

## REVIEW

# Stem cells as vehicles for orthopedic gene therapy

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Adult stem cells reside in adult tissues and serve as the source for their specialized cells. In response to specific factors and signals, adult stem cells can differentiate and give rise to functional tissue specialized cells. Adult mesenchymal stem cells (MSCs) have the potential to differentiate into various mesenchymal lineages such as muscle, bone, cartilage, fat, tendon and ligaments. Adult MSCs can be relatively easily isolated from different tissues such as bone marrow, fat and muscle. Adult MSCs are also easy to manipulate and expand *in vitro*. It is these properties of adult MSCs that have made them the focus of cell-

mediated gene therapy for skeletal tissue regeneration. Adult MSCs engineered to express various factors not only deliver them *in vivo*, but also respond to these factors and differentiate into skeletal specialized cells. This allows them to actively participate in the tissue regeneration process. In this review, we examine the recent achievements and developments in stem-cell-based gene therapy approaches and their applications to bone, cartilage, tendon and ligament tissues that are the current focus of orthopedic medicine. Gene Therapy (2004) 11, 417–426. doi:10.1038/sj.gt.3302197 Published online 15 January 2004

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## Introduction

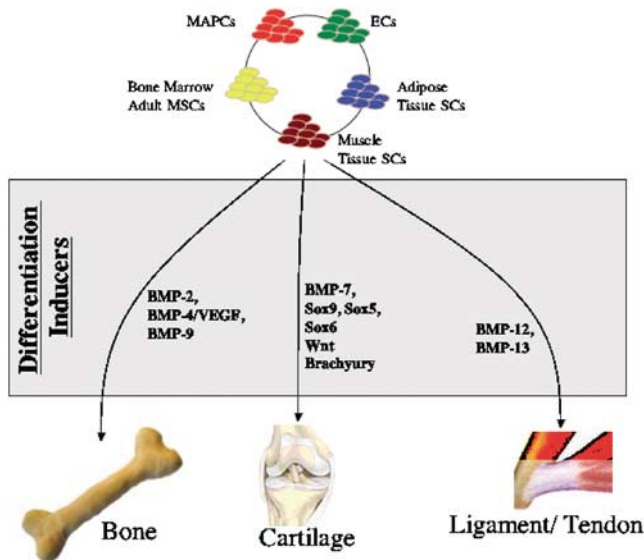
Stem cells are a distinct population of cells that form the source of tissues. Two main features characterize stem cells of all types: self-renewal ability, and the ability to give rise to differentiating cells. Stem cells can be further divided into two major groups. The first group constitutes the embryonic stem cells (ES), which together with the totipotent zygote present a cell population able to give rise to a multitude of cell types and tissues.<sup>1</sup> The second group constitutes adult stem cells, which reside in adult tissues and give rise to differentiated, tissue-specialized cells. These cells are responsible for the regenerative capacities of tissues. Generally, adult stem cells present a more limited range of differentiation lineages compared with ES cells. In recent studies done by Jiang *et al*<sup>2</sup> it was reported that multipotent adult progenitor cells (MAPCs) that were copurified with MSCs from adult marrow, can differentiate at the single cell level, not only into mesenchymal cells, but also to cells with visceral mesoderm, neuroectoderm and endoderm characteristics *in vitro*. In light of this, adult cells are preferable for therapeutic purposes as they are considered safer for transplantation with lesser proliferation capacity and tumorigenicity than ES cells. In addition, adult stem cells are more easily directed to specific lineages than ES cells which can give rise to a wide range of tissues following local transplantation.<sup>3</sup> Skeletal tissues such as bone, cartilage, tendon and ligament, which are the focus of orthopedic medicine, vary in their ability to self-regenerate. Whereas bone

tissue is considered to have high regeneration capacity and ligament tissue a somewhat lesser degree; cartilage tissue is considered to have a very low self-repair ability.<sup>4,5</sup> All these tissues originate from similar common stem cells. As much as MSCs possess a higher self-repair ability, their capability to recruit surrounding adult MSCs from their local environment is higher too.<sup>6</sup>

Adult MSCs are stem cells residing in a variety of adult mesenchymal tissues. Readily isolated from the bone marrow and expanded in culture,<sup>7</sup> they were shown to differentiate into various mesenchymal lineages including bone, cartilage, adipose, muscle and tendon.<sup>8</sup> Their accessibility and ease to manipulate *in vitro* has made bone-marrow-derived adult MSCs natural candidates for orthopedic gene therapy studies and the focus for the development of therapeutic approaches in orthopedic therapy. However, bone-marrow-derived adult MSCs are not the only stem cells found to differentiate to various skeletal tissues. Stem cells from other tissues, such as muscle and fat, were also found to have similar properties.<sup>9</sup> The aim of the present review is to outline the main features and properties of stem cells and the different strategies developed for their use in orthopedic therapeutic modalities directed to three main skeletal tissues: bone, cartilage and ligament (Figure 1).

