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ADULT STEM CELLS APPLIED TO TISSUE ENGINEERING AND REGENERATIVE MEDICINE

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Abstract – Regeneration takes place in the body at a moment or another throughout life. Bone, cartilage, and tendons (the key components of the structure and articulation in the body) have a limited capacity for self-repair and, after traumatic injury or disease, the regenerative power of adult tissue is often insufficient. When organs or tissues are irreparably damaged, they may be replaced by an artificial device or by a donor organ. However, the number of available donor organs is considerably limited. Generation of tissue-engineered replacement organs by extracting stem cells from the patient, growing them and modifying them in clinical conditions after re-introduction in the body represents an ideal source for corrective treatment. Mesenchymal stem cells (MSCs) are the multipotential progenitors that give rise to skeletal cells, vascular smooth muscle cells, muscle (skeletal and cardiac muscle), adipocytes (fat tissue) and hematopoietic (blood)-supportive stromal cells. MSCs are found in multiple connective tissues, in adult bone marrow, skeletal muscles and fat pads. The wide representation in adult tissues may be related to the existence of a circulating blood pool or that MSCs are associated to the vascular system.

Key words: Stem cell; Cell therapy; Tissue engineering; Regenerative medicine; Chondrogenesis; Osteogenesis.

INTRODUCTION

Stem cells (SCs), whether derived from embryos, fetuses, or adults, seem poised to dominate the next frontier of human regenerative medicine and cellular therapy. Over the last 15 years, major advances have been made in the isolation, the culture and the induction of differentiation of SCs from various sources. SCs have now been identified in every major tissue and organ of the human body. Concomitant with these discoveries intense efforts are made to understand the molecular mechanisms underlying the decision of SCs being in mitotic dormancy, to undergo self-renewal, or to differentiate terminally. An understanding of these molecular mechanisms would help realize the tremendous therapeutic potential of SCs.

To this end, state-of-the-art technologies have been developed to interrogate genome-wide gene expression in SCs in an effort to establish the cause-effect relationship between the biological states of SCs and the molecular signatures that they manifest. Recent studies

Abbreviations: ADSC, adipose-tissue-derived stem cell; **BM**, bone marrow; **EPC**, endothelial progenitor cell; **ESC**, embryonic stem cell; **HSC**, hematopoietic stem cell; **MAPC**, multipotent adult progenitor cell; **MSC**, mesenchymal stem cell; **NSC**, neural stem cell; **PCL**, poly caprolactone; **PLLA**, poly L-lactic acid; **SC**, stem cell; **TE**, tissue engineering.

uncovered novel mechanisms by which SC fate is regulated, implicating the participation of SC-specific microRNAs (1) and the fate reprogramming factors that can act cell autonomously (2). In addition to the discovery of new genes, the functions of definitive stem cell markers such as ***Nanog***, ***Oct4***, and ***Sox2*** seem to be rapidly elucidated. Continued discoveries in the cell and molecular biology of SCs will facilitate their application, the most exciting of which would be in regenerative medicine and cell therapy.

SCs and progenitors are present predominantly in all normal tissues (3-6). SCs are defined, in general, as resting cells (not actively proliferating) that are present in small numbers in normal tissues. They share two typical features: the ability for asymmetric cell division and self-renewal (7, 8). During these processes, a SC is activated by some signal or by some particular event to leave its normal resting state and to divide. However, the result of this cell division provides two daughter cells which are not identical. One daughter cell proliferates symmetrically, often for many cell divisions, to produce an abundance of progeny referred as being the progenitors. These progenitors subsequently differentiate to form a mature tissue. In contrast, the second daughter cell returns to the original resting state of the mother cell until a new activating signal or event occurs. It retains a SC phenotype and all of the capacities of the original mother cell in a process referred as being the self-renewal. This process is critically important, because if daughter cells become the progenitors, then the pool of SCs would be depleted progressively with each activation event. Such an outcome would rapidly deplete the normal tissues of its existing SC population, resulting in an insufficient number of cycles to support ongoing tissue remodelings and repairs required for long-term health.

Adult human SCs have been isolated from a wide variety of tissues and, in general, their differentiation potential may reflect the local environment. They lack tissue-specific characteristics but under the influence of appropriate signals they can differentiate into specialized cells having a distinct a phenotype differenet from that of the precursor. It may be that SCs in adult tissues are reservoirs of reparative cells, ready to mobilize and differentiate in response to wound signals or disease conditions. Little information is currently available about the biology of endogenous SC populations in adults and their precise role in tissue repair or regeneration. This may be due in part to the lack of useful cell-specific markers. What is clear, however, is the ease with which these cells can be isolated and expanded in culture through many generations while retaining the capacity to differentiate. Recent progress in the isolation and characterization of these cells has led to the development and to various tests of therapeutic strategies in a variety of clinical applications.

The chronic shortage of donor organs and tissues for transplantation has provided the impetus for intense research in the field of tissue engineering (TE). Unlike pharmacology and physiotherapies they are mainly palliative, TE and cellular therapy seek to augment, replace, or reconstruct damage of diseased tissues (9). Tissue engineering is an emerging field that offers outstanding opportunities for regenerative medicine. The most common concept underlying TE is to combine a scaffold or matrix, living cells and/or biologically active molecules to form a "TE construct" to promote the repair and regeneration of tissues. The scaffold supports cell colonization, migration, growth and differentiation, and often guides the development of the required tissue or acts as a drug delivering vehicle.

