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Stromal cells

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1. ABSTRACT

Stromal cells, or mesenchymal stem cells, are adherent clonogenic cells that can form colonies. They are mainly isolated from bone marrow but can also be found in umbilical cord blood, adipose tissues and amniotic fluids. These stem cells are easy to culture *in vitro*, and can differentiate into osteoblasts, chondrocytes, or adipocytes when stimulated appropriately. When seeded on a natural (titanium, ceramics, collagen fibers, silk, *etc*.) or synthetic (PLLA, PLGA, *etc*.) biomaterial scaffold, they adhere and differentiate to form a new tissue. Many studies have also explored stromal cell differentiation in bioreactors to form a 3-dimensional culture. This review focuses on the biomaterials used for tissue engineering with stromal cells.

2. INTRODUCTION

Mammalian bone marrow contains two types of adult stem cells. The first, the hematopoietic stem cells (HSC), are responsible for producing blood cells throughout life, and gives rise to different lineages. The second type are the stromal or mesenchymal stem cells. Although less well characterized than HSC, stromal cells were first studied because of their important role in formating the hematopoietic microenvironment (1). We now know that stromal cells have the potential to differentiate to form skeletal tissues, neural tissues, muscles, *etc*.

Recent findings indicate that stromal cells seeded on appropriate scaffolds can be used to regenerate certain tissues. The natural or synthetic scaffold provides the

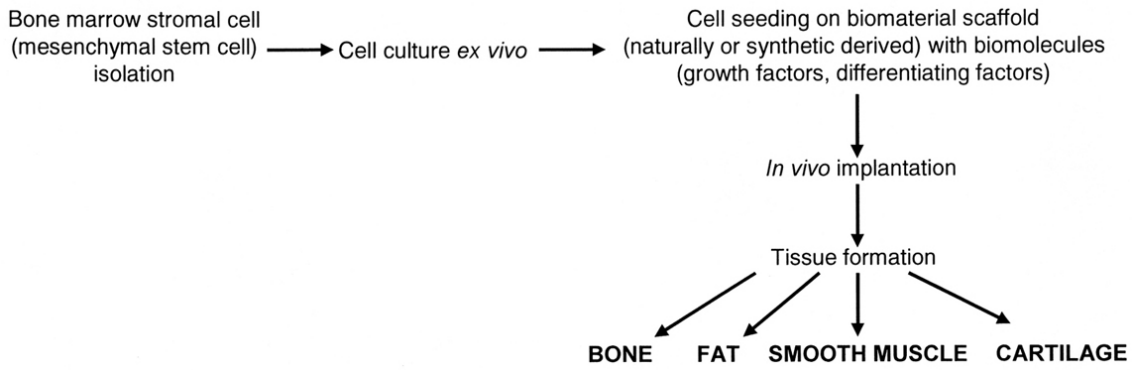

Figure 1. Tissue engineering approach to organ repair using stromal cells (124). Stromal cells are isolated from bone marrow and expanded *ex vivo*. They are then seeded on an extracellular matrix fabricated from natural or synthetic biomaterials and incubated with appropriate growth factors or differentiating factors to stimulate their proliferation and differentiation into a given tissue. The scaffold-stromal cell constructs are then implanted *in vivo*.

microenvironment in which they proliferate, and differentiate in response to appropriate differentiating factors (Figure 1) (1).

3. INTERACTIONS BETWEEN STROMAL CELLS AND THEIR ENVIRONMENT, AND CELL-CELL INTERACTIONS IN THE REAL *IN VIVO* ENVIRONMENT

3.1. Stromal cells

3.1.1. Denominations used to refer to stromal cells

Mesenchyme is an embryonic connective tissue arising mainly from the mesoderm (middle layer) of the early embryo. Mesenchyme differentiates to form blood vessels and blood-related organs such as the spleen, and connective tissues (bone, cartilage, fat, tendon, muscle, hematopoietic-supporting stroma and nerve tissue). Bone marrow is the source of multipotent stem cells that give rise to progenitors for mesenchymal tissues. That is why these cells are frequently referred to as mesenchymal stem cells, or mesenchymal progenitor cells. We will use the terms stromal cells (SC) or bone marrow stromal cells (BMSC).

3.1.2. Sources of stromal cells

Stromal cells are generally isolated from an aspirate of bone marrow (BM) taken from the superior iliac crest of the pelvis in humans (2-4). There are other sources of stromal cells. Lee *et al.* reported that the mononuclear cell fractions from cryopreserved umbilical cord blood (UCB) contained stem cells with the same characteristics as BM-derived stromal cells (same cell-surface antigen profile and multi-differentiation capacity) (5-6). Similar results were obtained by Wang *et al.* who also showed that UCB-derived SC can support the *ex vivo* expansion of HSC (7). UCB-derived SC have the potential to form skeletal muscle, neural cells and hepatocyte-like cells (8-11). Thus, the UCB is an alternative source of SC although differently expressed genes may reflect differences in their origins (12).

