REVIEW

Tomorrow's skeleton staff: mesenchymal stem cells and the repair of bone and cartilage

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Abstract. Stem cells are regenerating medicine. Advances in stem cell biology, and bone marrow-derived mesenchymal stem cells in particular, are demonstrating that many clinical options once thought to be science fiction may be attainable as fact. The extra- and intra-cellular signalling used by stem cells as they differentiate into lineages appropriate to their destination are becoming understood. Thus, the growth stimuli afforded by LIF, FGF-2 and HGF, as well as the complementary roles of Wnt and Dickkopf-1 in stem cell proliferation are evident. The ability to direct multi-lineage mesenchymal stem cell (MSC) potential towards an osteogenic phenotype by stimulation with Menin and Shh are important, as are the modulatory roles of Notch-1 and PPAR γ . Control of chondrocytic differentiation is effected by interplay of Brachyury, BMP-4 and TGF β 3. Smads 1, 4 and 5 also play a role in these phenotypic expressions. The ability to culture MSC has led to their use in tissue repair, both as precursor and differentiated cell substitutes, and with successful animal models of bone and cartilage repair using MSC, their clinical use is accelerating. However, MSC also suppress some T-cell functions in transplanted hosts, and could facilitate tumour growth, so a cautious approach is needed.

INTRODUCTION

Regenerative medicine is at an exciting time: it might be said that stem cells are regenerating medicine. The cell biology of the stem cells in many tissues and organs is becoming better understood, and *in vitro* culture methods have improved to the extent that many cell types are now routinely grown that were once thought impossible. Recent data suggest that some adult bone marrow (and other sources') stem cells retain considerable plasticity and may differentiate into a variety of cell types both *in vitro* and *in vivo* (reviewed in other articles in this issue). In addition, it is clear that the ability to grow human embryonic stem cells in culture means that most of the body's cell types should become available in the foreseeable future. Such basic studies have

led to an explosion of applications in the field of stem cell research, where efforts to differentiate precursor cells into functional cells for tissue engineering are beginning to show much progress. This means there will be essentially three autologous cell choices for the tissue reconstructor:

- extract and grow stem, or other, cells from the host's tissue of origin;
- encourage the host's own bone marrow-derived stem cells to fulfil this task;
- use embryonic stem cells from cloned individuals for their own therapeutic benefit.

For each of the above there are also allogeneic equivalent options. All this is predicated on the ability to actually grow the desired cells of choice. As we shall see, this is still highly variable in itself, and on top are the uncertainties of directing the cells to become the precursors and substantive cells of the tissue and organ required. Luckily for us, cells are more intelligent than they look, and will respond to appropriate environmental cues with a change in cell behaviour, which we attempt to subvert for our benefit. Despite any current challenges, the promise for medical repair is so huge that there is a clear hope, if not presumption, that all difficulties will be overcome in the near future.

With the ethical problems associated with human embryonic stem cells (de Wert & Mummery 2003), whether they be self-cloned or allogeneic, combined with the promise of autologous stem cell therapy given by adult bone marrow plasticity, it appears more likely that the adult course will be more often pursued, followed by the allogeneic adult bone marrow (BM) cells, and with the embryonic stem cell route more likely in last place, if for no other reason than consumer resistance. However, if this latter source of reparative cells became the most reliable and were available in sufficient quantities, then the diseased patient may have a different ethical standpoint. Whether this would be true for xenogeneic cell sources remains to be seen. Needs must, perhaps.

It is now evident that the classical tissue specificity and lineage restriction of stem cells is now challenged in several areas (see other articles in this issue). It is the prospect of multiple usage that fuels much research into the control and differentiation of many stem cell types, and those from the bone marrow in particular, as they seem to possess the greatest potential in post-foetal life. It can surely only be a matter of time (and expense) before many tissues are reconstructed, either *in vitro* or within the recipient, using their own bone-marrow-derived cells. As this article hopes to demonstrate, this goal is already being addressed with some speed in the bone and cartilage areas, notwithstanding the advances already achieved by using the patient's own, but not stem, cells in these areas.

BONE MARROW MESENCHYMAL STEM CELLS

This review will concentrate on post-2000 literature describing the role of mesenchymal stem cells (MSC) in osteocyte and chondrocyte biology, and will describe their current utility in the context of bone and cartilage repair, including reference to tissue engineering. Not all experiments described are 'pure', in so far as some reports deal with human cells xenografted into sheep or NOD-SCID mice, but these results may yet give insights into the processes involved, and are included where it is thought relevant.