Hence, TE can be defined as an effort to create or to induce the formation of a specific tissue in a specific location through the selection and manipulation of cells, scaffolds, biologic stimuli (10), and vascular support (angiogenesis and/or vasculogenesis), on which the TE paradigm is based on (Fig. 1). The right knowledge of these interactions will create exciting new opportunities that might be useful in a broad array of clinical applications. As a logical consequence, today, a great number of multi-disciplinary groups with different backgrounds (Fig. 2) focus on various problems associated with TE, including cell isolation, characterization, and manipulation of cell proliferation/differentiation for stem cell therapy, design and elaboration of appropriate biomaterials as well as for development of bioreactors to enlarge tissue/organ engineering as a strategy to be applied in regenerative medicine.

Expansion/Transformation of SCs

A fundamental bottleneck that must be overcome to exploit SCs for TE is the adequate supply of cells. This problem will become more

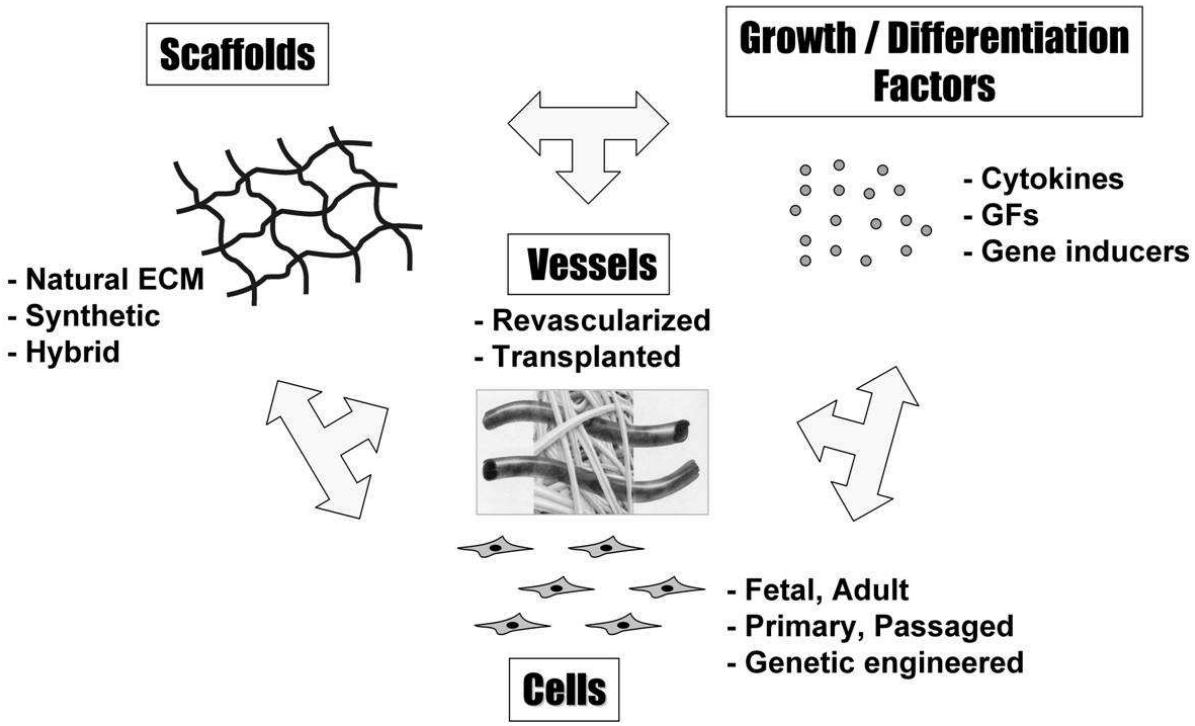

Figure 1. Graphical illustration showing the principles underlying tissue engineering.