## Bone marrow adult MSCs for bone gene therapy

Since adult MSCs and osteoprogenitors are relatively easy to isolate from the bone marrow and culture *in vitro*, it is conceivable to use them as vehicles for the delivery of therapeutic genes *in vivo*, a strategy known as cell-mediated gene therapy.<sup>10,11</sup> Most gene therapy studies



**Figure 1** Stem cells may be genetically induced to differentiate into various skeletal tissues.

directed to bone healing attempt to induce bone formation in a model of bone nonunion fractures or defects. Indeed, some studies have utilized primary adult MSCs and cell lines for the expression and delivery of osteogenic genes inducing bone formation.<sup>12–15</sup> These studies implemented various types of MSCs including cell lines such as C3H10T1/2 and primary marrow-derived stem cells for the delivery of bone morphogenetic protein-2 (BMP-2). The delivery of growth factors of the BMP family is often employed in these studies, since these factors promote osteogenic differentiation and bone formation.<sup>16,17</sup> In particular, BMP-2 is commonly used because it is a highly osteoinductive agent, well studied and known to induce bone *in vivo* in ectopic and orthotopic sites.<sup>18–26</sup> Other members of the BMP family, such as BMP-4 and -9, are also used for stem-cell-mediated gene therapy.<sup>27–31</sup> The hypothesis of these studies is that healing of bone defects can be achieved by long-term production of osteoinductive agents in the vicinity of the defect, inducing new bone formation. Adult MSCs, according to this rationale, serve as vehicles for gene delivery that are relatively easy to isolate, manipulate *ex vivo* and successfully engraft *in vivo*.

Bone-marrow-derived adult MSCs are good candidates for gene therapy directed to bone regeneration, not only because of their accessibility, but also because they form the source stem cells for osteoprogenitors and osteoblasts, the bone forming cells in the bone environment.<sup>6</sup> Osteogenic differentiation begins with the commitment of the undifferentiated adult MSC to the osteogenic lineage, giving rise to committed osteoprogenitor cells that gradually differentiate into mature osteoblasts.<sup>6</sup> We have previously postulated that utilizing genetically engineered MSCs for bone-cell-mediated gene therapy may have a particular advantage.<sup>10</sup> When these cells are engineered to express osteogenic growth factors such as recombinant human (rh) BMP-2, upon transplantation *in vivo*, the expressed transgene exerts its effect not only on host mesenchymal tissue (paracrine effect) but on the engineered adult MSCs as well

(autocrine effect). Thus, the engrafted, engineered MSCs also differentiate and contribute to the bone formation process. We have postulated that these dual autocrine and paracrine effects may promote bone formation to a larger extent than any other cell type merely exerting a paracrine effect. Using murine C3H10T1/2 mesenchymal stem cell lines that were engineered to express rhBMP-2, we were able to demonstrate their increased osteogenic potential over non-MSCs engineered Chinese hamster ovary (CHO) cell lines that also expressed rhBMP-2.<sup>10</sup> Engineered MSCs were able to heal murine nonunion radial defects to a greater extent than nonosteogenic CHO cells, despite the fact that these cells secreted more rhBMP-2 protein than the engineered MSCs.

Utilizing adult MSCs as vehicles for gene delivery has an additional benefit over direct *in vivo* delivery of proteins or genes. Engineered adult MSCs can potentially engraft into the damaged tissue *in vivo* and express the therapeutic genes for long periods, whereas local, one time administration of genes or protein will have a limited time effect. BMP family members are known for their ability to induce bone formation *in vivo* and repair bone defects when applied locally in the injury sites.<sup>32,33</sup> In order to test the efficiency of stem-cell-mediated gene therapy compared with BMP-2 protein delivery, Moutsatsos *et al*<sup>34</sup> have tested the extent of bone tissue produced by engineered MSCs (C3H10T1/2) expressing rhBMP-2 compared with local administration of a high dose of rhBMP-2 in murine radial nonunion defect. The authors have found that the engineered MSCs produced significantly more bone tissue than was produced following local administration of rhBMP-2 protein.

MSC or osteoprogenitor cell-mediated gene therapy holds yet another advantage over protein delivery or even other types of gene delivery. When analyzing the healing process in bone defects following transplantation of MSCs engineered to express rhBMP-2, an interesting pattern is observed. Engineered MSCs produce bone in an organized manner by augmenting new cartilage and bone on top of the defect edges, forming continuous regeneration between the original defect edges and the newly formed bone.<sup>10,34,35</sup> In comparison, rhBMP-2 protein delivery or the implantation of non-MSCs (CHO cells) expressing rhBMP-2 resulted in the formation of diffuse osseous foci with no continuity to the original bone.<sup>10</sup> This phenomenon can be attributed to the ability of MSCs to localize and orient themselves to particular sites in the defect area following transplantation. We have found that MSCs localized mainly to the edges surrounding the defect rather than being randomly distributed.<sup>10</sup> Apparently, being stem cells and progenitors, MSCs can respond to local factors and developmental signals that direct and guide their orientation in the transplantation site and affect the healing process in a manner similar to that in development. Liechty *et al*<sup>8</sup> demonstrated that human adult MSCs possessed these characteristics by showing that these cells were able to engraft in various fetal mesenchymal tissues following systemic administration *in utero* in sheep. Moreover, human adult MSCs were able to localize into the osteoprogenitor layers of calvarial bone when transplanted subcutaneously adjacent to the calvaria in SCID mice.<sup>36</sup>