MULTIPLE INPUTS AND OUTPUTS: WHAT ARE MSC?

MSC are defined both by positive and negative phenotypic staining and behaviour. At the primary level, using bone marrow aspirates, adult peripheral and neonatal cord blood, they are

non-haemopoietic stem cells, and so by phenotypic definition they are negative for several of that cell type's markers. In addition, their phenotype is further defined by positivity for other surface antigens, and these two phenotypes are summarized in Table 1.

It is generally the case that the presence of a marker on one lineage implies its absence on another, though this is not absolute, and many markers show dynamic expression during differentiation.

A further feature of each BM-derived stem cell is its ability to give rise to several further cell types. The list of these 'transdifferentiation' or plastic lineages is probably not complete, but includes several listed below (Table 2). The HSC-derived cell types have been reviewed recently (Poulsom *et al.* 2002).

Some of these cell types and factors in their control of differentiation are depicted in Fig. 1 (after Caplan & Bruder 2001). Figure 2 shows the phenotypes of typical cultures of cloned murine MSC grown in control, adipogenic, chondrogenic and osteogenic media.

It is true to say that it is still difficult to predetermine the outcome of a transdifferentiation experiment, and MSC will obey their own rules, of which we are mostly ignorant. Nevertheless, the guiding principle for their utility stems (pun intended) from their reaction to local stimuli, in particular that of tissue damage. This is already being exploited clinically in many settings, and with mixed results, but generally encouraging. There are several factors which will guide MSC in their cell fate decisions, among which are:

- Damage (wounding, necrosis, inflammation, immunoreactions, pathogens, tumours).
- Homing (including chemotaxis and interactions with ECM, integrins, CXCR-4, SDF-1).
- Multipotentiality (how far along one lineage are such cells already committed).
- Proliferation (can enough cells be generated locally or be 'imported').
- Interactions with and/or induction by local cells.

GENERAL LINEAGE OUTCOMES USING MSC

Many experiments using MSC describe situations where there are several cell types derived from the input cells. Thus, Liechty and co-workers reported on human MSC which were transplanted into foetal sheep before and after the development of the immune system. The input cells ended up contributing to a wide range of tissues, including cartilage, fat, cardiac muscle, as well as bone marrow and thymic stromas. Such engraftment was persistent for over a year, even when done after immunocompetence had arisen in the host sheep (Liechty *et al.* 2000). Furthermore, there was an increase in MSC-derived cells if tissue damage occurred (Mackenzie & Flake 2001). These results suggest that the clinical use of (autologous) BM MSC may be able to contribute to the correction of many mesenchymal diseases, including osteogenesis imperfecta and muscular dystrophy, which may be diagnosed *in utero*.

Prockop *et al.* have performed interesting experiments using MSC in combination with the conditioned medium from heat-shocked pulmonary epithelial cells. The MSC underwent a transition to an epithelial morphology, and also were able to fuse with the epithelial cells, including nuclear fusion (Prockop *et al.* 2003).

This may be a culture artifact, but may yet throw light on the observations in other experiments where BM-derived cells have apparently transdifferentiated, but did in fact fuse with host cells. There seems to be a propensity for this to occur in the liver, and other large cell types such as Purkinje cells (for discussion see Alvarez-Dolado *et al.* 2003 and Alison *et al.* 2004).

Table 1. Phenotypic markers of MSC and HSC (after several authors)

MSC		Surface receptors	
Surface antigens		IL1-R, IL-3R, IL4-R, IL-6R, IL-7R, LIFR, SCFR, G-CSFR, VCAM-1, ALCAM-1, LFA-3 IFN γ R, TNF1R, TNF2R, TGF β 1R, TGF β 2R, bFGFR, PDGFR, EGFR	
CD13		Cytoskeletal proteins	
CD29		α -Smooth muscle actin	
CD44		GFAP	
CD49a		ECM components	
CD71 (transferrin receptor)		Collagen types I, III, IV, V and VI	
CD90		Fibronectin	
CD105 (SH2; Endoglin)		Laminin	
CD114 (c-kit)		Hyaluronan	
CD166		Proteoglycans	
HLA-ABC		HSC	
Glycophorin A		Surface antigens	
gp130		CD11a (LFA-1 α)	
ICAM-1/2		CD11b (Mac-1)	
Mab 1740		CD14	
p75 NGF-R		CD34	
SH3		CD45	
SH4		CD133	
Stro-1 (human only, antigen undefined)		ABCG2	
HLA class II-ve		cKit	
Secreted proteins		Sca-1	
Interleukins: 1 α , 6, 7, 8, 11, 12, 14, 15			
LIF, SCF, FLT-3 ligand, GM-CSF,			
G-CSF, M-CSF			