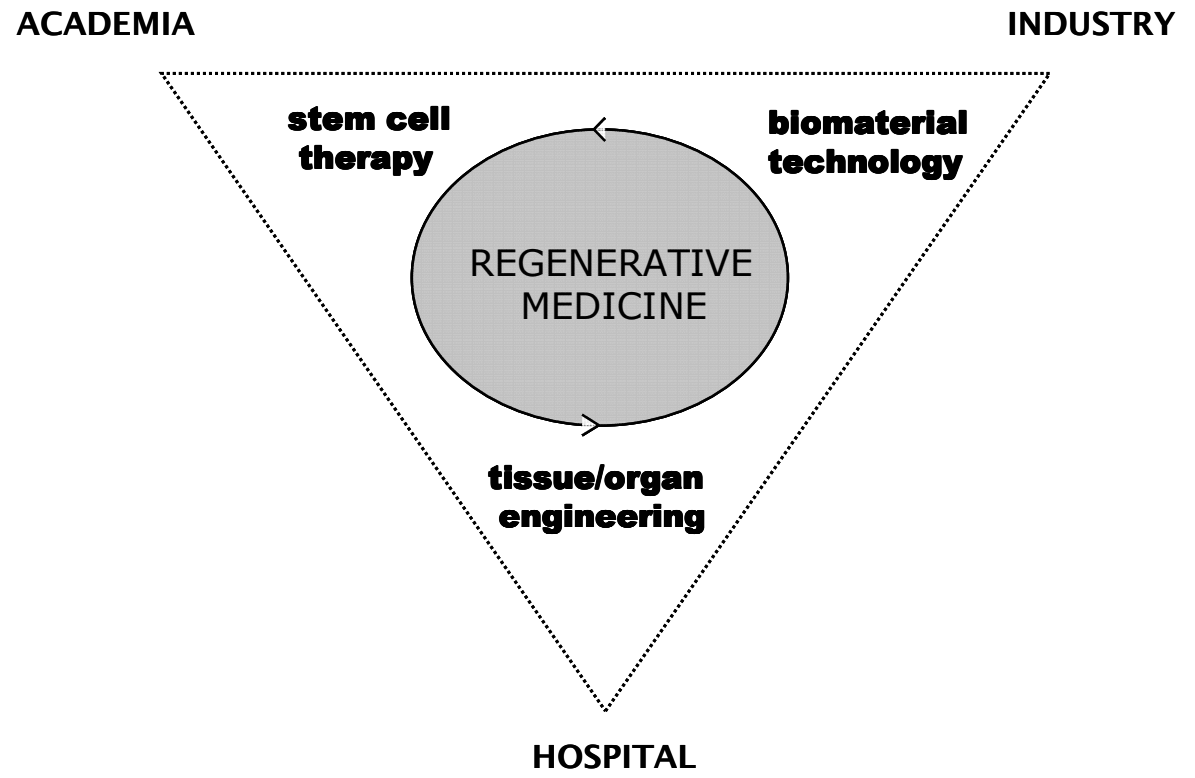

Figure 2. Graphical illustration showing the multidisciplinary and complexity of interactions within the context of regenerative medicine.

critical when the engineering of bulk tissue or complex organ is considered, particularly when autologous tissue production is desired. Such goals would necessitate the maintenance of large quantities of undifferentiated cells to provide sufficient starting material. The long doubling time of most types of SC weighs directly on this problem. There are different types of SCs with a broad variety of their doubling time (Table 1): for instance the doubling time of SCs ranges from 36 hr for human embryonic SCs (ESCs) to an estimated 45 days for human hematopoietic SCs (HSCs).

Table 1. Doubling time of human stem cells. ESC, embryonic stem cell; HSC, hematopoietic stem cell; MSC, mesenchymal stem cell; NSC, neural stem cell; EGC, embryonic germ cell.

Human stem cells	Average doubling time
ESC	35 h
HSC	45 weeks
MSC	1.3-16 days
NSC	4 days
EGC	3.2 days

Although it is generally believed that human ESCs can divide indefinitely, there is evidence to suggest that other SC types are submitted to Hayflick's limit when cultured ***in vitro***. Human mesenchymal SCs (MSCs) appear to show signs of senescence after the ninth passage in culture with a decline in differentiation potential from passage 6 (11). The recent identification of a population of adult MSCs (multipotent adult progenitor cell, MAPC) with a self-renewal and multipotent differentiation potential very similar to that of ESCs, raises hope for a source of renewable autologous SCs. These cells can be expanded ***in vitro*** up to 120 cell divisions without losing their SC potential (12). However, as these cells are less frequently encountered, extensive ***in vitro*** expansion would be required to obtain a sufficient number of cells for therapeutic purposes.

As a general concept we can assume that undifferentiated cells have greater tumorigenic potential than highly differentiated cells (Fig. 3). However, as a result of their slower growth rate and absence of telomerase activity ***in vitro***, MSCs are presumed to have a lower risk for tumor formation compared with embryonic SCs. However, it was recently reported that human adult MSCs from adipose tissues underwent spontaneous transformation and formed osteosarcoma cells after long-term culture (13). Therefore, and before the clinical studies start, there is a need to carry out appropriately designed toxicologic/tumorigenic assay protocols to demonstrate the long-term efficacy/safety of SC-based TE. As these problems are applied to highly sensitive areas, the development of novel therapeutic opportunities will need the approval of the government agencies and they will have a critical issue in the context of human therapies availability (Fig. 4).

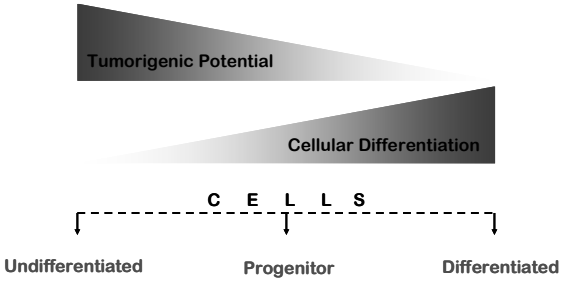

Figure 3. As a general concept we can assume that undifferentiated cells have greater tumorigenic potential than differentiated cells.

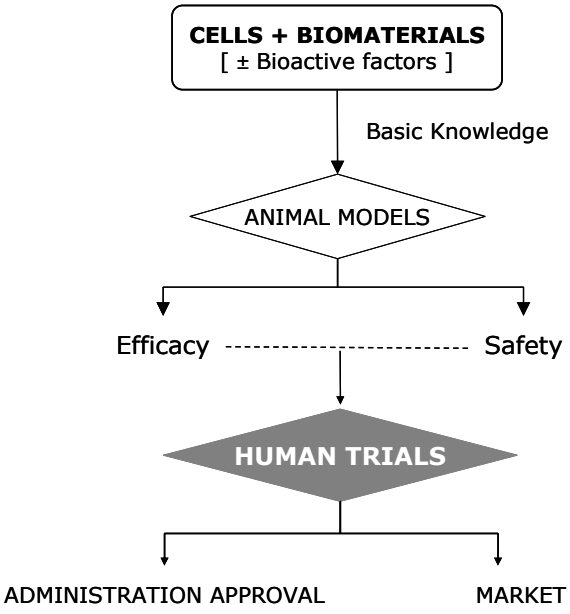

Figure 4. A logical pathway to put cell therapies into the clinical market after assays for toxicology and tumorigenic potential demonstrate in animal models the long-term efficacy/safety of the SC-based TE. Efforts to analyze and assess the efficacy/safety of using human SCs in the clinical setting are of vital importance.