Other sources include a small population of circulating mesenchymal precursor cells, but the yield varies with the methods used for isolating the peripheral blood mononuclear cells and the culture conditions (13-15). Lastly, SC have been isolated from human adipose tissue (16-17). The markers on adipose tissue-derived stromal cells are similar to those on BMSC and these cells can also differentiate into osteoblasts, adipocytes and chondrocytes. Amniotic fluid, placenta and other fetal tissues [21] are also possible sources of SC (18-21).

3.1.3. Characterization of stromal cells

Friedenstein *et al.* first isolated adherent cells that were clonogenic, non-phagocytic and able to form colonies (fibroblast colony forming units, CFU-F) from the BM stroma of newborn rodents (22). These CFU-F can proliferate extensively *in vitro*, but are essentially in a non-cycling state *in vivo* (23). The incidence of CFU-F in BM suspensions varies from one species to another (24). Some mitogenic factors, such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), transforming growth factor beta (TGF-beta), and insulin-like growth factor (IGF-1) regulate the proliferation of CFU-F (25-27).

Most SC have been isolated using their capacity to adhere to a plastic support. However, macrophages, endothelial cells, lymphocytes, and smooth muscle cells can also adhere and contaminate stromal cell preparations. Stromal cell enrichment could be realised with relatively deprivational medium, only containing serum. The cultures are morphologically heterogeneous (containing narrow spindle shaped cells, large polygonal cells, and tightly packed cuboid cells). CFU-F assays also show that the fraction is heterogeneous with different colony sizes, cell morphologies, and differentiation potentials (28-29).

SC are distinguished from hematopoietic cells by their lack of CD34, CD45, CD14 and HLA-DR and specific markers of SC have been identified and used to prepare a monoclonal antibody, Stro-1 that is specific for clonogenic SC (30-32). A subfraction of stromal cells bears the Stro-1 antigen and vascular cell adhesion molecule 1 (VCAM/CD106). These cells can differentiate to form cells with the characteristics of adipose, cartilage and bone cells *in vitro*, and form human bone tissue after transplantation into immunodeficient SCID mice (3, 33). Thus, stromal cell precursors, including stromal stem cells that can

Table 1. Biomolecules that have an effect on stromal cells

Molecules	Observations			Effects/Observations	Ref
	In vivo	*In vitro*			
		2D	3 D		
HGF, Onc M		2D		Induce hepatic differentiation	11
TGF-beta3		2D		Induces chondrogenic differentiation	44
beta-GP, Dex, aP		2D		Induce osteogenic differentiation	44
IBMX, Dex, IM		2D		Induce adipogenic differentiation	44
aP, Dex			3D	Enhance mineralization	46
NIC, beta-ME		2D		Induce pancreatic islet beta-cells differentiation	50
BMP-2			3D	Dose-dependently stimulates extracellular matrix calcification.	81 82
Dex		2D		Induces the osteogenic differentiation of rat SC	89
BMP-2	*In vivo*			Stimulates osteogenic differentiation Promotes bone formation	90 91
GDF-5	*In vivo*			Enhances *de novo* bone formation by BMSC seeded on HA	92
bFGF			3D	Enhances the mineralization of rat BMSC on a hyaluronic acid based polymer scaffold	101
O_2: hypoxia			3D	Induces marrow SC to migrate and form capillary-like structure	103
bFGF			3D	Induces BMSC proliferation, migration, and tubulogenesis	104
RGD			3D	Enhances the adhesion of human BMSC to silk fibers.	113
TGF-beta1			3D	Enhances chondrogenic differentiation of human marrow stroma-derived cells seeded on PLLA/alginate scaffold	119
O_2: anoxia (< 0.02 % O_2)		2D		Induces osteogenic differentiation and runx2 synthesis by SC inhibited	133
TGF-beta3-BMP6 TGF-beta3-IGF1			3D	Stimulate human SC to form chondrocytes	134
TGF-beta1			3D	Enhances rat marrow SC proliferation and osteoblastic differentiation	135
		2D		Reduces production of the chemokine stromal cell derived factor 1, and then inhibits cell migration and adhesion	136
TGF-beta1-CDMP1 (GDF-5)			3D	Synergically induce chondrogenesis	137
BMP-2		2D	3D	Increases osteopontin and VEGF synthesis by rabbit BMSC on a PLGA scaffold, induces ECM secretion Midly stimulates collagen I and VEGF expression and greatly stimulates osteopontin synthesis by SC on a PLGA film	138
Collagen I gel			3D	With mechanical stimulation, induces bovine BMSC differentiation into ligament-like cells	139
Denatured collagen		2D		Enhances the proliferation and differentiation of human adult BMSC	140
DMSO and BHA		2D		Induce marrow SC to form neural stem cells, and then differentiate into neuron-like cells	141
Oxysterols		2D		Regulate lineage-specific differentiation of SC in favour of osteoblastic and against adipogenic differentiation	142

Abbreviations: TGF-beta1: transforming growth factor beta-1; BMP-2: bone morphogenetic protein 2; DMSO: dimethyl sulfoxide; GDF-5: growth/differentiation factor-5; bFGF: basic fibroblast growth factor; beta-GP: beta-glycerophosphate; Dex: dexamethasone; aP: ascorbate-2-phosphate; IBMX: isobutylmethylxanthine; IM: indomethacine; HGF: hepatocyte growth factor; BHA: butylated hydroxyanisole; Onc M: oncostatin M; beta-ME: beta-mercaptoethanol; NIC: nicotinamide; RGD: Arg-Gly-Asp

differentiate to form several mesenchymal lineages are found only in the Stro-1^+ fraction of adult human bone marrow (3, 34).