Further details can be found in the following references: Caplan & Bruder 2001; Bonnet 2002; Conrad *et al.* 2002; Erices *et al.* 2002; Zhao *et al.* 2002; Bonnet 2003; Deschaseaux *et al.* 2003; De Ugarte *et al.* 2003a,b; Hou *et al.* 2003; In'T Anker *et al.* 2003; Parsch *et al.* 2003; Prockop *et al.* 2003; Roufosse *et al.* 2003.

Table 2. Output phenotypes of MSC

1	Adipocyte/lipocyte
2	Cardiac myocyte
3	Chondrocyte
4	Endothelial cell
5	Fibroblast
6	Myofibroblast
7	Osteoblast
8	Pericyte
9	Skeletal myocyte
10	Tenocyte
11	Thymic stroma

Interested readers are referred to selected reviews and papers on these matters: 1, MacKenzie & Flake 2002, Martin *et al.* 2002, Ringe *et al.* 2002; 2, Lovell 2004; 3, Caterson *et al.* 2002, Ekholm *et al.* 2002, Quintavalla *et al.* 2002, Ringe *et al.* 2002, Sakai *et al.* 2003; 4, Al-Khaldi *et al.* 2003, Roufosse *et al.* 2003; 5, Direkze *et al.* 2004; 6, Direkze *et al.* 2003; 7, Petite *et al.* 2000, Moutsatsos *et al.* 2001, Tsutsumi *et al.* 2002, D'Ippolito *et al.* 2002, Quintavalla *et al.* 2002, Ringe *et al.* 2002, De Kok *et al.* 2003, Diefenderfer *et al.* 2003, Endres *et al.* 2003; 8, Shi & Gronthos 2003; 9, Camargo *et al.* 2004; 10, Awad *et al.* 2003; 11, Liechty *et al.* 2000.

Figure 1. MSC progression to osteocytic and chondrocytic lineages and some influencing factors. Several lineages from Table 2 are omitted for simplicity. The timing of each lineage event is unclear, and is left undefined. How many steps exist between each level in a lineage is unknown, and there may well be bi- or tri-potentialities in any population, which may even reverse. Maturation is presumed to be tissue or organ specific, dependent on local cues, whereas prior steps may be possible during the circulatory or homing phases. Factors may not act simultaneously or continuously, and may act in more than one lineage.

IMMUNOLOGICAL EFFECTS OF MSC

Much of the excitement surrounding stem cells in general, and MSC in particular, is predicated on their general applicability, and there are some indications that this may be true. It is well to be aware of recent immunological data on MSC that cast a shadow over this assumption, and may even give cause for concern over their use. It is emerging that MSC may suppress some immune responses, and may also be capable of facilitating tumour growth.

Thus, in 2003, Krampera *et al.* reported that MSC can inhibit naïve and memory T-cell responses independently of HLA class expression and γ -interferon stimulation or even the presence of antigen presenting or CD4⁺ T cells/CD25⁺ cells, in a so-called non-cognate manner. The inhibitory effects were proportional to MSC cell numbers, and was reversible, *in vitro*, on their removal (Krampera *et al.* 2003).

Figure 2. Murine MSC in culture. (a) Untreated mouse bone marrow-derived MSC clone 2 cells after 3 weeks of culture. Phase-contrast. (b) Adipocytic differentiation. Oil Red O stain for intra-cellular lipid droplets. (c) Chondrogenic differentiation in micromass culture. 5- μ section, PAS/diastase with haematoxylin counterstain. (d) Osteogenic differentiation. Alizarin Red stain for deposits of calcium salts. Original magnifications 200 $\times$. Scale bar 100 μ . (Rao, unpublished results).