Human bone-marrow-derived adult MSCs present the same features as murine adult MSCs and are expected

to have the same benefits as those described above. However, if stem cells engraft and respond to local signals, what possible advantage does genetic engineering have? This question is stressed in the case of human adult MSCs which were previously found to regenerate bone *in vivo* upon local transplantation to bone defect, even without any genetic engineering.<sup>37</sup> On the other hand, additional studies have shown that human adult MSCs cannot form bone when engineered to express the LacZ gene or when they are not engineered at all.<sup>35</sup> It was found that human adult MSCs infected with adenoviral vector encoding human BMP-2 were able to differentiate into osteogenic cells both *in vitro* and *in vivo* forming cartilage and bone tissues, and healing nonunion defects created in nude mice.<sup>35</sup> Human MSCs infected with adenoviral vector encoding the LacZ reporter gene were not able to form bone or cartilage *in vivo*. The type of carrier employed for the human cells when delivered *in vivo* can explain this discrepancy. It was found that the ability of human adult MSCs to form bone is dependent upon osteoinductive carriers such as HA/TCP (hydroxyapatite/tricalcium phosphate) that are nonbiodegradable.<sup>38</sup> Consequently, genetic engineering of human adult MSCs may elicit the osteogenic potential of adult MSCs regardless of the carrier type, with the use of biodegradable carriers.<sup>35,39</sup> Therefore, human adult MSCs possess therapeutic potential even without genetic engineering. However, this potential is greatly dependent upon carriers promoting the osteogenic differentiation of adult MSCs; these carriers are often nondegradable. Also, their osteogenic potential is greatly enhanced upon genetic engineering with osteogenic growth factors such as BMP-2. One can safely assume that in large bone defects, 'naked' human adult MSCs will not be sufficient to induce repair compared to genetically engineered cells.

All the above studies demonstrate the unique features that are present in adult MSCs obtained from the bone marrow, granting them additional advantage for use in bone gene therapy and gene delivery aside from their accessibility. These stem cells can serve as 'smart' vehicles that, in addition to expressing the transgene in specific areas of the damaged tissue, can also actively participate in the bone formation process.

### Various sources of stem cells for bone gene therapy

Adult MSCs that reside in the bone marrow are the natural stem cells for bone formation. However, this does not exclude the use of other sources of stem cells, from other mesenchymal tissues, that upon introduction of appropriate osteogenic genes can engraft in bone tissue and differentiate to bone forming cells. The most prominent cells studied in this regard are muscle stem cells.<sup>40-42</sup> Muscle tissue contains stem cells with the ability to differentiate into osteoblasts under the influence of a proper osteogenic factor such as BMP-2.<sup>9</sup> These cells, although originating from murine muscle tissue, following engineering to express rhBMP-2, are able to differentiate into osteoblasts and osteocytes and can heal critical nonunion bone defects in the calvaria.<sup>40</sup> Likewise, engineered cells from human skeletal muscle were shown to have osteogenic potential as well, when engineered to express rhBMP-2 both *in vitro* and

*in vivo*.<sup>41</sup> In both studies adenoviral vectors were implemented for gene delivery. As muscle tissue is part of the MSC differentiation pathway, these results are not surprising. BMPs can alter the differentiation pathways of muscle progenitors towards the osteogenic pathways.<sup>17</sup> Muscle-derived stem cells are very convenient to utilize since they can be isolated from muscle by way of a needle biopsy and expanded *in vitro*.

Fat tissue stem cells can also respond to BMP signaling by converting from the lipogenic differentiation pathway towards the osteogenic differentiation pathway.<sup>43,44</sup> The isolation of adult stem cells from fat tissue is relatively easy. Therefore, it is possible to use stem cells from fat tissue origin for bone gene therapy as well. Osteogenic differentiation of adipose-derived stem cells was induced by transfection with rhBMP2.<sup>45</sup>

MAPCs that were copurified with MSCs from adult marrow, differentiated not only into mesenchymal cells, but also into cells with mesoderm, neuroectoderm and endoderm characteristics *in vitro*. *In vivo*, MAPCs engrafted and differentiated to the hematopoietic lineage and to the epithelium of liver, lung and gut. No contribution was observed in skeletal tissues, where low turnover is maintained in the absence of damaged tissue.<sup>2</sup> Nevertheless, as MAPCs proliferate at a high rate, they may also be considered as another source for cell-mediated therapy.

ES cells originating from the inner cell mass of an embryo in the blastocyst stage have a wide differentiation potential both *in vitro* and *in vivo*. They can give rise to a variety of cell types including neural cells, cardiomyocytes, vascular cells and hematopoietic cells.<sup>46</sup> ES cells were also found to differentiate into osteogenic cells *in vitro*.<sup>47</sup> However, there are still no data on ES cell osteogenesis *in vivo*. The problem here is that ES cells differentiate into various cell types, both *in vitro* and *in vivo*, so to obtain purified cell lineage is very difficult, compared with bone-marrow-derived MSCs. Additionally, obtaining human ES cells encompass major ethical issues.<sup>48</sup> Moreover, it was recently found that MAPC copurified with adult MSCs obtained from the bone marrow possess a pluripotent range of differentiation that includes all cell types in the embryo.<sup>49</sup> This pluripotent differentiation potential of adult MSCs is no less than that of ES cells. For these reasons, one can expect that ES cells will not be the main target cells for gene therapy applications in orthopedics in the future whereas, the safer and pluripotent adult MSCs obtained from skeletal tissue make a more realistic choice.

