

Adult mesenchymal stem cells: Potential for muscle and tendon regeneration and use in gene therapy

M. Pittenger, P. Vanguri, D. Simonetti, R. Young

Osiris Therapeutics, Inc., Baltimore, Maryland, USA

Abstract

The expansion potential and plasticity of stem cells, adult or embryonic, offer great promise for their use in medical therapies. Recent provocative data suggest that the differentiation potential of adult stem cells may extend to lineages beyond those usually associated with the germ layer of origin. In this review, we describe recent developments related to adult stem cell research and in particular, in the arena of mesenchymal stem cell (MSC) research. Research demonstrates that transduced MSCs injected into skeletal muscle can persist and express secreted gene products. The ability of the MSC to differentiate into cardiomyocytes has been reported and their ability to engraft and modify the pathology in infarcted animal models is of great interest. Research using MSCs in tendon repair provides information on the effects of physical forces on phenotype and gene expression. In turn, MSCs produce changes in their matrix environment in response to those biomechanical forces. Recent data support the potential of MSCs to repair tendon, ligament, meniscus and other connective tissues. Therapeutic applications of adult stem cells are approaching clinical use in several fields, furthering the possibility to regenerate damaged and diseased tissue.

Keywords: Stem Cells, Mesenchymal Stem Cells, Adult Stem Cells, Differentiation, Tissue Regeneration, Myogenic, Cardiac, Gene Therapy, Tendon

Introduction

Clearly, one of the most exciting recent developments in biology and medicine is the many reports that stem cells exist in the adult that can regenerate damaged and diseased tissue. Many adult tissues are known to contain stem cells that provide for the new cells for the normal tissue turnover that is known to occur. As examples, this includes liver parenchymal cells that can regenerate large segments of that organ, the regenerative satellite cells resident in skeletal muscle, several epithelial cell types such as the intestinal crypt cells that provide for the continued turnover of the intestinal epithelia. Stem cells also have been isolated from the embryo that can likely generate many tissues^{1,2} and the isolation of the human embryonic stem (ES)³ and embryonic germ (EG)⁴ cells brings this prospect closer to reality. While there

has been some effort to direct ES cells to different lineages⁵, the indication that differentiation of ES cells can be controlled in a predictable manner is not yet evident and various cell types are seen in the same culture dish. Almost concurrent with the human ES and EG cell reports were several publications describing stem cells from adult tissues⁶⁻⁸. Our own work characterized human bone marrow-derived cells that could be greatly expanded to a homogeneous population (or grown clonally), would differentiate to several discrete lineages and fulfilled the criteria to be called mesenchymal stem cells⁶ (Figure 1). Another report offered evidence that while hepatocytes have great ability to regenerate functioning liver tissue, there are also stem cells in blood with this capability⁷. A provocative study utilized neural stem cells to repopulate the hematopoietic lineages and rescue a lethally irradiated mouse, perhaps one of the first reports that stem cells may have great plasticity beyond the lineages for which they were first characterized⁸. Particularly important is the predictability and uniformity of the isolation process from one preparation of stem cells to the next. In this regard, the adult stem cells isolated from bone marrow continue to offer promising

*Corresponding author: Mark Pittenger, Osiris Therapeutics, Inc., 2001 Aliceanna Street, Baltimore, MD 21231, USA
E-mail: mpittenger@osiristx.com*

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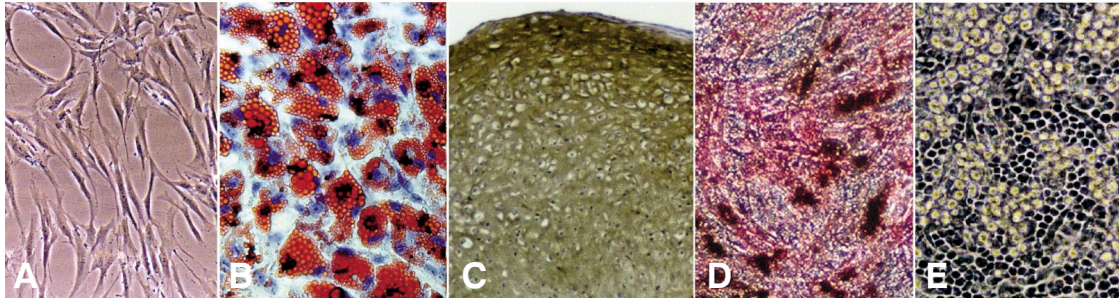


Figure 1. Human mesenchymal stem cells isolated from bone marrow taken from the iliac crest can be induced to differentiate to several lineages. A) phase micrograph of log phase hMSCs B) Confluent hMSCs can be readily differentiated into adipocytes as shown here with the lipid vacuoles stained with oil red O C) A micromass of hMSCs can be induced to become chondrocytes as shown here, stained with anti-type II collagen D) Monolayers of hMSCs differentiate to osteoblasts and elevate expression of alkaline phosphatase (red stain) and accumulate calcium deposits (dark areas) E) hMSCs can serve a stromal function to support HSCs and their progeny which form "cobblestones" on top of the hMSCs (hMSCs are underneath and not visible).

results for tissue regeneration. The challenge is to characterize these cells, understand their regenerative potential, and to develop useful therapies. Whether the adult bone marrow-derived MSCs can be used to regenerate tissues outside the mesodermal lineage is also under investigation in several laboratories. Below, we briefly review the developments in this field that support the prospects for therapeutic use of MSCs and highlight work on muscle and tendon regeneration.