Further, Djouad and colleagues reported that MSC actually reduce the immunocompetence of a recipient host, in a murine model (Djouad *et al.* 2003). They showed that primary MSC and a cell line of MSC-like cells (C3H/10T1/2) both reduced the Con A or allogeneic cell activation of lymphocytes when mixed with spleen cells. This was a CD8 T-cell response and was dependent on MSC cell number. The MSC were themselves unable to elicit an immune response in allogeneic host animals. C3H/10T1/2 cells expressing human BMP2 were still able to differentiate into bone in allogeneic hosts. When C57BL hosts bore the syngeneic B16 melanoma, tumour growth was seen in 90% of mice at 30 days, while allogeneic hosts (C3H mice) suffered none, indicating their active immune system. However, co-injection of 5×10^5 B16 and MSC in C3H allogeneic hosts led to tumour formation in 57% of mice by 21 days, irrespective of whether the two cell types were injected at the same site, and even at a ratio of 1 : 100 MSC to B16 cells. This indicates the role of circulating factor(s) in tumour formation. If these results are transferrable to the human situation, this experiment suggests that when we want to use MSC clinically, particularly allogeneic cells, we need to be very cautious, particularly with those recipients who may be at risk for tumour growth (smokers, families with hereditary cancers, etc.). This clearly needs much further investigation.

EARLY EVENTS IN MSC DIFFERENTIATION

Several recent reports throw light on the early events in MSC differentiation. How each is inter-related remains unclear at present.

Beqaj *et al.* demonstrated that the Rho pathway was important in regulating the fate of MSC in bronchial myogenesis. They found that undifferentiated MSC expressed high levels of RhoA, but on contact with the extracellular matrix component laminin 2 this was down-regulated, serum-response factor (SRF) became nuclear instead of cytoplasmic and the cells began a myogenic differentiation towards smooth muscle. Once triggered, subsequent RhoA expression further committed the cells in the smooth muscle direction (Beqaj *et al.* 2002). It is such conditionality that makes unravelling the roles of these factors so fascinating.

Gregory and co-workers have reported that the wnt signalling inhibitor dickkopf (Dkk)-1 is a stimulator of proliferation of early growth phase human MSC in culture, an effect reversed by anti-Dkk antibodies. Dkk-1 also lowered cytoplasmic β -catenin, a downstream effector of the wnt pathway (Gregory *et al.* 2003). This is depicted in Fig. 1 as Dkk-1 relieving the proliferative inhibition of wnt signals on MSC.

Deschaseaux and colleagues claim that CD49a⁺-directly selected BM cells produce the colony forming unit-fibroblast cell (CFU-F) which in turn can differentiate into adipo-/chondro-/osteo- and 'stromacytic' types (see Fig. 1). These were mildly CD45⁺ at first, but this was lost on culturing (Deschaseaux *et al.* 2003). A simpler method for isolating MSC is by filtering the smallest cells from a mixed population of BM cells through a 3- μ seive. Such cells retain tripotentiality for adipo-, chondro- and osteocytic lineages (Hung *et al.* 2002; Tuli *et al.* 2003), and may be a method of choice if researchers rapidly need MSC unaffected by antibodies.

OSTEOGENESIS

Several reviews of MSC differentiation into osteocytic cells and their utility in tissue engineering have been published recently (Minguell *et al.* 2001; Dennis & Charbord 2002; MacKenzie & Flake 2002; Noel *et al.* 2002; Cancedda *et al.* 2003). It is clear from the mushrooming literature that the use of MSC clinically will continue to progress as much on a 'can-do' approach as on a more cautious 'how-and-why?' approach. This section will review some key aspects of our understanding of the how and why, but will also refer to the utility of some can-do experiments.

MSC GROWTH IN CULTURE

The growth of MSC *in vitro* has been well documented, and is of variable ease depending on the source of cells, with human MSC being one of the less difficult types to grow (reviewed in Dennis & Charbord 2002; Tocci & Forte 2003). Frequently used culture conditions include the following additives to stimulate osteogenic progression:

- basal medium + 20% FCS + 10^{-7} or 10^{-8} M dexamethasone;
- 10 μ M β -glycerophosphate;
- 50–200 μ M vitamin C phosphate.