### Angiogenesis and bone gene therapy

Most gene therapy strategies to facilitate bone regeneration, as was discussed above, focus on the delivery and expression of osteoinductive genes, such as members of the BMP family. Such growth factors promote osteogenic differentiation of adult MSCs, osteoprogenitors and osteoblasts. This approach is aimed at initiating and promoting the primary process that is responsible for osteogenesis. A different, novel approach was suggested recently that targets initiating secondary processes supporting new bone formation by promoting angiogenesis.<sup>29,34</sup> Angiogenesis was found to be closely correlated to enchondral bone formation during development.<sup>34,50</sup>

It was found that vascular endothelial growth factor (VEGF) couples the transition from cartilage to bone in developing bones. Moreover, it was found that applying TNP-470, an angiogenesis inhibitor, could markedly reduce BMP-2-induced ectopic bone formation in muscle tissue. Other studies have found a correlation between angiogenesis and GDF-5, a member of the BMP family, and SMAD5, a BMP signaling molecule.<sup>51,52</sup> Recently, our group reported an important finding linking genetically engineered adult MSCs expressing BMP-2 induced angiogenesis *in vivo* with new bone formation.<sup>34</sup> Increased blood vessel formation was observed, coupled with new bone and cartilage created in ectopic muscle tissue transplanted with engineered adult MSCs. Our study showed in chicken chorioallantoic membrane (CAM) assay that BMP-2 protein induced angiogenesis and may, in part, mediate the angiogenesis observed in transplants of genetically engineered adult MSCs. These studies clearly indicate the important supporting effect that angiogenesis and its mediator, VEGF, have on bone formation.

The next step to implementing angiogenic growth factors promoting bone formation was undertaken by Peng *et al.*<sup>29</sup> In this study, the authors implemented a combination of BMP-4 and VEGF both expressed in muscle-derived stem cells. It was found that VEGF alone expressed in muscle stem cells did not elicit any bone response. However, when expressed together with BMP-4, a synergistic effect of VEGF and BMP-4 was observed. Timing and the ratio between VEGF and BMP-4 expression were found to be most crucial in this study.<sup>29</sup> Once again, the importance of angiogenesis in new bone formation was demonstrated when the soluble VEGF antagonist Flt1 was shown to inhibit new bone formation elicited by BMP-4. These studies represent a new approach which utilizes genes that mediate osteogenesis as the primary mechanism, and advocates a secondary supporting mechanism to encourage a synergistic effect on new bone formation.

### Gene therapy approaches for bone systemic diseases

The most common pathology in bone addressed by gene therapy studies has been nonunion bone defects.<sup>10,12–15,34,35,39–41</sup> As discussed above, adult stem-cell-based gene therapy has successfully addressed this problem in animal models by utilizing adult MSCs of bone marrow or muscle origin. These were genetically engineered to express osteogenic growth factors, primarily members of the BMP family, and transplanted locally. Several studies have aimed at developing gene therapy platforms for systemic and metabolic bone diseases. These diseases present more complex pathologies since they require systemic repair and the involvement of different genes, some of which are not fully known.

Osteoporosis is a disease resulting in bone loss and osteopenia. Although the results of bone loss are the same, two types of osteoporosis are commonly recognized: Type I or postmenopausal osteoporosis and Type II or senile osteoporosis.<sup>53,54</sup> The pathophysiology is considered different for both types. Type I is related to increased osteoclastogenesis resulting in over-resorption of bone due to estrogen depletion. Type II is related to

decreased osteogenesis due to the senescence of bone marrow adult MSCs reflected by decreased number, proliferation and osteogenic activity.<sup>11,55–57</sup> Although osteoclastogenesis is increased in Type I osteoporosis, there is ample evidence of decreased osteogenesis as well.<sup>58</sup> It is therefore a rational approach to attempt to increase bone mass in osteoporosis by increasing osteogenesis. Indeed, we have shown that systemic administration of rhBMP-2 protein into osteoporotic mice with both Type I and Type II osteoporosis models has resulted in increased osteogenic potential of bone marrow adult MSCs leading to restoration of bone mass.<sup>11</sup> Moreover, we have shown that human bone marrow adult MSCs obtained from osteoporosis patients have increased osteogenic activity and proliferation following infection with adenoviral vector encoding rhBMP-2.<sup>35</sup> Furthermore, these engineered cells were able to form bone *in vivo* and regenerate nonunion defects in CD nude mice. These studies indicate the potential use of bone marrow adult MSCs engineered to express osteogenic growth factors such as BMP-2 for the treatment of osteoporosis. Since bone marrow adult MSCs are affected in both Type I and Type II osteoporosis, it is conceivable to target these cells for gene therapy applications.

