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Development of human fetal mesenchymal stem cell mediated tissue engineering bone grafts

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1. Fracture treatment and bone tissue engineering

Although bone is a dynamic and well-vascularized tissue with innate healing and remodeling capacities (Salgado et al., 2004) , up to 10% of the bony fractures are complicated by non-union (Einhorn, 1995; Hayda et al., 1998; Marsh, 1998; Salgado et al., 2004; Bongso, 2005) , which require additional treatment with bone grafts in order to achieve defect union and healing. In fact, bone grafts has become the second most transplanted tissue in the world after blood, with approximately one million cases of bone graft transplantation occurring in United States alone annually (Salgado et al., 2004; Bongso, 2005).

Bone grafts can be categorized into three types: autografts, allografts, and synthetic grafts, with their relative frequency of use illustrated in Figure 1 and a comparison of their advantages and disadvantages in Table 1. *Autografts* are harvested from a secondary site from the patient's own body, commonly the iliac crest. This strategy has been described initially by Chutro and later Phemister in early 20th century (Connolly et al., 1991) , and has been considered the gold standard for many decades. This is because the use of autografts results in the the best clinical outcomes by providing both osteogenic cells and essential osteoinductive factors required for bone healing. However, the use of autografts has been limited by its availability, difficulty in fashioning of grafts to fit defects, an unpredictable efficacy and complexity of two surgeries (Banwart et al., 1995; Fowler et al., 1995; Goulet et al., 1997; Salgado et al., 2004; Hollinger et al., 2005). Futhermore, significant donor site morbidity such as chronic pain, hypersensitivity, infection and paraesthesia occur in up to a third of patients (Prolo and Rodrigo, 1985; Damien and Parsons, 1991; Arrington et al., 1996; Lane et al., 1999). ***Allografts***, bone grafts harvested from another donor (mainly from cadavers), are an alternative with enhanced flexibility of graft size and shapes. However,

they introduce the possibility of immune rejection and pathogen transmission. Moreover, processing techniques, such as demineralization, strip the tissue of osteoinductive factors necessary for stimulating bone repair, resulting in impeded healing times, as compared to autografts (Parikh, 2002) . ***Synthetic grafts*** are made from metals or ceramics, which can be fashioned to different shapes and sizes and are non-immunogenic, but thus far have been hampered by their poor speed of healing and inability to remodel in tandem with the natural healing process (Salgado et al., 2004).

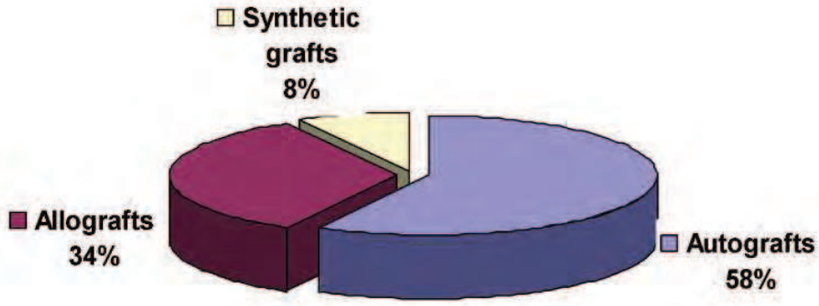


Fig. 1. Breakdown of the current used bone grafts: autograft, allograft and synthetic graft.

	Healing	Remodelling	Immuno-genicity	Availability	Surgery simplicity	Others
Autografts	+++	+++	-	+	+	donor-site morbidities
Allografts	++	++	+	+++	+++	disease transmission
Synthetic grafts	+	0	-	+++	+++	fatigue, fracture, wear
Tissue engineered grafts	+++	+++	-	+++	+++	

Table 1. Comparison of different bone grafts

Thus, in order to fulfill this increasing but unmet clinical need for effective bone grafts, recent research efforts have turned to a tissue engineering approach to develop tissue engineered grafts, which not only have off-the-shelf availability in various shapes and sizes (like allografts and synthetic grafts), but have the ability to stimulate rapid bone healing and undergo remodeling, achieving a better or similar clinical outcome than or as autografts (Table 1).