Strategies for Stem Cell-Based Tissue Engineering

Stem cells-based TE offers clear merits over conventional TE strategies using mature cells. Conventional replacement therapies using autografts, allografts, or xenografts suffer from a host of drawbacks such as scarcity of donor

source, donor site morbidity, risk of lateral transmission of pathogens, and graft-versus-host rejection. In contrast, SC-based approaches circumvent these drawbacks, yet introduce the advantages of scalability. A major unmet challenge in TE has been the synthesis of complex grafts that are comprised of multiple cell types. Stem cell-based TE provides one approach to this challenge. This concept was demonstrated by the engineering of an articular condyle with both cartilaginous and osseous components by differentiation of a single population of MSCs in a polyethylene glycol-based hydrogel scaffold (14).

Adult Stem Cells with Potential for Tissue Engineering Applications

A number of stem/progenitor cells provide interesting subjects for research and are probable candidates for organ-specific TE, although there are some limitations to develop secure and useful protocols for use in regenerative medicine.

Hematopoietic Stem Cells (HSCs)

Despite almost three decades of extensive research into HSC expansion and self-renewal, a stable and reliable expansion system for human HSCs has yet not been achieved. This is probably due to the extreme sensitivity of true HSCs to their immediate micromilieu. Minute fluctuations in cytokine concentrations, oxygen tension, temperature, and cell-extracellular matrix interactions are sufficient to set in motion irreversible differentiation cascades that lead to depletion of HSCs in culture (9).

Neural Stem Cells (NSCs)

In mammals, adult neurons lose their proliferative potential. The central nervous system, therefore, has limited regenerative capacity when inflicted with lesions resulting from trauma, stroke, or neuropathological conditions. Clinical trials using transplantation of fetal brain cells to treat neurodegenerative diseases such as Parkinson's disease has raised questions regarding the effectiveness of this strategy (15). Repair of neurological injuries in the central nervous system is complicated by the presence of natural inhibitors of nerve regeneration, notably neurite outgrowth inhibitor and myelin-associated glycoprotein. Thus, a subset or therapeutic strategies for spinal cord injury is focused primarily on creating a permissive environment for regeneration by targeting these inhibitory proteins.

The peripheral nervous system retains limited capacity for self-repair in the injuries so that this capacity is rather small. Larger injuries, however, require nerve grafts usually harvested from other parts of the body. TE using NSCs provides a viable and practical alternative for cell therapy of the central and peripheral nervous system (16). SCs with the ability to differentiate into neurons, astrocytes and oligodendrocytes have been isolated from rat spinal cord (17), and implantation of neural SCs in an adult rat model of spinal cord injury resulted in long-term functional improvement (18). ESCs are capable of forming dopamine neurons in an animal model of Parkinson's Disease (19).

Endothelial Progenitor Cells (EPCs)

Neovasculogenesis, or the formation of blood vessels postnatally, is now thought to be attributed mainly to the activity of EPCs. Ever since their isolation from peripheral blood mononuclear cells was first reported (20), EPCs have been identified from various sources including bone marrow (BM) (21), umbilical cord blood (22), vessel walls (23), and fetal liver (24). Resident EPC populations in BM constitute a natural reservoir of cells that can be rapidly mobilized upon acute demand following major vascular insult (25).

The potential application of EPCs for therapeutic vasculogenesis is widely recognized (26). Direct infusion of endothelial stem/progenitor cells from various sources for neovascularization has been evaluated extensively in preclinical and clinical studies (27). Early strategies for developing vascular prostheses focused on the delivery of angiogenic growth factors such as vascular endothelial growth factor, fibroblast growth factor-2, and DNA encoding these factors to induce ingrowth of microvessels from the host vasculature ***in situ***. ***In vitro*** preendothelialization was supposed to create an antithrombogenic barrier for the devices, thereby preventing thrombus occlusion. Artificial grafts were seeded with differentiated endothelial cells (ECs) (28) or ECs in combination with other cell types such as smooth muscle cells (29).

Adipose-Tissue-Derived Stem Cells (ADSCs)

Adipose-tissue-derived stem cells (ADSCs) display much the same surface markers as MSCs

with the exception of the presence of VLA-4 expression and the absence of the expression of its receptor, CD106. Consistent with this phenotypic similarity, the two cell types exhibit an almost indistinguishable differentiation repertoire. Under suitable culture conditions, ADSCs differentiate along classical mesenchymal lineages, namely adipogenesis, chondrogenesis, osteogenesis, and myogenesis (30). The interest in ADSCs lies primarily in their potential as an alternative to BM MSCs. Although they occur at frequencies comparable to those of their BM counterparts, the extraction protocol for ADSCs is deemed less invasive than that for BM harvest. Additionally, these cells may prove valuable in treating conditions associated with BM failure.