An opposite approach directed at blocking osteoclastogenesis was suggested by Goater *et al.*<sup>59</sup> to prevent the loosening of prosthetic implants due to bone resorption. The authors engineered synovial fibroblasts to express osteoprotegrin (OPG), a receptor activator of NF-kappaB ligand (RANKL) receptor antagonist that counteracts the osteoclast differentiation action of RANKL. Engineered fibroblasts were able to inhibit osteoclastogenesis induced by debris in mouse calvaria. This approach can be easily duplicated and applied to the bone marrow in osteoporosis utilizing adult MSCs as vehicles for OPG expression. However, in this approach adult MSCs serve merely as vehicles for OPG delivery and do not have any therapeutic properties of their own, as is the case when bone formation is desired.

Another interesting approach directed mainly towards age-related bone loss, Type I osteoporosis, was suggested by Yudoh *et al.*<sup>60</sup> The authors approach was directed towards the pathological mechanism of senescence affecting bone marrow osteoblasts, resulting in decreased proliferation of these cells related to reduced osteogenesis and consequently bone loss. In order to overcome senescence, the authors transduced human osteoblasts and osteoblastic cell lines that displayed the senescent phenotype with the telomerase reverse transcriptase (hTERT) gene. The forced expression of hTERT resulted in increased telomerase activity in these cells and consequently elevated replication capacity and delayed senescence. It is the author's suggestion to further use this approach for cell-based gene therapy for osteoporosis.

Osteopetrosis is a genetic disease that results in the opposite phenotype from osteoporosis. Excessive bone is formed in this disease, eliminating the bone marrow from the bone compartment and eventually resulting in death due to lack of sufficient hematopoiesis.<sup>61</sup> Osteopetrosis is caused by a decrease in osteoclastogenesis, which can be due to a genetic mutation of essential growth factors for osteoclast development such as colony-stimulating factor-1 (CSF-1). The op/op mouse

carries a genetic defect in CSF-1 and serves as a model for osteopetrosis. Abboud *et al* have suggested over-expression of soluble forms of CSF-1, specifically in osteoblasts, as a potential model of gene therapy for osteopetrosis.<sup>62</sup> To corroborate the notion that expression of CSF-1 by osteoblasts can reverse the osteopetrotic phenotype, the authors have created a transgenic op/op mouse that harbors the CSF-1 cDNA under the control of the osteoblastic specific osteocalcin promoter. The authors reported that within 5 weeks of birth, the osteopetrotic phenotype was completely reversed to the wild-type phenotype. One can assume that utilizing an *ex vivo* approach with bone-marrow-derived adult MSCs would be beneficial, as stem cells reside for long periods in the bone marrow and may express CSF-1 long term.

Osteogenesis imperfecta is a genetic disease that affects the quality of the bone formed in the body. Due to a mutation in the collagen originating in one of its procollagen genes, the resulting assembly and production of mature collagen fibers is impaired.<sup>61</sup> In order to overcome the genetic mutation, the delivery of the correct form of procollagen gene must be achieved. The *oim* mouse model with a defect in Pro $\alpha$ 2(I)-chain gene presents an osteogenesis imperfecta phenotype. Niyibizi *et al*<sup>63</sup> have proven the therapeutic potential of stem-cell-based gene therapy in osteogenesis imperfecta by using infected *oim* mice bone-marrow-derived adult MSCs in culture with adenoviral vector encoding for the correct Pro $\alpha$ 2(I) cDNA. The authors reported that the corrected gene was not only expressed *in vitro* in these cells, but was also able to assemble correctly with other procollagen chains, forming a stable type I collagen fiber composed of Pro $\alpha$ 1(I) and Pro $\alpha$ 2(I) in the correct ratio of 2:1.

Finally, all of the above studies demonstrate that bone marrow adult MSCs play a crucial role in the pathophysiology of systemic and metabolic bone diseases such as osteoporosis and osteogenesis imperfecta. However, even in diseases where the pathophysiology is not related specifically to adult MSCs, as in osteopetrosis, their unique properties make them good candidates for cell-mediated gene therapy.

### Vectors and promoters

The relatively easy isolation and expansion *in vitro* of adult MSCs from the bone marrow and other skeletal tissues has made them readily available for genetic manipulation with various vectors. The most common vectors used are classical adenoviral vectors.<sup>35,40,41,63</sup> However, adenoviral vectors do not integrate into cell genome and therefore, have limited expression time. Retroviral vectors have also been used for transducing adult MSCs and osteoprogenitors but with relatively poor results.<sup>14</sup> Modifications of retroviral infection techniques were suggested to improve the transduction rate of adult MSCs. Kuhlcke *et al*<sup>64</sup> showed positive results when tissue culture vessels were preloaded with retroviral vectors by low-speed (1000 g) centrifugation. Adult MSCs were also effectively transduced with retroviral vectors pseudotyped with vesicular stomatitis virus (VSV).<sup>65,66</sup> Human bone-marrow-derived adult MSCs were found to be highly susceptible to infection by VSV-pseudotyped retrovirus, achieving transduction

rates of greater than 81%.<sup>66</sup> Transduction with VSV did not alter the proliferation and differentiation potential of bone-marrow-derived adult MSCs, confirming the safety of these vectors.