2. Cell based strategy vs. Growth factor based strategy

Tissue Engineering was first coined by Langer and Vacanti in 1993 as "an interdisciplinary field of research that applies the principles of engineering and the life sciences towards the development of biological substitutes that restore, maintain, or improve tissue function"(Langer and Vacanti, 1993). Since then, several different bone tissue engineering (BTE) approaches have been proposed and explored. In essence, they can be classified into

two main categories of a cell or a growth factor based strategy. Both approaches seek to create an optimized bone growing bed with a biological microenvironment suitable for bone regeneration through introducing a critical mass of osteogenic cells onto the scaffold matrix and modulating intercellular communication and cell-matrix interactions. However, the manner in which the introduction of osteogenic cells is different. Cell-based approaches supply exogenous osteogenic cells while relying on the autologous growth factors from the repair site to stimulate bone regeneration, whereas growth factor-based approaches rely on the delivery of growth factors to recruit osteogenic cells from the local environment into the repair site. (Kimelman et al., 2007; Lanza et al., 2007).

The use of growth factor-based BTE approaches has been shown to be efficacious in small animal models. However, the translation from small animal models, through large animal models, to humans has proven to be difficult and the optimal dose of growth factors for human clinical therapy is difficult to determine, because of the large variation of efficacy between small to big animals (up to 100 fold). Another problem relates to the problem of develping an effective delivery and release approach, given the short half-life of the majority of the growth factors are (Hollinger et al., 1996; Bruder and Fox, 1999). In addition, growth factor-based approaches work indirectly by attracting and stimulating the proliferation and differentiation of local osteogenic stem cells to repair bone defect, therefore its effectiveness will be greatly compromised in the absence of sufficient osteogenic stem cell pool (Service, 2000). In contrast, a cell-based approach works independently of the presence of local osteogenic cells, and therefore is an attractive therapy for patients with a diminished pool of osteogenic progenitors such as those with severe trauma, diabetes, a history of tobacco use, irradiation, aging, osteoporosis or other metabolic derangements (Bruder and Fox, 1999). Furthermore, delivering the right quantity of different growth factors in a sequential manner reflecting the different phases of bone growth faces multiple technical challenges (Marsh and Li, 1999; Giannoudis et al., 2007). In contrast, cell-based strategies utilize osteogenic cells which secrete a wide spectrum of growth factors at physiological doses, establishing temporal-spatial microgradients necessary for effective bone regeneration (Rouwkema et al., 2008). Thus, cell based strategies have been pursued in our group.

Two distinct typical cell-based approaches have been proposed to use cultured osteogenic cells in combination with scaffold matrix to engineer bone grafts (Hollinger et al., 2005; Lieberman and Friedlaender, 2005; Lanza et al., 2007). The first approach is to seed cells on the scaffold matrix and the immediate implantation of the resulting construct (Figure 2 A). In the second approach, a period of in vitro culture is performed for cellular expansion and osteogenic pre-differentiation (Figure 2 B), which has been shown to result in significantly higher degree of bone defect healing compared to an immediate implantation approach (Mendes et al., 2002; Sikavitsas et al., 2003; De et al., 2006; Dudas et al., 2006) . In this delay implantation approach, osteogenic cells are seeded into three dimensional highly porous biodegradable scaffolds with the desired shape to fit the bone defect. These cellular scaffolds are then cultured and matured in a suitable *in vitro* environment for cellular expansion and proper pre-differentiation to obtain bone-like constructs with extracellular mineralized matrix and pre-differentiated cells. Finally, the grafts are implanted into the defect to induce and direct the growth of new bone with the controlled degradation of the scaffold matrix (Figure 2B).