Basal media include Dulbecco's modified Eagle's medium (DMEM) (Devine *et al.* 2001; Martin *et al.* 2002), DMEM-low glucose (Romanov *et al.* 2003), α -minimum essential medium (MEM) (Quirici *et al.* 2002), as well as mixtures such as DMEM-LG plus MCDB201 (60–40%) (Lennon *et al.* 1995). Output readouts are usually alkaline phosphatase activity and Von Kossa or alizarin red staining for extracellular calcium deposition. The MSC usually need about 2–3 weeks' stimulation before the endpoint assays are positive. It is not yet clear why dexamethasone and vitamin C can be used at such widely different concentrations.

Recent advances have shown that MSC respond differentially to HGF and Vitamin D3. D'Ippolito and colleagues reported that adult human vertebral (T1–L5) BM-derived MSC proliferated 2.5-fold faster in 10 ng/ml HGF than control cells, whereas 10 nM D3 inhibited growth by 50% but raised alkaline phosphatase (AP) activity 8-fold in cultures up to 18 days. Combining the factors resulted in a 1.5-fold growth enhancement and a 13-fold rise in AP activity, and only this succeeded in producing a mineralizing phenotype (D'Ippolito *et al.* 2002). These findings will have great utility in future osteogenic studies of MSC cells.

It is reasonable to ask whether the common stimulators of osteoblastic differentiation exert their effects via some action on specific intra-cellular pathways. Recent observations include the repressive effects of peroxisome proliferator activated receptor (PPAR) γ (Lecka-Czernik *et al.* 2002; Shindo *et al.* 2003) and menin (Sowa *et al.* 2003) on the ability of MSC to undergo osteogenic transdifferentiation in culture. There are several natural agonists for PPAR γ and these can have different effects on MSC readout phenotypes, which may have an influence on BM delivery of precursor cells into the circulation, or the relative balance of fat and bone cells on the inner bone surfaces. Thus, adipogenesis from MSC was promoted by the 9- and the 9,10-dihydroxy-octadecenoic acids (HODD, Fig. 1), whereas the similar 9,10-epoxy-octadecenoic acid (EODD, Fig. 1) had no effect, but the latter did inhibit the osteogenic phenotype. Menin is the protein product of the multiple endocrine neoplasia type I gene, and the lack of this gene results in cranial bone hypoplastic defects. Sowa *et al.* tested the role of this protein in cultured murine MSC-like C3H10T1/2 cells. Alkaline phosphatase (AP) and BMP2-induced osteocalcin expression were both reduced by menin antisense mRNA treatment, in contrast to the adipocytic (oil red O, PPAR γ) and chondrocytic (alcian blue, collagen IX) differentiation markers, which were not affected. Menin could be co-immunoprecipitated with Smad 1 or 5 in the osteogenic restricted line MC3T3-E1, indicating dual control of differentiation. However, here, antisense mRNA to menin had the opposite effects from those on MSC: increased AP, osteocalcin and mineralization activities. It is also clear that the developmentally regulated gene sonic hedgehog (Shh) is responsible for modulating several genes in the MSC-like cell line C3H10T1/2 (Ingram *et al.* 2002). These include up-regulation of seven genes (thrombomodulin, GILZ, BF-2, Nr4a1, IGF2, PMP22, LASP1) and the repression of four genes (SFRP-1, SFRP-2, Mip1- γ , Amh). It is not clear how these genes co-operate in influencing MSC differentiation, but may have effects on survival (IGF2) and apoptosis (GILZ). Indeed, the latter protein inhibits adipogenesis by suppressing PPAR γ 2 expression in glucocorticoid-treated cells (Shi *et al.* 2003). IGF proteins are involved in many cellular processes, but are significant in the suppression of apoptosis (Vincent & Feldman 2002).

TISSUE ENGINEERING OF BONE

Many papers have shown the potential of MSC to become osteoblasts and contribute to bone formation. Recent advances include the ability to grow MSC in culture and graft them into large

excisional defects in sheep long bones in a mineral matrix containing ground coral exoskeleton (mostly calcium carbonate on a charged organic matrix). The rationale for using this matrix is that it already has an open porosity similar to bone, and is biologically compatible and resorbable. The combination of MSC plus matrix resulted in bone union with lamellar cortical medullary canal morphogenesis in three out of seven operations (Petite *et al.* 2000), whereas fresh BM cells in coral matrix did not result in union at all. The authors were not able to quantify the proportion of surviving MSC in their constructs, as no cell tracing methodology was used. These results contrast with previous reports using hydroxyapatite and hydroxyapatite-tricalcium phosphate ceramics, which did not have sufficient remodelled strength or porosity and were prone to breakage (Bruder *et al.* 1998).