An interesting and novel approach was undertaken by Peng *et al*<sup>67</sup> for enhancing the secretion of BMP-4 transgene from transduced bone marrow adult MSCs *ex vivo*. The authors used an MFG-based, VSV-G pseudotyped retroviral vector. In order to increase BMP-4 secretion, the authors created hybrid constructs encoding BMP-4 peptide linked to a BMP-2 propeptide sequence. Replacement of the BMP-4 propeptide region with that of BMP-2 resulted in increased secretion of BMP-4 by engineered MSCs following transduction.

Achieving high expression is not the only goal of stem-cell-based gene therapy applications in orthopedics. Limiting the expression in terms of level and duration is desired as well. It is particularly important in orthopedic medicine, since in many applications more than one course of therapy is required to achieve tissue healing. One way to ensure limited expression of the transgene is the use of tissue-specific promoters. Osteoblast-specific promoters will ensure expression in the bone marrow zone where active synthesis of bone matrix occurs. Stover *et al*<sup>68</sup> used the collagen1 A1 promoter sequence in order to achieve osteoblastic-specific expression. Expression of the marker gene regulated by the tissue-specific promoter, was limited to osteoblasts both *in vitro* in adult MSC cultures and *in vivo* in chimeric embryos containing ES cells engineered with the same construct. A similar approach for using the osteocalcin promoter was undertaken by Abboud *et al*<sup>62</sup> where osteoblasts were engineered to express CSF-1 for the treatment of osteopetrosis. Limiting expression to osteoblasts is important to prevent expression in non-skeletal cell types and is required more in genetic skeletal diseases or systemic metabolic diseases such as osteopetrosis and osteoporosis. For skeletal defects due to injury, trauma or time-limited diseases this approach is less relevant, as it maintains long-term expression in osteoblasts.<sup>62</sup>

Fine tuning of transgene expression and temporal control on the duration of expression may be critical in the future development of gene therapy applications for orthopedic medicine. To accomplish this goal we have suggested the use of tetracycline-regulated promoters to manage transgene expression in our model MSC line, C3H10T1/2, expressing rhBMP-2.<sup>34</sup> In a Tet-off promoter system, where tetracycline inhibits BMP-2 transgene expression, we were able to show that engineered MSCs expressed rhBMP-2 under appropriate tetracycline regulation *in vitro* and *in vivo*. *In vitro*, BMP-2 regulation by tetracycline controlled the osteogenic differentiation of the engineered MSCs. In conditions promoting BMP-2 expression, osteogenic differentiation of engineered MSCs was induced, whereas this did not occur in the presence of tetracycline. *In vivo* experiments, where engineered MSCs were transplanted both ectopically in muscle tissue and in radial nonunion bone defects, we found that bone formation and nonunion defect regeneration were both dependent on tetracycline control. Tetracycline administered to mice transplanted with the engineered cells completely inhibited bone formation and defect regeneration otherwise observed in the absence of tetracycline. This study demonstrates the

potential of exogenously regulated promoters. Such promoters have the potential to allow us to control the time and level of transgene expression and therefore to modulate its biological effects in real time. Such constructs can be used both in chronic systemic and metabolic diseases, such as osteoporosis, that may need long-term gene expression and regulation, and for local injury and time-limited disease. In the latter case, regulated, nonintegrating constructs are preferable.

### *Stem-cell-based gene therapy applications for cartilage*

Cartilage, unlike bone, is a tissue known for its difficulty in self-repair. Cartilage is deprived of blood vessels and composed of chondrocytes that are embedded in the matrix they produced, rich in collagen II, IX, XI and proteoglycans. Cartilage is present mainly in joints where its function, together with the joint synovial fluid, is to form the interface between two adjacent bones and to allow movement. Cartilage degeneration can occur in injury as well as in chronic diseases like osteoarthritis and rheumatoid arthritis. In these conditions, repair of the cartilage is rarely achieved spontaneously. Although the etiologies of osteoarthritis and rheumatoid arthritis are different, with the latter being an inflammatory disease, the outcome of both diseases results in the loss of large portions of joint cartilage. Possible reasons for deficient self-regeneration of cartilage tissue are poor vascularization, insufficient stem cell recruitment and persistent chronic inflammation.<sup>69–71</sup> Cell-mediated gene therapy strategies for these diseases can be roughly divided into two approaches. The first is the delivery of genes that counteract the inflammatory process and the second is delivery of cells and genes that can promote chondrogenesis and new cartilage formation.

In the first approach the delivery of genes related to the inflammatory process were studied. As the cytokine IL-1 was found to play a key role in articular cartilage inflammatory process, several studies have focused on counteracting the activity of IL-1. The delivery of the secreted IL-1 receptor antagonist gene was the most common strategy employed.<sup>72,73</sup>

Another problem that was not answered in the studies directed against the inflammatory process was that even when successful, this approach can prevent further damage to articular cartilage but cannot restore already inflicted damaged cartilage.