The profile of adhesion molecules also varies from donor to donor and is influenced by the culture conditions (serum type) (35). BMSC also synthesize matrix proteins (vimentin, laminin, fibronectin, osteopontin), and some markers of myofibroblasts (alpha-smooth muscle actin, smooth muscle myosin heavy chain), neurons (nestin, Tuj-1) and endothelial cells such as MUC-18/CD146, endoglin/CD105, TGF-beta receptor III, integrins (beta1, alpha1, alpha5, and weakly alpha2, alpha3, alpha6, alphav, beta3, and beta4) (36-43).

3.2. Differentiation pathways

SC differentiate into various lineages of mesodermal origin, such as cartilage, bone, fat, tendon, muscle, myocardium, and marrow stroma, under a range of specific *in vitro* conditions.

A mixture of dexamethasone (Dex), beta-glycerophosphate (beta-GP) and ascorbic acid phosphate (aP) has been widely used to induce osteogenic differentiation, as shown by calcium accumulation and increased alkaline activity (44). Similarly, Dex plus isobutyl-methylxanthine (IBMX) and indomethacin (IM) are adipogenic factors (Figure 2, Table 1), causing SC to form cells containing lipid vacuoles that are stained with oil red O-solution (2, 41). SC can be stimulated to differentiate into chondrocytes for cartilage formation with TGF-beta3 and cell aggregation using a pellet culture system (Figure 2) (2). Cyclic compressive loading, alone or together with TGF-beta1, also promotes chondrogenesis (45). Differentiating stromal cells synthesize aggrecan, decorin and type II collagen (4).

The formation of cells with a vascular smooth muscle-like phenotype requires a high serum concentration, stretching, and TGF-beta (Figure 2) (36, 44, 46). Subsequent mechanical stimulation, especially compressive strain, also promotes the synthesis of smooth muscle cell-specific cytoskeletal proteins (47). And bFGF, dimethylsulfoxide (DMSO), beta-mercaptoethanol (beta-ME) and butylated hydroxyanisole (BHA) stimulate SC to form cells having a neuronal phenotype (31, 48). Qian *et al.* obtained cells with the morphology of neurons that produced neuron-specific enolase by incubation with DMSO, BHA, KCl, forskolin and hydrocortisone (49).

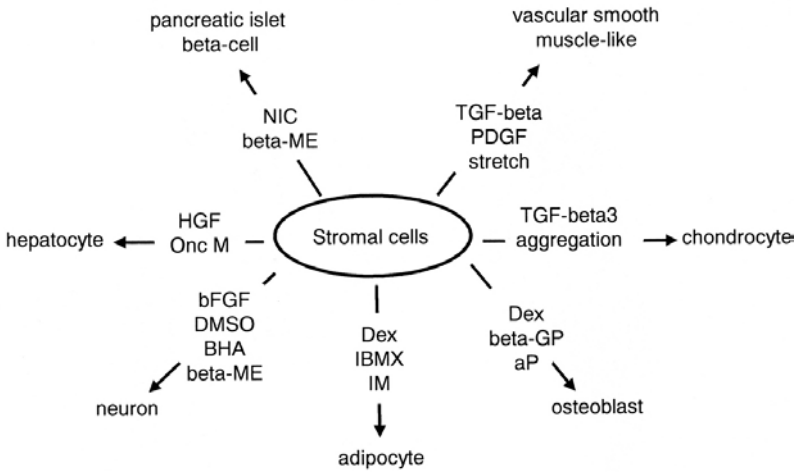

Figure 2. Effects of various molecules on stromal cell differentiation (36). Stromal cells can differentiate to form various lineages when treated with certain growth factors or molecules. Dex: dexamethasone; beta-GP: beta-glycerophosphate; aP: ascorbate-2-phosphate; IBMX: isobutylmethylxanthine; IM: indomethacine; bFGF: basic fibroblast growth factor; DMSO: dimethylsulfoxide; BHA: butylated hydroxyanisole; beta-ME: beta-mercaptoethanol; HGF: hepatocyte growth factor; Onc M: oncostatin M; NIC: nicotinamide; TGF-beta: transforming growth factor beta; PDGF: platelet-derived growth factor.

A new protocol was recently described that stimulates SC isolated from BM and UCB to form hepatic cells (11). The authors develop a two-step serum-free protocol using hepatocyte growth factor and oncostatin M, and obtained cuboid cells very like hepatocytes that bore appropriate markers (alpha-fetoprotein, glucose 6-phosphatase, tyrosine aminotransferase, cytokeratin-18, *etc*.) and had the functions *in vitro* (albumin production, glycogen storage, urea secretion, *etc*.) specific to liver cells.

Chen *et al.* caused SC to differentiate into pancreatic islet beta-cells using nicotinamide and beta-ME (Figure 2) (50). The differentiated cells had all the key properties including morphology (islet-like grape-like shape), high *insulin-1* mRNA content, and synthesized both insulin and nestin (a marker of pancreatic islet cell precursors).