Adult bone marrow has proven to be a reliable source of multipotential cells, first the hematopoietic stem cell (HSC) and now the mesenchymal stem cell (MSC).

Bone marrow stroma is a tissue composed of many cell types that fills the intramedullary space of bone. It contains osteoblasts, adipocytes, reticular cells, endothelial cells, macrophages, monocytes etc., as well as progenitors for these cell types. Bone marrow contains niches that provide support for the maintenance of hematopoietic stem cells, and the mesenchymal stem cells. Therefore, an important function of marrow stroma is to provide an environment that prevents the differentiation of stem cells. It is important to keep in mind that while bone marrow contains mesenchymal stem cells, clearly not all bone marrow stromal cells are stem cells.

Bone marrow can be drawn easily under local anesthetic from accessible sites such as the posterior iliac crest. While other tissues may contain MSCs, their collection is more difficult and/or traumatic for the donor. It is likely that such stem cells can be isolated from many tissues, but which tissues provide the most accessible cells that retain the greatest potential with the least trauma to the donor? Keeping in mind that we are only beginning to understand the potential of stem cells that persist in the adult, much evidence to date would recommend bone marrow MSCs. We routinely utilize a modified version of the isolation procedure first developed in the Caplan laboratory and this has been presented in detail elsewhere^{6,9,10}.

The process of tissue healing has always fascinated the observer. Literature on new tissue formation before 1900 comes largely from embryologists and from the accounts of

surgeons, often in military service. For the embryologist, there were few reagents available such as antibodies, purified proteins, or gene probes that we rely on heavily today for following cellular and molecular events during tissue remodeling. Moreover the first antibiotics, sulfonamides, were not available until the 1930s, so many *in vitro* experiments were hampered by the necessity to maintain absolute sterility. Therefore, many of these early investigations were performed *in vivo* where the immune system provided bacterial surveillance. The surgeons' descriptions of events in the ongoing healing process were largely observational at limited time points, and the process could not easily be followed longitudinally. Moreover, publications, including textbooks, made limited use of pictures and figures.

Studies on effects of exposure to radioactive substances in the 1940s led to the identification of spleen and bone marrow as the source of progenitor cells for the hematopoietic system¹¹⁻¹³. These early studies led to the development of marrow ablation and therapeutic bone marrow transplantation and the realization that there must be an isolatable hematopoietic stem cell (HSC). The search for greater understanding of the characteristics of this adult stem cell is 30-40 years old and continues to be a very active field at both the research and clinical levels¹⁴.

Early tissue transplantation studies revealed that cells responded to being placed in a new environment by altering their phenotype¹⁵. The transplantation of whole bone marrow to an ectopic site demonstrated the formation of bone and cartilage^{16,17}. In the 1960s, Alexander Friedenstein cultured cells from guinea pig bone marrow and studied the cells that became attached to the culture dish^{18,19}. These cells were mitotically very active and formed bone when implanted in syngeneic hosts. They were described as fibroblastic colony forming cells (FCFC) or osteogenic progenitor cells (OPCs), later more commonly referred to as colony forming units-fibroblastic (CFU-F). Experiments

placing these marrow-derived cells in diffusion chambers to separate them from the host tissue proved that the implanted cultured cells were the source of the new bone. These studies of cells cultured *in vitro* from bone marrow were the first to test their ability to differentiate *in vivo*. *In vivo* cell labeling with ³H-thymidine prior to isolation from marrow failed to detect labeled cells in subsequent colonies, showing that the cells giving rise to *in vitro* colonies were not cycling *in vivo*. Further work by Friedenstein and colleagues characterized similar cells from mice and rabbits, and, more recently, from humans²⁰. Dr. Friedenstein produced scientific findings in several areas during his career. With the continuing interest in marrow-derived stem cells, his legacy will certainly live on.

During the late 1970s and 80s, there was a period of characterization of the stromal support cells present in marrow responsible for maintaining cultures of HSCs. The culture of human bone marrow cells that could propagate as attached, fibroblastic cells was first described by Moore and co-workers²¹. Experimentally, treatment with high doses of ³H-thymidine at initial isolation of the cells failed to reduce their initial growth, suggesting the cells were non-mitotic or quiescent at first isolation. These authors characterized the cells' morphology and several of their expressed proteins. They were distinguished from endothelial cells on the basis of lacking Weibel-Palade bodies, expression of factor VIII or basement membrane collagens, or support of granulocytes and macrophages. However, there was not an attempt to characterize the ability of these cells to differentiate to other lineages.

