theory) or if the cells are driven into proliferative silence by changes within the surrounding tissue (extrinsic theory).

For practical reasons, aging of MSC tends to be studied in monolayer cultures of purified or enriched MSC. In reality however, MSC make up just a small part of the bone marrow, a complex network of cells and extracellular matrix which forms the bone marrow microenvironment maintaining a number of stem cell components including those of haematopoietic, mesenchymal, endothelial, and probably other lineage. Because of the methods used to isolate and characterise bone marrow (usually flushing out under pressure followed by the creation of a single cell suspension) the complex, three-dimensional structure of bone marrow (beginning with a central nutrient artery from which tributaries radiate out to form a capillary plexus, which is continuous with the bone marrow sinusoids) is often overlooked. In addition to stem cell compartments, the bone marrow microenvironment comprises mainly of haematopoietic cells, but there are also considerable numbers of bone marrow stromal cells, adipocytes, endothelial cells, and pericytes. The bone marrow environment itself is supported by a complex extracellular matrix consisting mainly of fibronectin, collagen (I and IV), heparin sulphate, chondroitin sulphate, and hyaluronan (Dorshkind, 1990). The development of MSC therefore takes place in an environment with multiple opportunities for interaction at both the cellular and matrix levels and all of these elements have been shown to modify MSC behaviour.

It has been suggested that in vivo MSC function as a part of multicellular compounds, containing endothelial cells, megakaryocytes (Miao et al., 2004) or adipocytes (Boiret et al., 2003). As both structures are likely to be associated with the bone marrow capillary

plexus or sinusoids, this is consistent with recent suggestion that MSC associated with the bone marrow microvasculature (Shi and Gronthos, 2003). Similarly, connective tissue proteins such as collagen or fibronectin have been shown encourage optimal MSC growth in culture and to retain their adipogeneic and osteoblastic differentiation potential (Matsubara et al., 2004; Mauney et al., 2004, 2005). In contrast, the effect of proteoglycans and glycosaminoglycans on MSC is relatively poorly studied despite the fact that they comprise a large proportion of the bone marrow (Oguri et al., 1987). In particular, chondroitin-6-sulphate is a good candidate for modulating bone marrow cell activity (Okayama et al., 1988). Proteoglycans and glycosaminoglycans can directly modulate MSC differentiation in vitro (Hegewald et al., 2004; Zou et al., 2005) and the disruption of their synthesis results in a reduction of MSC proliferation and viability in vivo (Bi et al., 2005). Interestingly, both are involved in the binding of haematopoietic cells to MSC (Siczkowski et al., 1992) and play a role in the interaction between these cells (Hidalgo et al., 2002). The presence of haematopoietic cells is necessary for the optimal MSC growth in vitro (Friedenstein et al., 1992). Thus, age-related changes in haematopoietic cells (Greenwood and Lansdorp, 2003; Van Zant, 2003) might impact on MSC function.

Changes in megakaryocyte numbers can be correlated with bone formation (Compston, 2002) and MSC CFU numbers (Bianchi Scarra et al., 1983). Collagen is known to become increasingly cross-linked with age. In vitro advanced glycation end products (AGEs) inhibit the adhesion and spreading of MSC lines on collagen (Paul and Bailey, 1999). Similarly, AGE-modified BSA inhibits proliferation and differentiation of MSC whilst inducing apoptosis and ROS production (Kume et al., 2005). Lastly, and maybe most importantly, the age-related effect of signalling and ROS and NO emissions has been reviewed above.

In summary, there are clear indications to support the extrinsic theory. Correspondingly, some studies claim that MSC transplants from aged donors are less effective (Rauscher et al., 2003), others disagree (Dressler et al., 2005). If the indications for extrinsic aging are salient, donor age should be less significant in comparison with recipient age. CFU formation in cultured bone marrow transplants from old mice implanted to young surpassed that seen when implanted to old recipients almost three-fold (Friedenstein et al., 1992). Content of nucleated cells in bone marrow transplants from SAMP (senescence accelerated) mice increased more than two-fold where mice with a normal aging rate were recipients instead of SAMP mice (Friedenstein et al., 1992). Parabiotic pairing of young and old mice significantly improved the regeneration of muscle in the old mice; it could be confirmed that this repair was carried out by aged, pre-existing satellite cells whose expression of Delta-1 was restored by the pairing (Conboy et al., 2005).

Overall, these data demonstrate that the bone marrow comprises multiple discrete but interrelated systems, all of which show age-related changes and all of which can impinge on the activity of MSC. The aging of MSC should therefore be carefully interpreted both in terms of intrinsic aging and in terms of the environment in which the MSC finds itself.