Differentiation is also regulated by genetic events, involving transcription factors. This leads to the concept of master regulatory genes whose expression activates progenitor cells along a particular phenotype pathway (34). Some master genes have been identified ; they include *MyoD*, *Myf 5*, *myogenin* and *MRF4* which regulate myogenesis (51-52). *PPAR-gamma2*, *C/EBP*, *retinoic C receptor-alpha* activate adipogenesis, while *PLZF* and *Cbfa-1* induce osteogenesis (53-57). Lastly, *Smad3, CBP/p300, SOX9* activate chondrogenesis (58).

Lineage repression can also lead to differentiation. For exemple, overexpression of the *NFAT* gene (nuclear factor of activated T cells) inhibits chondrogenesis, while repression of the *NFAT* gene is correlated with chondrogenesis induction (59). Overexpression of the *PPAR-gamma2* gene encoding adipogenic factor also represses *Cbfa-1* gene expression in osteogenic cells (60). Jun N-terminal kinase signaling seems to promote osteogenesis and repress adipogenesis by inhibiting the function of the adipogenic transcription factor, CRE-binding protein (61).

3.3. *In vivo* functions of stromal cells

3.3.1. HSC differentiation

Interactions between HSC and SC are important for hematopoiesis (62). SC communicate with each other *via* gap-junctions (GJ). Studies with lucifer yellow dye transfer showed that there are connexin 43 (Cx43) type GJ between BMSC (63). Cancelas *et al.* showed that various Cx are synthesized by stromal cells, and that a lack of Cx43 leads to hemopoietic defects *in vivo* (Cx43 knock-out mice) and *in vitro* (coculture systems) (64). However, Rosendaal *et al.* found no difference in the hematopoietic capacities of Cx43 wild-type and KO mice (65). Thus, the role of Cx43 GJ in hematopoiesis remains uncertain.

SC constitutively secrete several cytokines, such as IL-6, IL-11, leukemia inhibitor factor (LIF), macrophage colony-stimulating factor (M-CSF), stem cell factor (SCF), and Ftl-3 ligand (42, 66). These cytokines are relevant for HSC proliferation and differentiation. SC also express adhesion-related antigens (CD166, CD54, CD31, CD106) and integrins (CD49, CD29, CD11, beta4). These proteins allow the adhesion and proliferation of HSC. Thus, SC support hematopoeisis in long-term cultures and can support the expansion of clonogenic hematopoietic cells (31, 42, 67).

The adhesion of megakaryocytic progenitors to SC inhibits their differentiation to form megakaryocytes. Zweegman *et al.* showed that BM stromal heparan sulfate proteoglycans are involved in the binding of hematopoietic progenitors and megakaryocytopoiesis-inhibiting cytokines, suggesting that BM stromal proteoglycans reduce differentiation of HSC towards megakaryocytes (68). However SC enhance human myelopoiesis and megakaryocytopoiesis when transplanted into NOD/SCID mice together with human $CD34^+$ human HSC (69).

3.3.2. Host immune response

There is now good evidence that SC are hypoimmunogenic, which has led to their use in allogenic therapy.Haploidentical SC were recently used to treat a case of severe graft-versus-host disease (GVHD) after

allogenic stem-cell transplantation (70). The patient was given a transplant of blood stem cells and developed a GVHD. SC were injected intravenously, and they had a striking immunosuppressive effect. Maitra *et al.* suggested that SC directly inhibit the activation of alloreactive T lymphocytes (71). Aggarwal and Pittenger used coculture systems to show that SC alter the cytokine secretion profiles of dendritic cells, T cells, and natural killer cells (72). Dendritic cells in contact with SC produced less TNF-alpha and more IL-10, T cells produced less IFN-gamma and more IL-4, leading to a more anti-inflammatory or tolerant phenotype (72).

3.3.3. Homing mechanisms

Human SC transplanted into fetal sheep became embedded into various tissues (BM, spleen, thymus, liver) (73). SC can also migrate to the site of an injury and be engrafted to and repair damaged tissues, such as the meniscus or kidney (74-76). The mechanisms by which they home on a site are unclear. The chemokine monocyte chemoattractant protein-1 (MCP-1) may be involved (74).

4. INTERACTIONS BETWEEN STROMAL CELLS AND MATERIALS: BIOHYBRID SYSTEMS

Tissue engineering is a promising method for treating a patient who has lost a tissue or an organ. Dissociated cells can reassemble *in vitro* to form structures similar to the original tissue when supported by an appropriate environment. Exogenous three-dimensional extracellular matrices (ECM) are used to create this environment. The ECM must allow cell adhesion, proliferation and differentiation, and promote revascularization and innervation *in vivo*. Hence, the interaction of a scaffold like the ECM with cells is determined by its structure, the presence of pores, the pore size, geometry, and distribution, surface texture (roughness, pattern, orientation), and the surface chemistry (hydrophilicity, ionic charge, *etc*.). The ECM used in tissue engineering with stromal cells can be fabricated from either naturally derived or synthetic materials.