Adipose TE using ADSCs is currently being considered as a viable alternative strategy in plastic, corrective, and reconstructive surgery. Taking into account the results obtained by our colleagues (31) for the treatment of Crohn's fistula, we are currently investigating the potential of this cell type to develop into functional tenocytes. This cell application is directed to heal tendon and ligament injuries using biosutures, which are surgery resorbable sutures formatted with autologous ADSCS into a type I collagen gel (Fig. 5). The cells contract the gel and because the suture is loaded, cells oriented with regard to the biosuture which is aligned with the load axis within an Achilles tendon defect in adult rats.

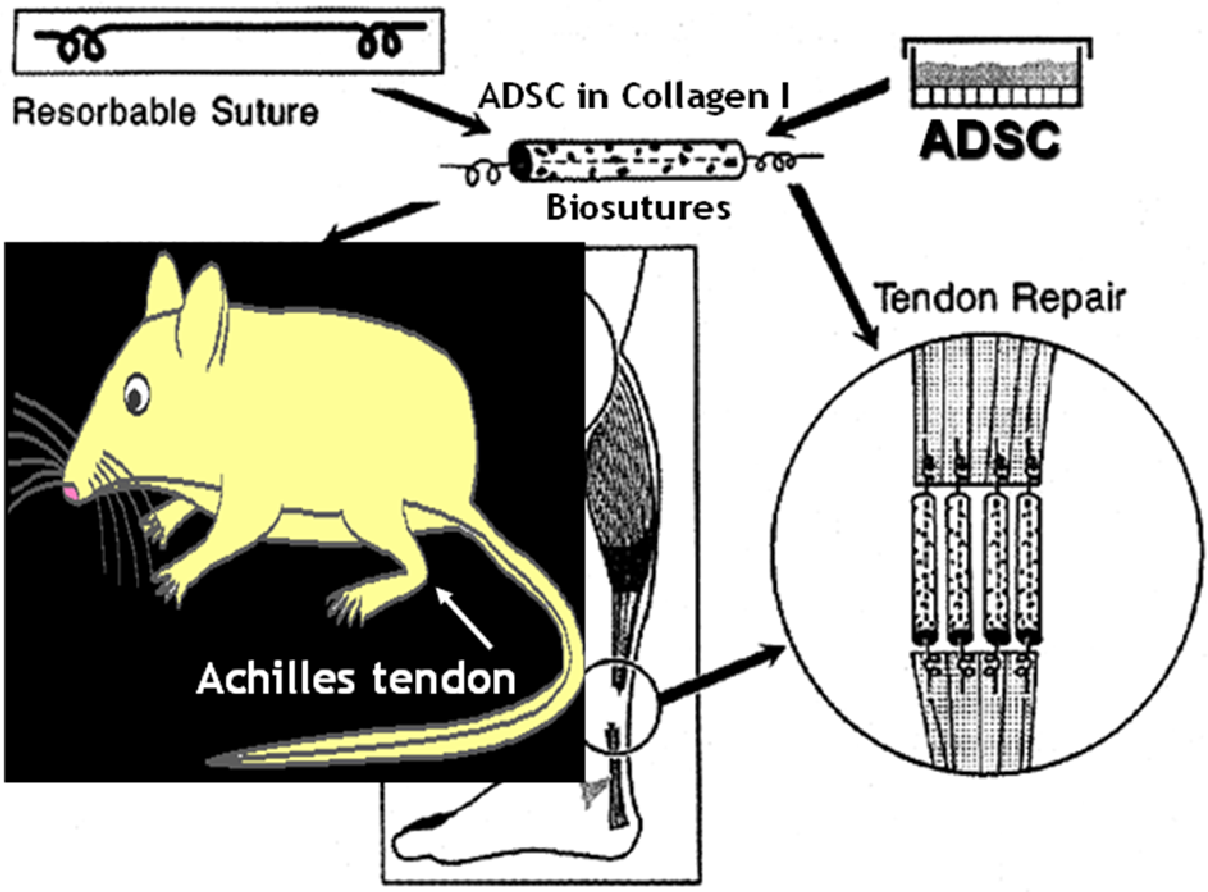

Figure 5. Construction of a composite formed from a resorbable suture held under tension around which a collagen gel with autologous ADSCs are loaded to treat tendon or ligament injuries. *Modified from Handbook of Stem Cells, Vol. 2, Chapter 28, Arnold I. Caplan, Mesenchymal Stem Cells, 299-308, Copyright 2004 by Academic Press. With permission from Elsevier.*

Other stem/progenitor cells with potential for TE applications

The recent report of the isolation of human renal progenitor cells from adult kidney (32) was set to launch a new branch of TE. End-stage renal failure is a catastrophic disease usually leading to death. Conventional treatments such as kidney transplantation and renal dialysis have by pity severe limitations and are often associated with considerable morbidity. Although the idea of a tissue-engineered kidney is not novel (33), the use of renal SCs could allow for the construction of a new organ ***de novo*** as well as for prospects for creating an autologous organ.

Tissue engineering of a functional pancreas has been an area of intense research for several decennies. Multipotent adult pancreatic progenitor cells identified recently (34) will provide momentum to make this goal achievable in the near future. Other newly discovered stem/progenitor cells that have broadened the cellular arsenal for regenerative medicine including liver (35), retinal structures (36), skeletal muscle (37), hair follicle (38), and dentine pulp (39) SCs.

Since our laboratory is focused on the use of adult MSCs for cell therapy and TE we are going to review in this chapter some data of their potentiality and applicability.