The work of Maureen Owen and colleagues extensively explored the osteogenic potential of cells cultured from bone marrow^{22,23}. Bone marrow was known to contain the HSCs and a variety of cell types, but isolated cultured bone forming cells were not well studied. It was clear that osteoblasts were present in marrow but what other cell types could be identified? Several reports described the osteogenic potential of isolated, cultured cells from bone marrow that were not pre-committed osteoblasts. These cells were also referred to as stromal stem cells. Whether other organs contained such cells was unclear and perhaps stromal stem cells from different organs differed in their properties. There was also the question whether each stromal stem cell was capable of producing a different stromal cell type, or more than one cell type. A working diagram initially proposed by Owen²⁴ and by Owen and Friedenstein²⁵ and further developed by Arnold Caplan²⁶ and included the stem cells that could give rise to all mesenchymal lineages –the mesenchymal stem cells or MSCs. Working with Caplan, Steve Haynesworth was able to isolate and characterize human cells from adult bone marrow that formed bone and cartilage in an *in vivo* setting²⁷. Some criticism over the term mesenchymal stem cell resulted in publications using the name mesenchymal progenitor cell

(MPC) to refer to the same cells²⁸.

Over the years, the fibroblastic cells from bone marrow isolated from different species and described by different laboratories have been given a variety of names. The isolation techniques, culture conditions, media additives, subculturing techniques, and assays vary among the laboratories studying these cells. It is likely that, similar to HSCs, the expressed surface molecules will depend on careful attention to many factors (composition of the medium, homogeneity of the cells, time in culture, cell density, time since last feeding, etc.). While the names likely describe similar cells, it is clear that the cultured cells studied in various laboratories are not identical. With this in mind we have extensively characterized the expression of surface proteins on the human MSCs with which we work⁶. It is likely, however, that a short list of surface molecules would be helpful to characterize the multipotential cells isolated in many laboratories. We suggest that a useful group of antibodies to characterize human MSCs by flow cytometry would be CD90, 29, 105, 73, 166, HLA-A,B,C (positive); CD 45, 54, 106 (positive but variable); CD14, 34, 117 (negative) where the analysis is done with 3 positives and 1 negative staining antibodies per tube and the blank consists of the control IgG run at the time of analysis. It should be recognized that a set of cell markers will not necessarily identify a stem cell or the outcome of the differentiation that is to follow (as this is dependent on many additional parameters), but it speaks to the identity and uniformity of the cells under study.

Recently, Catherine Verfaillie and colleagues have characterized cells from adult human bone marrow that grow in culture through extensive passaging, and differentiate to mesenchymal lineages but they have evidence for non-mesenchymal lineages. Under the right conditions, these cells also express genes normally found in neural or endothelial lineages. The authors have termed these cells mesenchymal progenitor cells (MPC) or multipotential adult progenitor cells (MAPC), both referring to the same cell population.

Myogenic potential of bone marrow-derived cells

That bone marrow-derived mesenchymal cells should differentiate to muscle was investigated by Caplan and colleagues who studied the *in vitro* differentiation of rat MSCs to myoblasts. They were able to demonstrate that 24 hours of 5-azacytidine treatment of rat MSCs in culture resulted in elongated myotubes that stained positively for myosin²⁹. Further, they were able to demonstrate in other work that mouse bone marrow-derived MSCs from an unaffected sibling could provide dystrophin to the muscle fibers of affected mice³⁰. More recently, Cossu, Mavillo and co-workers demonstrated active muscle regeneration *in vivo* with bone marrow-derived cells from the C57/MLacZ

transgenic mice that express the β -Gal gene downstream of a myosin light chain promoter³¹. This ensures continued expression of the transgene in muscle tissues. The healing muscle clearly showed the incorporation of the β gal⁺ donor cells. The results were similar if the bone marrow was first fractionated into adherent and non-adherent cell types and then injected. Further, if animals were irradiated and repopulated with the transgenic bone marrow cells first, regenerating muscle showed the presence of the β -Gal⁺ cells, further suggesting that recruitment from the bone marrow compartment through the circulation is a common mechanism. The bone marrow cells appeared to differentiate more slowly than similarly implanted muscle satellite cells, suggesting that the satellite cells are the first cells recruited to heal muscle damage, while bone marrow-derived cells may take part in prolonged regeneration. The potential for gene-modified bone marrow-derived cells or the more fully characterized MSCs is being evaluated in several laboratories (see below).

Recent work has characterized a subpopulation of muscle satellite cells in the mouse that actively exclude nuclear dyes and can be distinguished by flow cytometry as a "side population" or SP cells³². These SP cells have a tremendous ability to proliferate and provide myoblasts for muscle regeneration. They also appear to be able to differentiate to additional lineages and therefore represent another example of a multipotential stem cell that persists in adult tissue³³. Reasoning that the constant recruiting of myogenic progenitor cells necessary in muscular dystrophies would exhaust the satellite cell population in a muscle and require another source of progenitor cells, Gussoni and colleagues were able to restore dystrophin expression in the mdx mouse model by a bone marrow transplant, or by using the SP population from donor marrow³⁴. Male donor Y chromosomes could be detected in the muscle nuclei of the recipient females.