4.1. MATERIALS

4.1.1. Natural materials

4.1.1.1. Titanium

Most orthopedic and dental implants are metal based and titanium (Ti) and its alloys are the materials of choice. Many *in vitro* studies have shown that increasing surface roughness enhances stromal cell attachment, proliferation, and synthesis of differentiation markers(77-78). However, others have found no effect of surface roughness on stromal cell proliferation and differentiation (79). Sikavitsas *et al.* used SC seeded on titanium fiber meshes to demonstrate that increased shear forces enhance mineralized extracellular matrix deposition i.e., osteoblastic differentiation (80). Fluid flow-induced shear forces appear to be important stimuli of stromal cell differentiation. Cells incubated with a scaffold rotating at a low speed are stimulated to become attached and invade the titanium fiber mesh (81).

Titanium materials have also been widely used in association with differentiating factors, or natural matrix components. Van den Dolder *et al.* showed that recombinant human BMP-2 (100 and 1000 ng/mL) stimulates the differentiation (alkaline phosphatase activity) of SC and the formation of matrix in a dose dependent manner without altering cell proliferation (79, 82). Wang *et al.* also showed that low concentrations (10 ng/mL) of BMP-2 stimulate the development of the adipocyte phenotype, while higher concentrations (100 and 1000 ng/mL) stimulate formation of the chondroblastic and osteoblastic phenotype (83).

Some studies have also been done on titanium coated with hydroxyapatite (HA). Cells cultured on Ti-HA grow slowly (84). Torensma *et al.* also observed that only a subfraction of stromal cells attached to Ti-HA (35). Thus, cell recruitment onto HA ceramics is selective.

Van den Dolder *et al.* evaluated effects of coating titanium fiber meshes with fibronectin and collagen I (85). They found that fibronectin and collagen I coatings did not stimulate the proliferation and differentiation of stromal cells.

Lastly, Datta *et al.* investigated effect of ECM macromolecules deposited on titanium on SC (86). The SC were induced to differentiate by culturing them on titanium for 12 days in medium containing osteogenic complements (beta-GP, aP, Dex). These cells produced ECM. Fresh rat SC were then seeded onto a Ti/ECM construct with or without Dex. The SC grown on the Ti scaffold with ECM proliferated more and produced calcium deposits. Thus, bone-like ECM deposited on a Ti fiber mesh scaffold can induce osteoblastic differentiation of bone progenitor cells (86).

4.1.1.2. Ceramics

Calcium phosphate is an essential mineral in bone and teeth. Bioactive calcium phosphate ceramics, such as hydroxyapatite (HA) and tricalcium phosphate (TCP), are biocompatible and osteoinductive, and are currently used to support stromal cell differentiation.

4.1.1.2.1. Hydroxyapatite (HA)

SC can be seeded onto microparticles of HA and the resulting constructs implanted *in vivo* (87). Bone was formed only with 212 to 300 micrometers diameter particles. However, Magrufov *et al.* found no significant variation in the proliferation of SC on 3D-HA with the percentage of micropores (88).

A novel material for cell culture has been recently prepared. These transparent HA ceramics were made by the spark plasma sintering process (89). The shape of the seeded stromal cells can be monitored, as can their differentiation. These HA ceramics have shown that SC immediately become attached to the surface, so that proliferation and osteogenic differentiation are both excellent.

HA is also often used in association with recombinant proteins. Bone morphogenetic protein 2 (BMP-2) acts in synergy with HA ceramics to enhance the osteogenic differentiation of stromal cells (90-91). A new member of the BMP family, growth/differentiation factor-5 (GDF-5, BMP-14), can also be incorporated into

composites of SC and porous HA (92). Recombinant GDF-5 can stimulate dose-dependent *de novo* bone formation in rats by suitable implants, as demonstrated by high alkaline phosphatase activity and mRNA, and high synthesis of osteocalcin.

HA has been associated with chitin, the precursor of chitosan, an abundant natural polymer. Chitin accelerates wound healing and is biocompatible. Ge *et al.* fabricated HA in 25%, 50%, and 75% w/w fractions incorporated into chitin solutions (93). They caused SC from rabbit bone marrow to differentiate into osteoblasts with Dex *in vitro*, and then seeded them onto a porous HA-chitin matrix. The osteoblasts retained their phenotype on this matrix and proliferated. Implantation of this matrix loaded with cells and matrix alone promoted the ingrowth of surrounding tissues.

4.1.1.2.2. Tricalcium phosphate (TCP)

Kasten *et al.* assessed the capacity of resorbable ceramics such as TCP to support SC penetration, proliferation, and osteogenic differentiation (94). Almost half (41.5%) of the total cells seeded did not adhere to TCP. There were only small increases in alkaline phosphatase and osteocalcin production. However, injecting SC loaded onto TCP-fibrin glue (a composite of fibrinogen and thrombin) into rats led to the formation of mature bone (95).

The capacity of gelatin sponges (denatured collagen) incorporating TCP to act as a scaffold for SC has been evaluated (96). Gelatin sponges have interconnecting pores (180-200 micrometers). SC adhered to the sponges but their morphology varied. They became flattened on sponges with TCP and spherical on sponges without TCP. The best increases in alkaline phosphatase activity and osteocalcin content were obtained with sponges containing 25-75% TCP in stirred cultures (96).