Mesenchymal Stem Cells for Tissue Engineering

MSCs, which are located within the stromal compartment of BM and represent a very small fraction (0.001-0.01%) of the total population of nucleated cells in bone marrow (40), were first identified during the pioneering studies of Friedenstein and Petrakova (41), who isolated bone-forming progenitor cells from rat bone marrow. However, they can be isolated and expanded with high efficiency, and induced to differentiate to multiple lineages under defined culture conditions. They express a non-hematopoietic cell surface phenotype, consisting of CD34-, CD45-, HLA-DR-, while possess markers such as STRO-1, VCAM, CD13, CD29, CD44, CD90, CD105, and SH-3 (42). They have the capacity to differentiate into cells of connective tissue lineages, including cartilage, bone, fat and muscle. MSCs have also been isolated from the periosteum (43), trabecular bone (44), adipose tissue (45), synovium (46), skeletal muscle (47) and deciduous teeth (48). Adipose tissue has also been shown to contain multipotent SCs, which have the capacity to differentiate into cells of connective tissue lineages (49, 50). Osteoprogenitor cells have also been isolated from skeletal muscle in mice and humans (51). Being less invasive procedures, muscle biopsy and liposuction are hence attractive alternatives in cell-based tissue engineering strategies.

MSCs have generated a great deal of interest because of their potential use in regenerative medicine and TE. Currently this cell population is second to BM SCs in terms of clinical entry in Phase III clinical trials. Some striking examples of the therapeutic use of bone marrow-derived MSCs have been reported recently. These address a broad spectrum of indications, including cardiovascular repair, treatment of lung fibrosis, of spinal cord injury and bone and cartilage repair. Orlic et al. (52) showed that locally delivered BM cells can generate ***de novo*** myocardium, indicating that SC therapy can be useful in treating coronary artery disease.

Stamm et al. (53) demonstrated the practical utility of this approach in a study involving the delivery of BM cells into the infarct zone in patients following myocardial infarction. The result of this treatment was a dramatic improvement in global heart function. Deb et al. (54) have also shown engraftment of BM-derived cardiomyocytes in the adult heart following BM transplantation. Saito et al. (55) demonstrated that MSCs are tolerated in a xenogeneic environment while retaining their ability to be recruited in favour of an injured myocardium and undergo therefore differentiation into a cardiac phenotype.

In vivo differentiation of MSCs to a skeletal muscle phenotype has also been demonstrated. Gussoni et al. (56) showed that murine MSCs, injected into the quadriceps muscle of ***mdx*** mice, expressed dystrophin in association with the muscle fiber sarcolemma, and pointed towards a potential therapy for muscular dystrophy. Toma et al. (57) injected ß-galactosidase-expressing human MSCs into the left ventricle of CB17 SCID/beige adult mice, and found the labeled cells dispersed throughout the myocardium and expressing desmin, cardiac-specific troponin T, ß-actinin and phospholamdan, all indicative of differentiation of the engrafted cells into a mature myocardial phenotype. MSCs have also been shown by Ortiz et al. (58) to engraft at high levels in lung tissue following exposure to

bleomycin, and to offer protection against bleomycin-induced lung injury, including inflammation and collagen deposition. These observations have broad implications in the area of lung disease associated with environmental damage.

MSCs respond to and produce regenerative cytokines, replicate and have the capacity to differentiate, form structural matrix, and respond to mechanical demands to restore skeletal function. In addition, they play a role in providing the stromal support system for haematopoietic SCs in the marrow. In the area of orthopedic medicine there are also many examples of applications involving local delivery of marrow SCs. These include spine fusion (59), the repair of segmental bone defects (60) and craniotomy defects (61). Similar approaches have also been described for the repair of focal defects in articular cartilage (62) and tendon (63). In an animal model of osteoarthritis involving injury to the meniscus delivery of SCs by intraarticular injection resulted in engraftment of those cells on the meniscus, fat pad and synovium with regeneration of meniscal tissue and protection of the cartilage (64). The chondroprotective effects seen in these studies apparently derive from the regenerated meniscus since there is no evidence of direct engraftment of the implanted cells on the fibrillated cartilage.

While the therapeutic testing of these cells has progressed well, there are still many questions to be addressed concerning the role of endogenous populations of SCs in the adult, and the function of various SC niches. The role of MSC in bone formation continues to be defined, and manipulation of MSC has resulted in new strategies for bone regeneration (65-67). In addition, there are several aspects to the implanted cell-host interaction that need to be addressed as we attempt to understand the mechanisms underlying these therapies. Firstly, host responses to allogenic MSC therapy need to be defined. Secondly, little is known about the mechanisms that direct homing and engraftment of implanted cells and thirdly, the response of MSCs to local differentiation signals ***in vivo*** has not been clarified.

Tissue Engineering Constructs

Cell-based clinical therapies using MSCs involve at least three different approaches. First, TE strategies in which MSCs are incorporated into three-dimensional (3-D) scaffolds for the replacement of 3-D pieces of ***in vivo*** tissues; second, cell replacement therapy, in which genetic defects can be cured by replacing the mutant host cells with normal allogeneic donor cells; and third, where MSCs act as cytokine/growth factor mediators to stimulate reparative events or to inhibit degenerative events. Therefore, a scaffold or matrix, living cells and/or biologically active molecules, as well as blood vessels ingrowth, are used in variable strategies to form a "TE construct" to promote the repair and regeneration of tissues (Fig. 1).