Myogenic differentiation of stem cells in heart

The great mortality associated with heart disease has prompted research into the replenishment of the myocardium with additional cells, a procedure referred to as cardiomyoplasty. The majority of the work in this area has sought to utilize myoblasts but many cell types have been utilized. Recently, several articles have demonstrated that cells from bone marrow can engraft in the heart. Bittner et al. performed bone marrow transplants from male donors into affected mdx female mice and searched for the donor cells in muscle and heart tissues. They were able to detect the Y chromosome by fluorescence *in situ* hybridization (FISH) in the mdx animals, in skeletal and heart muscle, and these cells were often found to express myogenin, myf5 and dystrophin. Chiu and colleagues have reported the

engraftment of cultured syngeneic bone marrow stromal cells into the hearts of rats³⁶. The cells were labeled with the nuclear dye DAPI and then injected directly into the ventricle wall of recipients. The animals were sacrificed at times out to 12 weeks. At 4 weeks and beyond, tissue sections of the injected hearts revealed the presence of engrafted donor cells. The donor cells were seen to express sarcomeric myosin heavy chain and the gap junction protein connexin 43, suggesting functional coupling with neighboring host cardiomyocytes.

In another recent study, Orlic, Anversa and colleagues tested the ability of mouse bone marrow-derived cells to engraft in the damaged area of the ischemic mouse heart³⁷. They utilized bone marrow from transgenic male mice expressing the enhanced green fluorescent protein (GFP). The cells to be injected were not cultured *in vitro*, but were selected for the presence of c-kit^{pos} and the lack of several hematopoietic lineage markers (Lin^{neg}). Infarcts were created in female mice by coronary artery ligation and cells delivered to the region by direct needle injection. These selected cells engrafted in the infarct region of mice and developed into a robust myogenic tissue with highly developed vasculature. The implanted cells were identified by the presence of both the Y chromosome and expression of GFP. These authors further characterized the expression of cardiac transcription factors MEF2, GATA-4, CSX/NKX2.5 and gap junction protein connexin 43 in the reconstituted region, identifying cardiomyocytic differentiation. Injection of c-kit^{neg} Lin^{neg} cells failed to produce this myogenic tissue.

Over the past several years, we have been studying the potential of human and rat MSCs to engraft in the heart and to repopulate regions of the heart damaged by ischemia. We have utilized human MSCs placed into the SCID/beige mouse to test the ability of these cells to become cardiomyocytes³⁸. The approach was to inject the LacZ-tagged hMSCs into the ventricle chamber, where only a portion (~5%) are delivered to the myocardium by the coronary circulation. The hMSCs that enter the vessels do find their way out of the blood vessels and can be found dispersed in the ventricle wall, surrounded by cardiomyocytes. Due to the high metabolic demands of heart tissue and the rhythmic beating, the engrafted hMSCs are subjected to an environment that would be difficult to establish *in vitro*. The engrafted hMSCs underwent morphological differentiation to resemble the surrounding cardiomyocytes³⁸. The cells began to express muscle specific proteins including desmin, α -actinin, phospholamban, cardiac troponin T and myosin heavy chain. Significantly, the hMSCs expressed these proteins to levels that are indistinguishable from surrounding cardiomyocytes. In other experiments, we have created ischemic regions in the hearts of athymic rats and implanted the human MSCs directly in the infarct regions. We are able to detect the implanted cells