4.1.1.2.3. Animal bone or skeleton

Some studies have used xenografts from cattle and pigs, as their structures are similar to that of human bone. They must be sterilised to eliminate organics components and possible pathogens. Park *et al.* used heat-treated pig trabecular bone as a scaffold for rabbit SC and found that block of this bone were very porous (97). X-ray diffraction analysis showed that HA was the main component and that the calcium/phosphate molar ratio was closer to that of human trabecular bone than was the ratio for bovine bone blocks. Coating the bone blocks with collagen I increased SC adhesion in rotating cultures. The SC differentiated towards an osteogenic lineage in the presence of Dex and beta-GP. The authors concluded that the bone blocks are a very porous HA-based material whose stiffness is closer to that of human bone than is that of commercially available bovine xenografts (97).

A marine sponge skeleton has also been used as a scaffold (98). This material has a porous structure and was used to grow SC. The cells adhered to the skeleton, and the alkaline phosphatase activity and collagen I content indicated bone matrix formation. This matrix can also be used with recombinant human BMP-2 as osteogenic factor.

4.1.1.3. Biomolecules

4.1.1.3.1. Hyaluronic acid

Hyaluronic acid is a naturally occurring glycosaminoglycan found in ECM and essential for the maintenance of normal ECM structure. A new biodegradable polymer scaffold, based on hyaluronic acid, is now available (Hyaff®-11) in various configurations (sponge, non-woven, *etc*). It is biodegradable and immunologically inert (99-100). Rat BMSC seeded on a non-woven mesh Hyaff®-11 proliferated and mineralized when the medium contained Dex, beta-GP and b-FGF. Mineralization was stimulated by b-FGF and involved the entire scaffold (101). Curiously, cells grown on scaffold without b-FGF had a higher alkaline phosphatase activity than cells grown with b-FGF, but b-FGF also stimulated the synhesis of matrix proteins (osteocalcin, osteopontin and bone sialoprotein). The Hyaff®-11 scaffold was also mineralized *in vivo* (102).

4.1.1.3.2. Matrigel

Matrigel is a solubilized basement membrane preparation extracted from mouse sarcoma, a tumor rich in ECM proteins. Its major components are laminin, collagen IV, heparan sulfate proteoglycans and entactin.

Annabi *et al.* found that BMSC plated on 3D-Matrigel basement membrane and incubated under hypoxic conditions formed 3D capillary-like structure (103). This seemed to be regulated by paracrine and autocrine actions. b-FGF is the most potent stimulator of SC proliferation, migration and tubulogenesis (104).

Stromal-vascular fraction (SVF) is a heterogeneous cell population surrounding adipocytes in fat tissue, including mature microvascular endothelial cells. SVF cells cultured on Matrigel differentiate into adipocytes in adipogenic medium (105-106). Planat-Benard *et al.* cultured these cells on methylcellulose, which reveals the endothelial potential of progenitors, to test their endothelial differentiation capacity *in vitro* (106). A network of branched tube-like structures was formed. These structures were also found *in vivo,* when cells mixed with Matrigel were injected into mice. SVF may be progenitors of both adipocytes and endothelia (106).

Qian *et al.* showed that high density coating (50 microg/cm^2) of Matrigel provides a favorable substrate for the adhesion and proliferation of human SC, and differentiation to form neurons when cultured in neuron-induction medium (49).

4.1.1.3.3. Collagen

Dorotka *et al.* used a new multi-layer matrix of type-I, type-II and type-III collagens (107). They found that ovine BMSC became rounded up and synthesized type-I collagen, but not type-II collagen. These BMSC on the collagen matrix had increased glycosaminoglycan production. Sakai *et al.* evaluated the potential of autologous SC embedded in type-II collagen-based Atelocollagen® gel for treating intervetebral disc degeneration (108). The Atelocollagen® gel was a good carrier of SC that fostered the proliferation and differentiation of SC, plus matrix synthesis, on intervetebral disc.

Collagen is often used to coat scaffolds like PLGA, Ti and HA. A hybrid scaffold has been developped

in which collagen microsponges are formed within the knitted mesh of PLGA (109). Human SC adhered to and were uniformly distributed within the hybrid system. SC increased their production of type-II collagen and aggrecan mRNA when placed in a chondrogenic induction medium, but produced less type-I collagen mRNA. The results were confirmed by immunohistochemical staining. The authors suggest that the hybrid mesh stimulates the chondrogenic differentiation of SC because of the huge surface areas of the microsponges in the PLGA mesh openings. This structure facilitates cell adhesion and cell-cell contacts: cells formed aggregates. This hybrid system provided an environment similar to that of a micromass pellet system culture.

Type-I collagen adsorbed on microporous HA promotes attachment, spreading, and alkaline phosphatase activity of human BMSC (110).

4.1.1.3.4. Silk

Silks isolated from silkworms or spiders have important mechanical properties, and provide a remarkable combination of strength and toughness. They are a promising alternative biomaterial for use as matrices in tissue engineering (111).