In order to capture and expand a population of cells with chondro-osteogenic potential, our group has used type I collagen to develop an ***in vitro*** culture system for BM mesenchymal progenitor cells (Fig. 6), which includes a MSC compatible collagen-gel impregnated with a genetically engineered hTGF-ß1 fusion protein, bearing an auxiliary von Willebrand's factor-derived collagen binding domain (rhTGF-ß1-F2) (Fig. 7) (68). Such a device bestows on the factor the ability to bind specifically to type I collagen and the collagen binding domain of the rhTGF-ß1-F2 allows a slow release of the growth factor from the collagen matrix to which it is bound and therefore a longer half-life and better availability to the target cells. Several growth factors are being designed for different purposes.

A wide variety of natural and synthetic devices and matrices have been used as carriers to deliver MSCs, including poly L-lactic acid (PLLA), polycaprolactone (PCL), etc. There is simple evidence that the nature and properties of the scaffold and cell carriers play an important role in tissue engineering. It is important to emphasize at the outset that the field is still young and many different approaches are under experimental investigation. Thus, it is by no means clear what defines an ideal scaffold-cell or scaffold-neo-tissue construct, even for a specific tissue type. Indeed, since some tissues perform multiple functional roles, it is unlikely that a single scaffold would serve as a universal foundation for the regeneration of even a single tissue (69).

CONCLUSIONS

In the last ten years we have published reports suggesting that SCs have a promising potential to be utilized for regenerative medicine. For example, our laboratory has accumulated experience working ***in vitro*** with blood-, fat-, skeletal muscle- and BM-derived SCs,

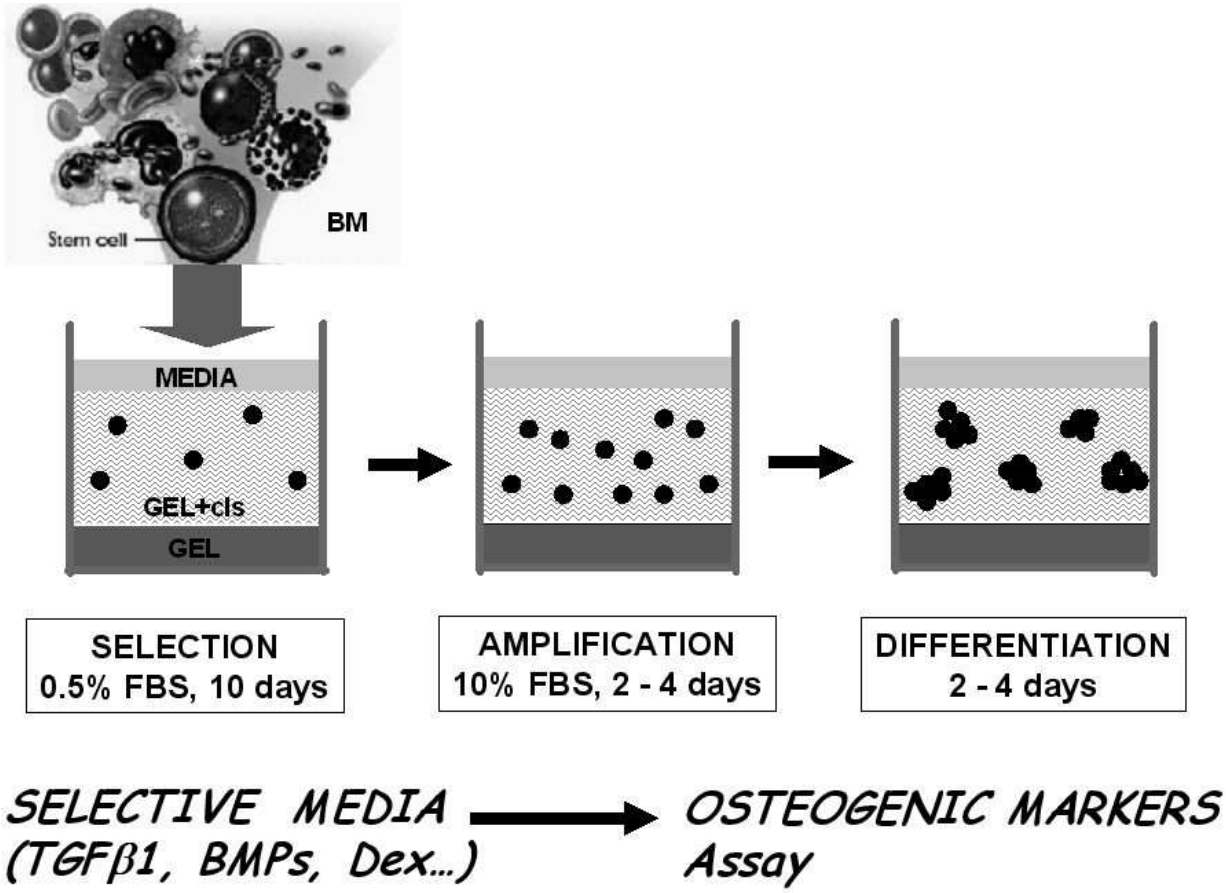

Figure 6. Collagen matrices prepared *in vitro* using type I collagen holding one of the growth factors shown in Fig. 7 in the appropriate doses and the bone marrow cells. The osteogenic potential is evaluated through the osteogenic lineage.