Despite the possibility that hydroxyapatite and tricalcium phosphate may be less than perfect for bone remodelling, there is a recent report which uses this material to compare allogeneic and autologous MSC in a canine model of bone repair (De Kok *et al.* 2003). Matrices were pre-loaded with MSC and both allotypes were equivalent in their seeding efficiency at the SEM level, with 75% of the porous surface covered. Cell-free and MSC-loaded matrices were compared after 4 and 9 weeks' implantation. Both autologous and allogeneic MSC produced the same amounts of new bone formation, and both were significantly better than matrices without cells. Interestingly, the allogeneic MSC did not produce an immune response, as judged by lack of circulating antibodies and infiltrating lymphocytes. This may be due to the low or zero level of HLA II molecules on the cell surface of unstimulated MSC. This can rise, however, to 90% positivity on γ -interferon stimulation (Le Blanc *et al.* 2003). Differentiation into osteogenic, chondrogenic and adipogenic cell types did not alter this lack of allo-stimulatory effect of MSC. The authors suggested that MSC can be transplanted between allogeneic individuals. These results should be viewed in relation to the possible immunological effects of MSC described above.

Such experiments are useful to show the potential of unaltered MSC. Other approaches are available where the MSC are transfected with a gene which may influence both the lineage restriction of the cells and the tissue forming ability of those cells. Thus, Moutsatsos and colleagues used MSC expressing an inducible bone morphogenetic protein (BMP)-2 under the control of exogenous tetracycline, and showed that these cells could both form and regenerate bone tissue *in vivo* and *in vitro* (Moutsatsos *et al.* 2001). Such methods show much promise for future clinical use of MSC in this area.

The role of cell-matrix and ECM interactions in bone regeneration is vital. In the α -1 integrin knockout (KO) mouse there is normal bone formation, but injuries are not healed well. Thus, Ekholm and colleagues reported that the α -1 KO had fewer proliferating MSC in culture, and produced less callus *in vivo* after a femur fracture. In addition, these mice expressed less mRNA for several cartilage-related genes, including collagens II, IX and X (Ekholm *et al.* 2002).

CHONDROGENESIS

Cells isolated from cartilage tissue can be grown in culture fairly readily. Typical ingredients for promoting this include:

- basal medium + 100 nM dexamethasone;
- 50 μ g/ml Vit C phosphate;
- 10 ng/ml TGF β 3;
- 40 μ g/ml proline.

Similar conditions are used for the direction of MSC towards chondrocytic cells. Other factors have been added recently to this list, such as FGF2 (Tsutsumi *et al.* 2001) and HODD (Lecka-Czernik *et al.* 2002). It is not yet clear how these agents interact with each other.

Once liberated from their ECM, the cells proliferate well and change their shape to a fibroblastic morphology. If re-embedded in type II collagen, the cells revert to a more chondrocytic type. Such behaviour has been exploited in the tissue engineering of new cartilage for orthopaedic repairs (reviewed by Minguell *et al.* 2001; Dennis & Charbord 2002; MacKenzie *et al.* 2002; Tocci & Forte 2003).

One of the limiting factors in MSC research is the ability to consistently grow sufficient cells for use in tissue repair. The *in vitro* cell biology of MSC is becoming better understood, and their need for growth factor support is now evident. Tsutsumi and co-workers reported that human, rabbit and dog MSC responded *in vitro* to basic fibroblast growth factor (FGF)-2 with increased growth rate and life span, particularly in low-density cultures, which suggests that many population doublings can be achieved. Critically, these stimulated cells retained their tri-lineage potential for chondrocytic, osteocytic and adipocytic phenotypes (Tsutsumi *et al.* 2001).

With stem cell plasticity being topical, it is of interest to note that adult differentiated cells may retain some plasticity. This was reported by Tallheden *et al.* who showed that human chondrocytes could be re-stimulated to express osteogenic and adipogenic phenotypes in culture in appropriate media, but that *in vivo*, in contrast to MSC, they would only express a chondrocytic phenotype, and would not express an osteogenic one (Tallheden *et al.* 2003).