Altman *et al.* prepared a silk fibroin cord and used it as a matrix; it retained its mechanical tensile strength for 21 days in tissue culture (112). Human BMSC adhered to it, spread and grew to form sheets of cells that synthesized ECM. The SC expressed ligament-specific markers (collagens I and III, tenascin-C). Silk fibroin is thus non-antigenic, biocompatible and capable of supporting stromal cell adhesion, growth, and differentiation when it is correcly extracted and purified. These authors also coupled RGD peptides to the silk matrix to enhance initial cell adhesion, increase cell density and ECM production (113). Hence, silk matrix to which RGD peptides are coupled significantly enhances stromal cell adhesion and collagen I production; the bone sialoprotein osteopontin and BMP-2 were synthesized (114).

Leastly, Karageorgiou *et al.* showed that BMP-2 immobilized on silk fibroin films stimulated osteogenic SC differentiation. The delivery of BMP-2 immobilized on the silk was more efficient than soluble BMP-2 (115).

4.1.1.3.5. Alginate

Alginate is a polysaccharide isolated from seaweed. It is a family of copolymers of D-mannuronate and L-guluronate, and forms gels in the presence of divalent ions such like Ca^{2+}.

Alginate beads have been successfully used to induce the chondrogenic differentiation of SC (116-117). Cells encapsulated in alginate beads and cultured in serum-free medium with TGF-beta1, Dex and aP, or with TGF-beta3, aggregated and formed cartilage. Yang *et al.* showed recently that an alginate bead culture system is more efficient and appropriate for differentiating human SC into chondrocytes than is the pellet culture system (118).

Natural molecules are often used in association with synthetic scaffolds to increase specific cell adhesion. For example, a PLLA/alginate amalgam plus TGF-beta causes SC to differentiate to form chondrocytes (119). Alginate optimizes the cell shape and confines growth factors and cells within the scaffold, while PLLA provides mechanical support.

4.1.1.3.6. Chitosan

The polysaccharide chitin is the most abundant biopolymer in nature after cellulose. It is the principal component of the exoskeletons of crustaceans and insects, as well as of the cell walls of some bacteria and fungi. Chitosan is a partially deacetylated polymer obtained from chitin that is non-toxic, biocompatible and biodegradable (120). It can thus be used as a biomaterial in tissue engineering (121).

Human SC cultured in a chitosan-poly(N-isopropylacrylamide) copolymer differentiate to form chondrocytes, as assessed by their aggrecan and collagen II production (122). The viability of SC grown on this copolymer is similar to that of SC on alginate, as is their capacity to differentiate to form chondrocytes, Chitosan-poly(N-isopropylacrylamide) is a stable gel *in vivo* that does not require Ca^{2+} ions, as does the alginate bead system.

Chitin has also been used with HA and Ti-calcium phosphate to form new bone (93, 123).

4.1.2. Synthetic materials

Poly(glycolic) acid (PGA), poly(L-lactic) acid (PLLA), and copolymers of poly(lactic-co-glycolic) acid (PLGA) are widely used in tissue engineering, and have been approved by the US Food and Drug Administration for clinical use (124).

Gugala *et al.* found that rat SC (isolated from bone marrow) spread uniformly on the surface of PLLA membranes, grew deep into the pores of porous membranes, and then formed a three-dimensional fibrillar network (125). These cells underwent osteogenic differentiation, as assessed by their alkaline phosphatase activity and mineral nodule formation, and produced an abundant extracellular matrix, as *in vivo*. Both non-porous and porous PLLA membranes supported the growth and osteoblastic differentiation of rat SC *in vitro*, but cells were more active and proliferated better on porous membranes than on the non-porous membranes.

Several groups have used BMSC seeded on PLGA scaffolds. Ouyang *et al.* used a knitted PLGA scaffold loaded with rabbit BMSC which they implanted in rabbits with defective Achilles tendons (44). The rabbits implanted with SC plus scaffold formed and remodelled tissue faster than rabbits with scaffold alone, and the resulting histology was similar to that of native tendon tissue. The regenerated tendons were composed of collagen I and III as is native tendon. Thus, a knitted PLGA scaffold loaded with allogeneic BMSC can be used to regenerate and repair gaps defect in Achilles tendons, and effectively restore their structure and function.

SC grown on PLGA scaffold have also been used to engineer smooth muscle (SM) tissue (37). Dog BMSC were collected, cultured, and had a SM cell-like phenotype