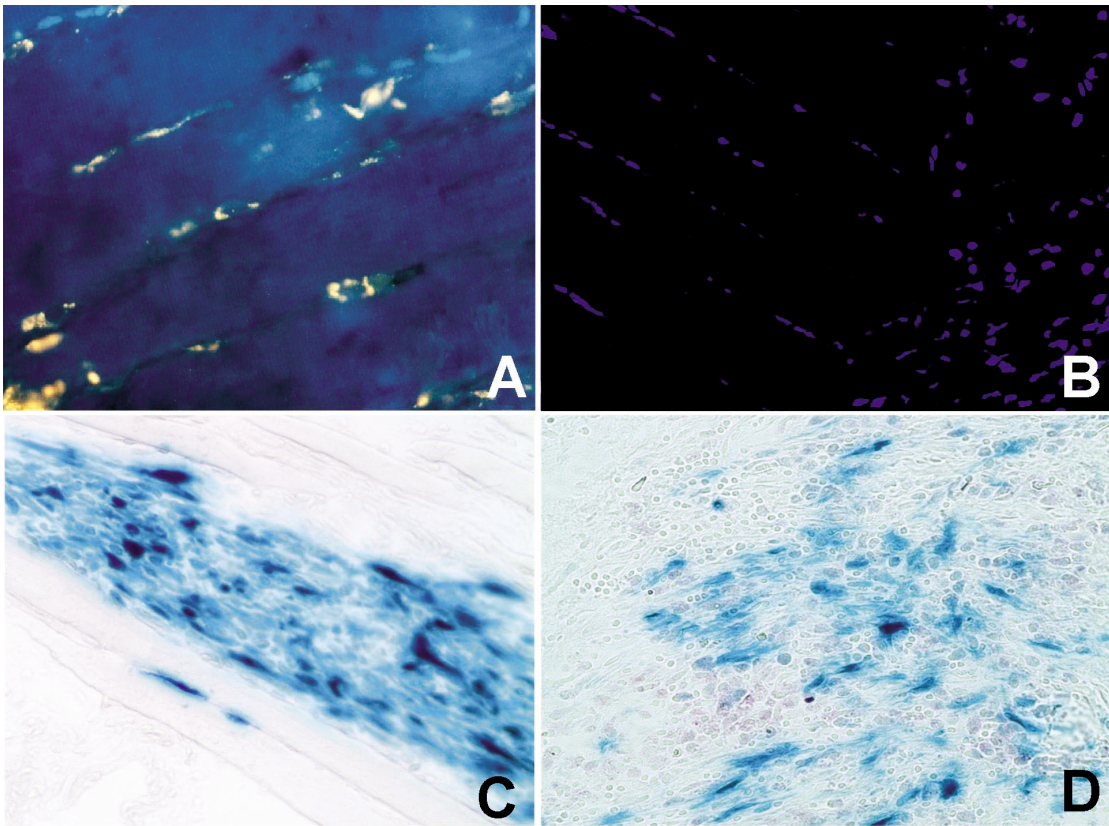


Figure 2. Rat MSCs isolated from bone marrow of Fischer rats were transduced with moloney leukemia retrovirus expressing b-galactosidase-IRES-Neo^R. Transduced or non-transduced cells were labeled with CM-DiI or DAPI and injected into the thigh or calf muscle of Fischer rats. After 3, 14 or 28 days, the animals were sacrificed and frozen sections of the muscle were prepared. The expression of β -Gal was detected with histochemistry using X-Gal as a substrate. Frozen sections were also observed by fluorescence microscopy for red (CM-DiI) or blue (DAPI) fluorescence. As seen in Fig. 2A and B numerous β -Gal- expressing cells are present in the muscle at day 3 (A) and day 14 (B). Fig. 2C shows Di I stained hMSCs at day 14 between muscle fibers away from the injection site. Fig. 2D shows DAPI-positive non-transduced MSCs in the muscle at day 28. Magnification 20 X.

within the infarct region and see low levels of immunostaining for sarcomeric proteins (Martin, Senechal and Pittenger, unpublished). With colleagues at Johns Hopkins Hospital Department of Cardiac Surgery, we moved to a porcine large animal model of ischemic heart failure (Shake et al., 2002). The size of the pig heart is similar to man and allows for *in situ* physiological measurements through the use of sonocrystals or echocardiogram. In animals analyzed as late as six months after cell implantation, porcine MSCs implanted in the infarct region appeared to prevent the pathological thinning evident in animals which did not receive MSCs. These results will be confirmed in additional animals and new animals included that utilize allogeneic porcine MSCs.

Genetically modified MSCs for gene delivery in muscle

Ex vivo gene modified cells are attractive for gene therapy as they avoid the risks associated with the delivery of viral or other potentially pathogenic vectors. To this end, hematopoietic and other stem cells, cell lines, fibroblasts,

keratinocytes, and myoblasts have been studied extensively. A major requirement for such cell-based therapy is the sustained expression of the gene without adverse effect on the recipient. The parameters to consider include availability and expansion capability of the cells, efficiency of gene modification, length of therapeutic gene expression *in vivo*, immunogenicity of the cells, and safety of the cells, which includes the absence of formation of tumors and ectopic tissues. The route of delivery of the cells plays a significant part and is based on whether systemic or local expression of the gene product is required. For the systemic expression of proteins, cells can be infused into the general or organ-specific circulation; or cells can be encapsulated or attached to various matrices and implanted subcutaneously. The more simple method of delivery is to directly inject the cells under the skin, intra-dermally or intra-muscularly.

Intra-muscular delivery is the optimal choice for myoblast-mediated cell therapy because of the obvious advantage of the myoblasts fusing with the host muscle cells to differentiate into muscle fibers. In addition, the inherent vascularity of the muscle makes it a useful depot to deliver

secreted proteins. Thus, genetically engineered myoblasts, or muscle-derived stem cells, have been used for replacing degenerating muscle as in Duchenne Muscular Dystrophy^{39,40} or in bone defects⁴¹. As a gene delivery vehicle, myoblasts were used to deliver growth hormone⁴², VEGF⁴³, Factor IX^{44,45}, erythropoietin⁴⁶, and glucocerebrosidase (GC)⁴⁷, to name a few. Another route of delivery has been to use myoblasts in implantable microcapsules to deliver gene products, for example, Factor IX⁴⁸.

Myoblast mediated delivery of glial cell line-derived neurotrophic factor (GDNF) was shown in an elegant study to prevent motoneuron loss and disease progression in a mouse model of amyotrophic lateral sclerosis⁴⁹. The transgene persisted for up to 12 weeks in the muscle and the expressed GDNF increased the size and number of motoneurons innervating the muscle where the myoblasts were implanted. Furthermore, behavioral tests showed improvement of motor function in treated mice.

Upon implantation of GC- transduced myoblasts into mouse muscle, the cells differentiated and fused with adjacent myofibers and expressed GC for up to 300 days. The secreted GC in circulation was taken up by macrophages in the liver and bone marrow⁴⁷.

Lee et al.⁴⁰ have isolated putative muscle-derived stem cells from the mdx mouse using a preplate technique to enrich a CD34+Bcl+ population. These cells have both myogenic and osteogenic properties and were shown to regenerate muscle in a dystrophic mouse. mc13 cells genetically modified to express Osteogenic Protein (OP-1) and delivered by intramuscular injection, accelerated healing of a skull defect in mice.