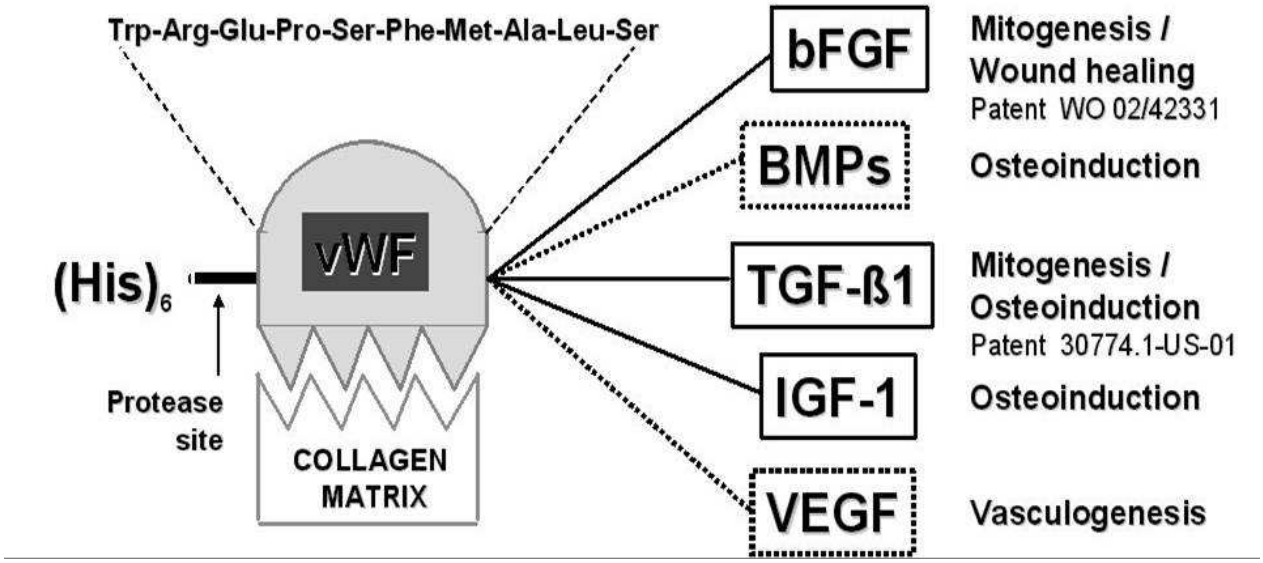

Figure 7. Genetically engineered growth factor fusion proteins in our laboratory, bearing an auxiliary von Willebrand's factor-derived collagen binding domain (decapeptide). Closed boxes indicate the growth factors obtained and currently being used. Open boxes point the growth factors under production.

performing ectopic implants (in mouse and rat), preclinical trial with small animals (rabbit, using an osteochondral defect model), with big animals (sheep, using a spinal fusion model), and with a pilot clinical trial in a patient affected by osteomyelitis (70) (Fig. 8). However, it is clear that there are several critical questions which have to be addressed in order to develop effective treatments.

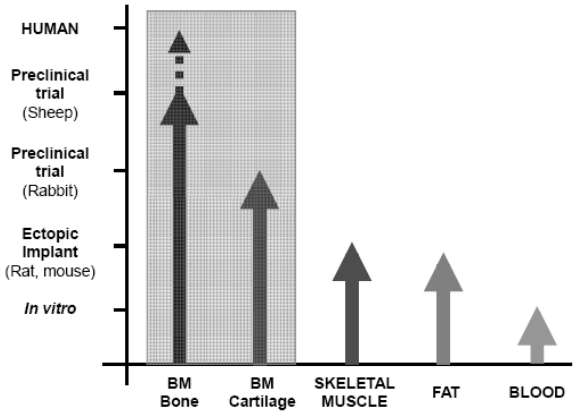

Figure 8. Experience working with MSCs derived from different origins as well as the current developmental status of our investigations in the field of skeletal repair. The shadowed area shows the research areas that are more developed in our laboratory.

First, in general SCs are a heterogeneous population of cells which contain cells at different stages of development. Although many molecules have been reported as cell-surface markers specific for the different types of SCs, it is still difficult to isolate highly purified SCs in most of the cases.

Secondly, it is critical to understand how to maintain the "stemness" of SCs following ***ex vivo*** expansion, and then how to direct tissue-specific regeneration in the presence of complex interactions with recipient tissues. In order to understand the stem properties of SCs in depth, identification of the local microenvironment or SC "niche" is essential for finding the markers to define a particular SC at early stages of development and to asses their functions.

Thirdly, tissue regeneration demands more predictable outcomes because most of the diseases or injuries consist of many relatively small and complicated components that have a huge impact on an individual's condition. To enhance or control the differentiation of SCs, proper application methods such as delivering methods, genetic manipulation, combinations of SCs with growth factors and suitable biomaterials have to be further improved. Although there are many challenges ahead of us in terms of utilizing SCs for TE, MSCs are one of the most promising postnatal SC populations for tissue repairing and regeneration for a wide range of organs.

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PS:Professor R.Wegmann to whom this work has been submitted for publication in his journal CMB®, has made a thorough lecture of this paper which is of high scientific standard. But as usually he has controlled the quality of the English language and has corrected many expressions and terms in order permit a better general understanding of the text and consequently to maintain the rigorous high standard of this journal .

Nevertheless the important main criticism I address to the authors is to not have respected the alphanumerical order of the literature as it was clearly indicated and recommended in the general "Notes to Authors". Such a lining is fundamental because it permits to find immediately out in the references if some important author has been mentioned or if a paper has been missed or not.

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