In order to repair cartilage, a strategy should be taken to promote chondrogenesis and to induce new cartilage formation. Delivery of factors that are known to promote chondrogenesis or to participate in cartilage homeostasis is one approach. Insulin-like growth factor-1 (IGF-1) is known to participate in cartilage homeostasis and matrix production. Several studies have implemented the delivery of the IGF-1 gene into cartilage.<sup>74–76</sup> Genes encoding other growth factors known to promote cartilage differentiation of chondrocyte progenitors have also been studied. For example, Lee et al<sup>77</sup> have utilized fibroblasts engineered to express transforming growth factor- $\beta_1$  (TGF- $\beta_1$ ) in rabbit knee joints with experimental cartilage defects. In this model, fibroblasts served as vehicles for the delivery of TGF- $\beta_1$  to host chondrocytes and chondrocyte progenitors. Others have expressed

BMP-2 in fibroblasts and chondrocytes for the promotion of cartilage formation in knee joint cartilage.<sup>78</sup> Cell-mediated gene therapy was applied in this setting specifically to overcome the disadvantages of direct viral gene delivery.

Cell-mediated gene therapy, however, can bestow greater benefit than just avoiding *in vivo* viral gene delivery, in particular by utilizing stem cells. Adult MSCs are potential candidates for cartilage repair as source cells for chondrocytes. Adult MSCs were shown previously to differentiate into chondrocytes *in vitro* and *in vivo*.<sup>6,8,79</sup> Engineering of adult MSCs to express chondrogenic genes has the potential to direct their differentiation towards cartilage *in situ* and hence to repair damaged cartilage tissue. By implementing this approach, Mason et al<sup>80</sup> have used periosteal adult MSCs, engineered to express BMP-7, to regenerate rabbit osteochondral defects. Engineered adult MSCs were seeded *ex vivo* onto polyglycolic acid carrier scaffolds and transplanted into osteochondral defects *in vivo*. Interestingly, similar to bone-marrow-derived adult MSCs, muscle-derived stem cells were also shown to differentiate into chondrocytes and engraft in articular cartilage following transplantation. This demonstrated the feasibility of using muscle-derived stem cells for cartilage-cell-mediated gene therapy.<sup>81</sup> ES cells were also shown to differentiate into chondrocytes *in vitro* when activated by BMP-2 and BMP-4.<sup>82</sup>

All the above studies implementing adult MSCs have used growth factors of the BMP family to induce cartilage differentiation. However, BMPs are not exclusive for cartilage differentiation. BMPs are known for their induction of bone tissue or enchondral bone tissue, that is, bone tissue preceded by cartilage formation.<sup>18,19</sup> Most likely, other local factors have interacted with BMP-7 in order to form cartilage *in situ* when engineered adult MSCs were transplanted locally into articular cartilage defects.<sup>80</sup> Otherwise, one would expect that newly formed cartilage would be transformed to bone eventually as a consequence of continuous BMP expression. Limited level and duration of BMP expression may provide a solution for this problem. Other possible solutions would be to engineer adult MSCs to express factors that are exclusive for inducing cartilage differentiation. Several candidate genes may be considered. All of these factors are signal transduction molecules. The role of Sox9 and other Sox members (Sox5, Sox6) in chondrogenic differentiation is well recognized.<sup>83</sup> Signaling molecules from the Wnt family were also found to associate with BMP-2 signal transduction pathways to enhance chondrogenic differentiation of MSCs.<sup>84</sup>

Recently, we have characterized a novel transcription factor from the T-box family (Brachyury) that induces chondrogenic differentiation only in MSCs.<sup>85</sup> When expressed in the C3H10T1/2 MSC line, chondrogenic differentiation alone was induced *in vitro*, reflected in the expression of chondrogenic markers, primarily of the early stage such as type II collagen. Moreover, transplantation *in vivo* of genetically engineered MSCs expressing Brachyury has resulted in ectopic cartilage formation in abdominal muscle. No osteogenic differentiation was observed *in vitro* or *in vivo*. These results demonstrating the exclusive chondrogenic differentiation induced in MSCs *in vitro* and *in vivo* are encouraging, and indicate the advantages of using

stem-cell-based gene therapy approaches for cartilage tissue regeneration, using specific cartilage factors such as Brachyury.

### Stem-cell-based gene therapy applications for tendon and ligament

Tendon and ligament tissues present clinical problems in orthopedics mainly due to injuries. Tendon and ligament are specialized connective tissues that physically connect muscles to bone or attach two bones together, respectively. When injured, the main concern is that impaired tissue will not be replaced by nonspecialized scar tissue.<sup>86,87</sup> Several studies have explored the avenue of gene therapy for the repair of ligament and tendon.<sup>88–92</sup> Bone-marrow-derived human adult MSCs were shown previously to differentiate and integrate into ligament tissue.<sup>8</sup> Here as well, adult MSCs are suitable candidates for stem-cell-based gene therapy. Different from bone and cartilage, with regard to muscle tissue as a source for cells, muscle-derived MSCs have not yet been utilized for the delivery of genes to ligament and tendon tissues. Instead, myoblasts, a more specific muscle cell progenitor, were employed. When engineered myoblasts were injected into the anterior cruciate ligament they integrated into the tissue and maintained transgene expression, but retained their muscle phenotype.<sup>93,94</sup>