We have investigated the use of mesenchymal stem cells for gene therapy as they meet many of the criteria described above. MSCs are self-renewing, and can be extensively expanded in culture. MSCs can be efficiently transduced with retroviruses⁵⁰, adenovirus³⁸, or adeno-associated virus (Byrne and Pittenger, unpublished) to express high levels of transgene product. The transgene expression is maintained over several passages and also after differentiation into adipocytes, osteocytes or cartilage⁵¹. Gene modified bone marrow stromal cells have been injected intravenously into the central nervous system, into intra-articular spaces, in large defects of bones⁵² or into the heart³⁸, to either deliver gene products for a systemic effect or for local therapy. The ability of MSCs to differentiate along several lineages is also attractive for gene therapy in a variety of tissues. The MSCs appear to take their signals from their immediate environment to undergo site-specific differentiation without evidence of ectopic tissue formation^{53,54}. Human MSCs injected into skeletal muscle show signs of differentiation into myoblasts, although the extent of engraftment and differentiation is still under investigation (Vanguri and Pittenger, unpublished). Since MSCs appear to engraft

better in injured or regenerating tissues⁵³, it is possible that injuring the muscle with toxins such as Notexin could provide an enhanced stimulus for MSCs to survive and differentiate into muscle cells as was seen, for example, with myoblast transplantation in monkeys⁵⁵.

We have investigated human MSCs transduced with several genes including human Erythropoietin (Epo) and soluble TNF receptor type II fused with a portion of the Fc segment of human IgG1 (sTNFR_{II}) in mice and rats. Intramuscular injection of Epo-transduced MSCs produced significant levels of human Epo in the blood and increased the hematocrits of the mice⁵⁶. sTNFR_{II}-MSCs injected intramuscularly resulted in significant increase of sTNFR_{II} in the blood⁵⁷. To investigate the fate of transduced MSCs in immunocompetent animals, we used the LacZ reporter gene and showed expression of β -Galactosidase (β -Gal) by donor cells for up to 28 days (Alur and Vanguri, unpublished). Rat MSCs isolated from Fischer rat bone marrow were transduced with a moloney leukemia virus retrovirus expressing β -Gal. β -Gal expression was analyzed by LacZ histochemistry of the cells, which showed transduction efficiencies of 50-70% *in vitro*. β -Gal or non-transduced cells were also labeled with a fluorescent Cell Tracker™ dye, CM-DiI or with a fluorescent nuclear marker, DAPI (4',6-Diamidino-2 phenylindole Dihydrochloride hydrate) and injected into the calf or thigh muscle of Fischer rats. Histological examination of the muscles showed that CM-DiI positive or DAPI-positive cells were present at the injection site, and away from the injection site in the inter-fascicular tissue among muscle bundles (Figure 2C). Many of the cells aligned themselves along the muscle fibers and were present up to 28 days as seen in Fig. 2D. Numerous β -Gal expressing cells were present at days 3 and 14 (Figure 2A and B). β -Gal expressing cells decreased with time, indicating a possible immune response against cells expressing the foreign gene product. Long-term expression of transgene could be improved by preventing the loss of transduced cells by expressing non-immunogenic autologous proteins, or preventing loss of transgene expression due to silencing of retroviral promoters. Clearance of cells may also be minimized by inducing regeneration in tissues to enhance engraftment by either making space for the cells or by inducing signals for proliferation and differentiation of cells. Silencing of retroviral long-term repeat (LTR) promoter expressing the foreign gene can result from methylation of sequences in the LTR or from positional inactivation of the integrated provirus by heterochromatin^{58,59}. Several modifications of retroviral vectors involving deletion of LTR elements, and use of either endogenous or tissue-specific promoters/enhancers were shown to avoid LTR-silencing⁶⁰. Chromatin insulators and matrix/scaffold attachment regions in conjunction with post-transcriptional regulatory elements have significantly improved transgene expression by retroviral vectors⁵⁹.

MSCs and tendon regeneration

Tendons function to transmit the contraction forces of the muscles to bone and thus facilitate skeletal motion. In doing so, tendon tissue must possess significant stiffness, strength, and at the same time a certain amount of elasticity⁶⁰. Repair of tendon and ligament tissues is a complex process and remodeling of the healing tissue may require long periods of convalescence to achieve biomechanical levels necessary for normal function of the tissue. Tendon tissue engineering has incorporated cells into the structural materials to enhance extracellular matrix production, promote growth factors via genetic engineering of the cells, and recruit endogenous cells to the site of repair⁶¹. Terminally differentiated fibroblasts or chondrocytes from various sources have been used in most of these designs.

Initial experiments using MSCs to regenerate musculo-skeletal soft tissues *in vivo* began with autologous MSCs suspended in a type I collagen matrix⁶². Wakitani et al. observed that in a free floating collagen gel, the cells deformed the gel in an unorganized fashion, contracting the matrix radially in all directions (unpublished observations). However, when we placed two fixed immobile posts in the collagen suspension, the cells contracted the collagen fibers parallel to the axis between the posts, causing the collagen fibers and the cells to be aligned along that axis⁶³. Awad et al. studied the effects of MSC concentration on the kinetics of this process, increasing the concentration of cells from 1 to 8 million per mL⁶⁴. These results demonstrated that increasing the cell concentration increased the rate of the contraction, but produced no additional benefits in the degree of organization of the cells or the matrix.