4. Collating findings

In collating current findings on age markers in MSC, the emerging picture is curiously consistent in its inhomogeneity. A few methodological difficulties in MSC analysis have

been discussed, and in the context of aging-related research further difficulties are presented by the inclusion of differently defined age groups, variations in sex and disease status of donors.

However, evidence suggests that many of the apparent inconsistencies are likely attributable to observations made in Section 1. In the absence of a clear understanding of what MSC are, and how to identify them, past investigations on aging 'MSC' would have been performed on many different subsets of stem cells with variable potentials and characteristics. This analysis is supported by the findings of Jiang et al. (2002), Pittenger et al. (1999), Schieker et al. (2004), Suva et al. (2004) and Yoon et al. (2005) related above.

In general, there is evidence for three distinct types of aging changes within MSC: (1) changes in quantity, (2) changes in quality (differentiation/regeneration capacity) and (3) changed mobilization capacity (compare Blazsek et al., 2000). For aging-related purposes, we can differentiate between MSC types, which are subject to the somatic cell-type constraints of senescence (''deteriorating MSC'') in that they (a) have a more limited differentiation potential, (b) do not have the ability for infinite self-renewal, (c) are limited to the Hayflick limit and (d) undergo senescence including inactivated telomerase. Other MSC types are less prone to senescence but seem to be subjects to aging (''persisting MSC'') and a few rare types seem to elude aging indefinitely (''perennial MSC'') (Table 5). In such an amalgam of cells that are subject to normal aging, resilient to aging (slowed aging), and virtually resistant to aging, gerontology will likely continue to encounter divergent data until selection methods are improved and standardised. Furthermore, there is strong evidence that aging of MSC cannot be reliably determined by in vitro 'monoculture'. Signalling and emissions form the surrounding tissue cannot only modulate MSC differentiation but also their regeneration potential during aging. MSC are both subjects to and causes of organismal aging. MSC are subjects to aging directly, as they experience time-related stress such as oxidative damage and genetic aberration (but might be particularly well equipped to deal with such onslaught) and indirectly, as surrounding tissue becomes quiescent in time, withholding activation stimuli, thus silencing the differentiation capacity of MSC. MSC are causes of aging at the tissue and organ level when their age-related inability to replenish progenitor cells leads to functional deterioration.

Table 5
Hypothetical classification of age-behaviour of different subsets in adult stem cells

	Somatic cells	Deteriorating MSC	Persistent MSC	Perennial MSC
Differentiation potential	None	Some	Some	Great
Senescence	Yes	Yes	Yes	No
Telomerase	Inactive	Inactive	Potential	Active
Self-renewal	No	No	Yes	Yes
Examples	Sozou and Kirkwood (2001)	Simonsen et al. (2002) and Stenderup et al. (2003)	Pittenger et al. (1999) and Schieker et al. (2004)	Jiang et al. (2002) and Yoon et al. (2005)

Table 6
Suggested markers for further investigation of aging in MSC

Senescence signals upregulated
p53 (Sharpless and DePinho, 2002)
p16 (Dimri et al., 1995; Garkavtsev et al., 1998)
RB tumor suppressor protein (Ohtani et al., 2004)
Numb (notch inhibited) (Conboy et al., 2003; Karanu et al., 2000)
NFk (Kletsas et al., 2004; Medicherla et al., 2002)
ICAM-1 (Kletsas et al., 2004)
Increases in damage
Frequency chromosome aberrations (Wojda and Witt, 2003)
Carbonyl content (Liu et al., 2004; Traverso et al., 2003)
TBARS content (Liu et al., 2004)
Lipofuscin content (Porta, 2002)
Mitochondrial mutations (Liu et al., 2004)
8-Oxo guanidine DNA mutations (Liu et al., 2004)
Decreased protection
Repair enzyme activities (Lombard et al., 2005)
Antioxidant enzyme activities (Edwards et al., 2003)
HSP expression upon stress (Verbeke et al., 2000)

Some established methods to measure age-related changes that could also be applied to investigate aging in MSC. References are typical examples with a preference for studies in stem cells and fibroblasts.

The full extend of the role of MSC in the aging process is only just beginning to emerge. Potentially, gerontological markers themselves could prove useful in identifying specific MSC subsets. While established methods such as CFU and ROS assays have proved unreliable, other established methods to measure age-related changes have yet to be applied to MSC. Table 6 lists a number of recommendations for future research in this regard, investigating both intrinsic and extrinsic factors, based on established data in other fibroblast-type cells and stem cells.

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