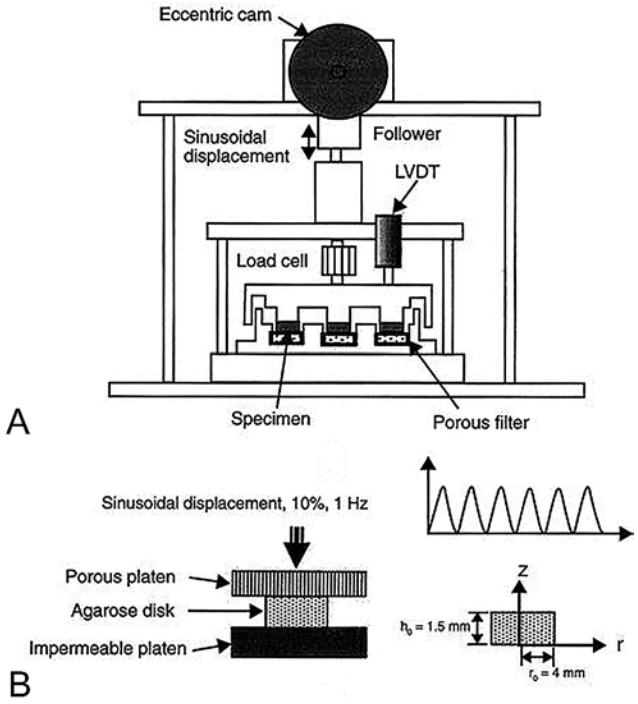

Figure 3. Bioreactor used to stimulate SC to differentiate into chondrocytes (45). (A): Bioreactor developed to mechanically stimulate agarose specimens under dynamic, unconfined compression. (B): Cells were caste in agarose disks, and the constructs placed in the bioreactor. A 10% magnitude sinusoidal compressive load was applied at a frequency of 1 Hz for 4 hours. Reproduced with permission from AlphaMedPress, Inc. (45).

and morphology. Immunohistochemical analyses revealed that these cells expressed SM cell-specific markers (SM alpha-actin and SM myosin heavy chain). SC on PLGA produced SM-like tissue when implanted in athymic mice (formed tissue expressed markers of SM cells). Thus, SC can be used to produce SM tissue and the porous PLGA scaffold enables SC to adhere and form three dimensional SM tissues.

PLGA has also been used to study the incorporation and release of molecules like aP and Dex that induce SC to differentiate into osteogenic cells (46). They found that aP and Dex loaded onto porous PLGA scaffolds were released over a period of one month and that SC cultured on these scaffolds actively deposited more calcium than did the control scaffolds.

Some studies have also been done on a diblock copolymer PLLA-poly(ethylene glycol) (PEG) (126). This copolymer enables cell adhesion and cell shape to be controlled. Marrow SC differentiated to an osteoblastic phenotype better on PEG-PLLA than on PLLA, PLGA, or tissue culture polystyrene, and had greater alkaline phosphatase activity and mineralisation.

4.2. Bioreactors used for stromal cell culture

Several bioreactors are used to culture SC seeded on synthetic or natural ECM. Some of these bioreactors are particularly useful for stimulating specific differentiation pathways.

One of the simpliest bioreactor is the spinner flask. In it, scaffolds are attached to a needle hanging from the lid of the flask. The scaffolds are immersed in medium and the whole stirred with a magnetic bar in the bottom of the flask. This bioreactor has been used for generating bone and cartilage (127-129).

Another bioreactor is the rotating wall vessel, originally designed by NASA to simulate the effects of microgravity. The reactor is composed of two concentric cylinders. Cells in scaffolds are seeded in the space between the two cylinders and perfused continuously with medium. Gas exchange occurs through the stationnary inner cylinder *via* a membrane, while the second cylinder rotates. Cell-scaffold constructs are maintained in a state of constant free-fall by adjusting the speed at which the outer cylinder rotates: centrifugal force then balances the force of gravity. Rotating wall vessel reactors have been used to induce bone tissue formation from SC and cartilage (128, 130). This system encourages cell agregation, which is a feature of chondrogenesis.

The third type of bioreactor is the flow perfusion bioreactor. Medium is pumped continuously through the internal porous network of scaffolds. Bancroft *et al.* designed a flow perfusion bioreactor for marrow stromal osteoblasts seeded on titanium fiber meshes (131). The cells produced a highly calcified matrix, had enhanced alkaline phosphatase activity, and secreted osteopontin even in the absence of Dex (132). This suggests that flow perfusion culture alone can stimulate the osteogenic differentiation of rat SC.

The mechanical environment can influence the differentiation of SC along the chondrogenic pathway. Huang *et al.* observed that SC subjected to cyclic compressive loading in a bioreactor expressed more

chondrogenic genes (collagen II, aggrecan) than did the control cells, suggesting that this mechanical stimulation promotes chondrogenesis of rabbit BMSC in the absence of cytokines (45). The authors developed a bioreactor to mechanically stimulate SC (Figure 3).

5. CONCLUSIONS

Stromal cells seeded on a range of 3D-scaffolds based on naturally occurring materials or synthetic polymers will adhere, proliferate and differentiate to form many tissues (bone, cartilage, fat, smooth muscle, *etc*.). The differentiation pathway is dependent upon the microenvironment afforded by the specific scaffold in which the cells are seeded. As stromal cells are readily isolated from adult bone marrow or UCB and can then be expanded, they represent a promising tool for tissue engineering.

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Abbreviations: aP: ascorbate-2-phosphate; BM: bone marrow; BMP2: bone morphogenetic protein 2; BMSC: bone marrow stromal cells; CFU-F: fibroblast colony forming unit; Cx: connexin; Dex: dexamethasone; ECM: extracellular matrix; GJ: gap-junction; HA: hydroxyapatite; HSC: hematopoietic stem cells; PLGA: poly(lactic-co-glycolic) acid; PLLA: poly(L-lactic) acid; RGD: Arg-Gly-Asp; SC: stromal cells; SM: smooth muscle; TCP: tricalcium phosphate; Ti: titanium; UCB: umbilical cord blood

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