Archambault et al. tested the hypothesis that, just as muscle contraction exerting a tensile load on the embryonic tendon may lead to maturation of molecular composition and matrix structure, MSC gene expression may be altered and the composition of collagen types in the matrix changed when a cyclic load is applied. We subjected human MSC/collagen gel constructs to cycles of tension to model the effects of tensile forces on *in vitro* construct maturation⁶⁵. The constructs were fixed in an apparatus that applied a 10% longitudinal strain for 5 seconds at 0.1 Hz over 30 minutes followed by a rest period of 90 minutes, repeated continuously for 15 days. Structural changes were evident in the cycled tissue as compared to static tension as early as 24 hours. Histologically, the cycled tissue demonstrated collagen fiber organization parallel to the long axis of the tensile loading with an alignment of crimp patterns that was not evident in the static constructs. In addition, the MSCs produced increased amounts of collagen type VI. Collagen type VI is associated with repairing connective tissues and with the early maturation of normal tendon tissue^{66,67}.

The ability of MSCs to organize the structure of collagen

in vitro prompted the hypothesis that MSCs could organize a biocompatible implant that, when implanted *in vivo*, would result in early structural organization and gains in biomechanical function. To test this, we implanted the static-tensioned, MSC/collagen implants described above into one-centimeter defects of the Achilles tendon of rabbits⁶³. Histological samples from MSC-treated groups at the early 4 week time point exhibited greater organization of the collagen fibers parallel to the axis of tension and improved orientation of the cells parallel to the axis of stress. Biomechanically, tendons implanted with MSCs consistently had significant improvements in the structural and material properties compared to controls. In a kinetic comparison of 4, 8, and 12 week groups, MSC-loaded collagen gels achieved twice the rate of increase in structural and mechanical properties compared to controls.

In another study, Awad et al. labeled MSC's with a fluorescent cell surface molecular marker, prepared static-tensioned, MSC- contracted collagen constructs, and implanted the constructs in patellar tendon defects of rabbits⁶⁸. They identified labeled cells in the repaired central third of the patella at 12 weeks post-operatively. Moreover, when compared to the normal healing process of scar tissue in the sham-operated, contralateral limb, MSC-loaded tissues were again 58% stronger and 51% stiffer (significant at $p < 0.05$) than natural repair tissues at 12 weeks post-surgery, increasing to 174% stronger and 183% stiffer at 26 weeks post-op. Such significant improvements in function, in combination with Archambault's data on modulations of gene expression and protein synthesis, further indicate adult bone marrow-derived MSCs have potential as a cellular component in tissue engineering of tendons and ligaments.

MSCs and meniscal regeneration

Investigators have sought to produce a prosthetic meniscus based on synthetic materials or resorbable biomaterials, but the rigors of the biomechanical loads on meniscus combined with the avascular nature of the tissue leads to breakdown of the meniscal implant and osteoarthritis of the adjoining articular cartilage. Meniscus has a very limited capacity for self-repair due to limitation of the blood supply to the outer one-third, or "red zone", of the meniscus. Some early but promising results have enlisted tissue engineering with biologic matrices⁶⁹ and chondrocyte-seeded prostheses⁷⁰.

The meniscal structure is further complicated by the duality of the cellular and extracellular matrix composition⁷¹. Fibrocytes in a fibrocartilaginous matrix are found in the outer third, while chondrocytes synthesizing hyaline cartilage are predominant throughout the inner region. This composition of the matrix is well-adapted to the dual biomechanical forces exerted on the tissue; in addition to overall compression, there is circumferential tensile forces due to translational shear on the outer one third⁷². Tissue

engineered implants utilizing MSCs offer great promise for a dual composite tissue such as the meniscus, given the cells' potential to differentiate to both fibroblastic and chondrocytic lineages.

Murphy et al. injected GFP-labeled goat MSCs into the intra-articular joint space of meniscectomized goats to assess cell distribution in the stifle⁷³. They recovered viable cells from joints one week after injection and found cells adhered to soft tissue surfaces throughout the joint. In particular, they identified MSCs in the regenerated soft tissue, or neomeniscus, emanating from the synovial lining at the site of meniscectomy.

In a second study, they injected GFP-labeled MSCs into operated stifle joints of goats 6 weeks after meniscectomy with ACL resection to further destabilize the joint⁷⁴. The neomeniscal tissue developed on the tibial plateau had a hyaline-like appearance in 4 of 6 knees, and in 7 of 9 joints at 3 month and 6 month time points, respectively. Immunohistochemistry demonstrated these hyaline-like tissues to contain cells expressing type II collagen.