Hatakeyama *et al.* (2003) reported on the role of bone morphogenetic protein (BMP) 4 in the control of Smad proteins in transdifferentiation of MSC-like C3H10T1/2 and the chondrogenic MC615 cells in culture. The MSC-like cells did not produce a chondrogenic phenotype without BMP4, whereas the already-committed cell line did, as measured by type II collagen and Sox-9 expression or alcian blue staining for ECM. The Smad involvement was also differential, in that the MSC-like cells showed synergy in the activity of Smad 4 with Smad 8 in this phenotypic effect. By contrast, chondrogenic cells showed Smad 1 or Smad 5 synergy with Smad 4 (Hatakeyama *et al.* 2003) (see Fig. 1).

Cell tracing is a useful ability when using MSC *in vivo*, to be certain that the cells infused persist, and to help ascertain whether fusion or proliferation has occurred. It is equally important to be able to show that the tracability does not of itself detract from the ability of the MSC to differentiate into the cells of study. Quintavalla and colleagues showed that goat iliac crest MSC retained this ability when loaded for 2 h with 10 μ M fluorescent tracer dye Cell Tracker Orange (5-(((4-chloromethyl)benzoyl)amino)-tetramethylrhodamine; CMTMR). Chondrogenic phenotype was induced in both pelleted aggregates of MSC and cells seeded into gelatin foams, using high glucose DMEM containing 1% ITS, 100 nM dexamethasone, 50 μ g/ml vitamin C phosphate, 40 μ g/ml proline, 1 mM sodium pyruvate and 10 ng/ml TGF β 3. Osteogenic differentiation was produced using 10 nM dexamethasone, 50 μ M Vitamin C phosphate, 10 mM β -glycerophosphate and 100 nM prostaglandin E2. These culture conditions resulted in undiminished osteo- and chondrogenesis in 21 day cultures. During this time, the dye fluorescence on FACS was reduced 100-fold, but was still detectable above background at 28 days. Full-thickness MSC-gelatin foam implants were made into the trocheal groove and medial condyle of the knee joint and their *in vivo* differentiation followed for up to 2 weeks. By 2 days *in vivo*, only 27% of the cells in the implants remained CMTMR positive, while the histology of 2-week implants showed little bone or cartilage formation or retention of labelled MSC. The authors concluded that the gel-foam needs to be of higher resilience to digestion before it can be considered a useful vehicle for MSC implantation (Quintavalla *et al.* 2002).

A common disease for which there is as yet little the medical profession can do is the degeneration of intervertebral discs and the ensuing back pain. A recent report suggests that MSC in a type II collagenous matrix may be able to decelerate this process, if not yet reverse it. Sakai and co-workers described experiments using autologous rabbit iliac crest MSC transfected with adenovirus containing LacZ driven by the CAG promoter (cytomegalovirus IE enhancer, chicken β -actin promoter and rabbit β -globin polyadenylation signal). MSC were seeded into Atelocollagen, a proprietary bovine type II collagen preparation which has no N- and C-globular domains which house the main antigenic sites of the molecule. Lumbar nuclei pulposi were aspirated and replaced 2 weeks later with the collagen matrix containing autologous LacZ-positive MSC ($10^6/\text{ml}$). Discs were examined at 2, 4 and 8 weeks. Control discs without cell or matrix replacement showed progressive vacuolation of nucleus pulposus cells over time, inner annulus fibrosus collapse at 4 weeks and extensive fibrosis and disc flattening by 8 weeks. The MSC/collagen grafted discs showed little alteration from unoperated discs, with intense ECM staining using Safranin O for extracellular proteoglycans in the areas containing the now spindle-shaped MSC-derived cells, which remained LacZ positive up to 4 weeks. Whether by 8 weeks there were no surviving inoculated MSC cells was not clear, yet the discs appeared remarkably normal (Sakai *et al.* 2003).

CONCLUSIONS

Much more information is now available on what controls the way MSC differentiate in many environments, from transcription to growth factors. Gene chip analyses are helping decipher the many pathways involved in these transitions. It is clear that there is, akin to many scenarios, much built-in redundancy in control mechanisms and stimuli to attain a similar outcome. Whether these are capable of simple substitution remains to be seen. It is more likely that each bestows a form of conditionality or synergism onto the cells, which may have a phenotypic readout or not, depending on the situation (say immune challenge, wounding). Understanding these subtleties will bring great advances to cell and tissue therapies for genetic or somatic diseases.

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