Here too, as with bone and cartilage, the question arises regarding what genes can induce ligament and tendon tissue formation. BMP-2 was implemented in this setting at the bone–ligament interface to promote the integration of genetically engineered anterior cruciate ligament grafts to bone.<sup>88</sup> Are there any specific genes that promote ligament and tendon tissues exclusively? Two candidate genes were reported to have such a function and both are members of the BMP family. Lou *et al*<sup>89</sup> have engineered the C3H10T1/2 MSC line to express the novel BMP-12 gene. The authors could not find any osteogenic differentiation of these cells *in vitro*. However, when transplanted into muscle *in vivo*, engineered MSCs were found to form tendon and ligament ectopically with no sign of bone tissue formation. Moreover, they recently delivered the BMP-12 gene via an adenoviral vector, directly into injured ligament tissue and found that BMP-12 expression augmented its repair.<sup>89</sup> Additionally, Helms *et al*<sup>91</sup> have found that the adenoviral gene delivery of BMP-13 ectopically to muscle tissue also resulted in ligament-like tissue formation in the muscle with no sign of bone or cartilage formation. Unlike potent osteoinductive factors such as BMP-2, neither BMP-12 nor BMP-13 were found to promote osteogenesis in myoblasts.<sup>92</sup> However, both are involved to some extent in promoting cartilage growth and homeostasis.<sup>89,95</sup> Despite the evidence that correlates BMP-12 and BMP-13 to cartilage development, it is possible that these genes can only promote chondrogenesis by already established chondrocytes and cartilage tissue, as shown by Gooch *et al*,<sup>95</sup> whereas their effect on adult MSCs is exclusive to promoting ligament and tendon differentiation. Thus it is reasonable to assume that adult MSC-based gene therapy strategies will be effective in ligament and tendon tissues. The above studies clearly indicate that combining the properties of adult MSCs from different sources to specific genes that

promote ligament and tendon differentiation can provide a promising stem-cell-based gene therapy approach for ligament and tendon regeneration.

### Future prospects

As is evident from the above reviewed studies, adult MSCs offer a great advantage for stem-cell-mediated gene therapy directed towards orthopedic medicine. Adult MSCs can be isolated from various tissues, the most common of which are bone marrow, muscle tissue and fat. Although many studies on marker selection of adult MSCs have been performed, the various selected adult MSC subtypes are indistinguishable.<sup>96</sup> Some evidence indicates that adult MSCs may even be retrieved from the blood.<sup>97</sup> Together with molecular studies directed at finding distinct molecular markers of adult MSCs, future development may provide us with a reliable technique for purifying and expanding *in vitro* circulating adult MSCs which are more easily retrieved than those from other tissues.

Since most of the studies performed on nonunion defect models were highly successful, it is expected that large animal studies are to follow, possibly leading towards the first clinical trials. It is expected that additional cell-mediated gene therapy studies involving other genes with osteogenic potential will be undertaken.<sup>98,99</sup> As adult MSCs have multilineage differentiation potential, further studies are expected to examine the advantages of adult MSC in terms of their ability to differentiate along various lineages for the regeneration of skeletal tissues. Investigations may evolve following other studies elucidating the signal transduction pathways of mesenchymal tissue differentiation and identifying novel genes that can trigger lineage-specific differentiation of adult MSCs.

The increasing understanding and recognition of the complexity of skeletal tissue formation has led to the discovery of mechanisms that support skeletal tissue development and/or regeneration. This has been exemplified by the discovery of the important role of angiogenesis in bone development and regeneration as discussed above. Moreover, this has addressed the complexity of bone regeneration and its mechanisms. By expressing both BMP-4 and VEGF a synergistic effect between the two mechanisms of osteogenic differentiation and angiogenesis was achieved. Expressing several genes that are applied for different specific mechanisms in order to promote skeletal tissue development and regeneration should be in the scope of future gene therapy strategies applied for skeletal regeneration. Such complex approaches should also pave the way for the development of adult MSC-based therapeutic applications for systemic and metabolic bone diseases, like osteoporosis, which have several mechanisms involved in their pathophysiology.

Finally, it is expected that engineered adult MSCs combined with specially designed polymeric scaffolds will soon be utilized for skeletal tissue engineering both *in vivo* and *ex vivo*. Combining adult MSCs with a particular growth factor gene that directs their differentiation and that triggers the process of tissue formation is a good approach to engineering tissues. Here too, the properties of adult MSCs that enable them to differenti-

ate and express growth factors can be exploited for the purposes of tissue engineering.

To conclude, adult MSCs as reviewed here can have a wide range of applications for orthopedic medicine. Their differentiation ability, easy manipulation *in vitro* and relatively easy accessibility from various tissues enables them to become major building blocks for the design and development of therapeutic applications to all skeletal tissues concerned in orthopedics. It is expected that the use of adult MSCs will expand to other tissues and will acquire an important place in regenerative medicine.

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