In a more complex tissue engineering approach, Walsh et al. permeated autologous, bone marrow-derived MSCs throughout a Type I collagen sponge and applied this construct to a partial anterior medial meniscectomy model in rabbits⁷⁵. These authors found that the MSC/sponge construct consistently developed more abundant regenerated tissue than sponge alone or the empty spontaneously repaired defects, at 6, 12, and 24 weeks post-operation. Cell/sponge constructs also produced more fibro- and hyaline cartilage in the regenerated tissue at the later time points as compared to the thin fibrous collagen fibers that marked the sponge-alone specimens. However, due to the biomechanical shortcomings of the matrix scaffold utilized in these experiments, MSC-treated menisci had breakdown of the anterior attachment to the intermeniscal ligament site and similar degree of osteoarthritis on the joint surface as the sponge-alone group. Thus, while the addition of MSCs to the matrix provided some histological structural advantages, the scaffold lacked the initial tensile strength necessary to secure the construct.

Another tissue engineered prosthesis was devised by Angele et al., who cultured autologous rabbit MSCs on a hyaluronan-ester/gelatin composite matrix for replacement of the complete meniscus⁷⁶. After 14 days' incubation in a chondrogenic medium, they implanted these cell-seeded matrices into the medial meniscectomized knees of the donor rabbits. When evaluated by immunohistochemistry and *in situ* hybridization at 12 weeks post-implantation, they found that the matrix composites had fibrocartilage containing both collagen types I and II. While such techniques require further scrutiny of biomechanics at much longer time points, these studies certainly offer a promising future for incorporating MSCs into tissue engineered prostheses for musculoskeletal therapies.

Clinical outlook

The prospects for using bone marrow-derived MSCs clinically to treat a variety of degenerative and disease states is quite real. Cellular therapeutics is a promising field to augment traditional approaches and the new small molecule drugs resulting from efforts into genomics. As we learn more about the stem cells we all possess within our tissues we will understand why we are not able to regenerate tissues ourselves. Several clinical studies involving human MSCs have begun and results have been reported recently from three studies that speak to the safety and potential for MSC based therapy.

An early IND safety study completed by investigators at University Hospital in Cleveland reported no adverse effects in patients who received autologous MSCs. The cells were isolated from a small bone marrow aspirate and expanded *ex vivo*, before being reinfused⁷⁷. A follow-up study tested the ability of infusion of MSCs, which are known to produce many hematopoietic cytokines and can support HSCs *in vitro*, to enhance the hematopoietic recovery of advanced breast cancer patients undergoing bone marrow transplantation⁷⁸. This study utilized co-infusion of autologous culture-expanded MSCs and autologous peripheral blood progenitor cells in 28 patients and results reported early hematopoietic recovery based on neutrophil and platelet counts.

Osteogenesis imperfecta (OI) is a devastating disease resulting from mutations in the type I collagen gene and results in multiple skeletal abnormalities, fragile bones and attendant complications. Following preclinical studies from the Prockop group suggesting that MSCs could engraft in animal models of OI, Horowitz, Prockop and colleagues performed bone marrow transplants from HLA matched siblings into 5 pediatric patients⁷⁹. The 3 patients with confirmed engraftment demonstrated bone growth and mineral density comparable to healthy weight-matched controls and a significant decrease in spontaneous bone breakage.

Conclusions

It is clear that MSCs can be developed as powerful vectors for the delivery of recombinant genes. With improved retroviral vectors and delivery methods, the skeletal muscle can serve as a suitable depot for gene delivery. The ability to easily obtain, transduce and expand these cells also makes it convenient to bank several doses of the cells that can be injected at frequent intervals into different muscle sites. MSCs have been infused into patients and appear quite safe in early clinical trials. There is also accumulating evidence that human MSCs can be used allogeneically, without the need for extensive immunosuppressive drugs⁸⁰ (and McIntosh et al., submitted).

The potential for MSC regeneration of musculoskeletal tissues is immense. Directing these cells in specific tissue engineered applications has many challenges ahead but much work has been started. The eventual outcome of MSC differentiation is influenced by the *in situ* milieu, extracellular matrix, cytokines, growth factors, and physical forces. One of the challenges of any orthopedic application will be to identify the proper biocompatible matrix, one that will withstand the immediate structural forces, provide for cell differentiation along the appropriate lineage path and be resorbed at rates proportional to the rate of increasing strength of the newly formed matrix.

Arguably, it would be best if the *in vitro* cultured cell represented the cell as found in the body. On the other hand, a well-characterized cell that behaved in a predictable and safe manner would have great utility, even if it differed in a discernible trait from its counterpart *in vivo* (most drugs differ from the natural compounds they mimic). A recurrent finding in many recent reports in which MSCs are introduced either i.v. or by direct injection is that the proportion of introduced cells in the vicinity of injured tissue is high, suggesting signaling or homing to sites of injury. While many of the cytokines and growth factors produced at the site of tissue damage are known, it is not known what is the attractant for MSCs. This is of high importance as it speaks to the nature of the stem cell regenerative response and might give clues to what breaks down as we get older and no longer heal so readily. Is it that the stem cells no longer respond, are in short supply, cannot heed the signal or is the trauma signal no longer strong enough to attract stem cells or elicit the needed response? It is likely too, that further understanding ES and EG cells will enhance our understanding of adult stem cells. Such far-ranging questions bring back the reality that understanding stem cell-based tissue regeneration will require insight and diligence and the efforts of many research groups.

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