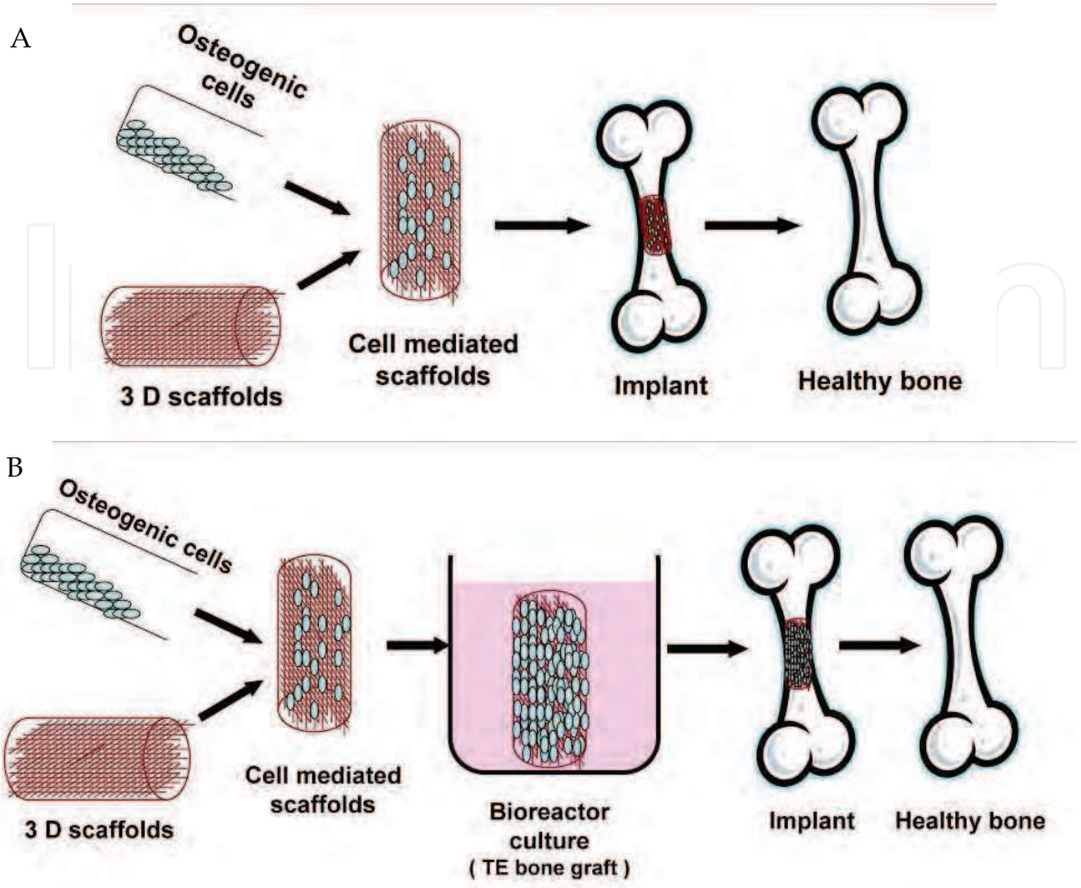


Fig. 2. Two cell based BTE approaches.

3. Polycaprolactone based scaffolds for BTE application

To generate an effective TE bone grafts, three dimensional (3D) scaffolds are required to function as a supportive matrix for cell proliferation, extracellular matrix deposition and consequent bone in-growth and at the same time define the overall shape of the tissue engineered transplant (Laurencin et al., 1999). Moreover, the 3D scaffolds should have rigid mechanical properties to protect the defect area from collapse of surrounding tissue, and proper macroporous structure to prevent the invasion of fibrous tissue but allow the infiltration of blood vessels. Scaffolds should also actively contribute to the regenerative process by enhancing the proliferation and osteogenic differentiation of the cells grew on their surface through the cell-scaffold interaction (Agrawal and Ray, 2001).

Our group has extensively investigated the use of Fused Deposition Modeling (FDM) technique to fabricate the porous three dimensional (3D) scaffolds for BTE applicaiton (Hutmacher et al., 2001; Zein et al., 2002). A polycaprolactone and tricalcium phosphate composite material was selected for the scaffold fabrication. Poly (ε-caprolactone) (PCL) is a semicrystalline, bioresorbable polymer belonging to the aliphatic polyester family. It is highly processable, with low glass-transition temperature (–60˚C) and melting point (60˚C) but high decomposition temperature of 350˚C. It has been previously exploited for drug delivery material (Coombes et al., 2004) and demonstrated great biocompatibility with US

Food and Drug Administration (FDA) approval for its use in several medical and drug delivery devices. It has slow degradation kinetics of only seven percent over a six month in vivo (Lam et al., 2008) , and thus is suitable for utilisation in load bearing bone grafting applications, which requires a prolonged period of mechanical support. In addition, chemical hydrolysis as opposed to enzymatic reactions is responsible for degradation of polymeric chains *in vivo*; thus degradation does not vary from patient to patient.

However, PCL is not without drawbacks, as with all synthetic polymers, PCL implants experience fibrosis and gradually become isolated from the surrounding bone. Consequently, they do not adhere to bone and this has been a critical problem in their use in bone repair (Kokubu et al., 2003). In addition, a common problem to biodegradable polyesters family is that the bulk release of acidic degradation products can cause acidosis (Ciapetti et al., 2003). This led to the search for additional compounds to modify PCL for BTE applications.

β-tricalcium phosphate (TCP), a synthetic ceramic material, is one such compound. Firstly, TCP is bioactive and can generate a carbonated hydroxyapatite layer on its surface that is equivalent chemically and structurally to the biological mineral of nature bone, known as the determining step for the biointegration (Kokubo et al., 2003). Secondly, TCP is osteoconductive and can promote the cellular adhesion, function and expression leading to formation of new bone and a uniquely strong bone-TCP biomaterial interface. Finally, TCP is reported to be osteoinductive, and capable of binding and concentrating endogenous bone morphogenetic proteins from the circulation (LeGeros, 2002). However, pure TCP is a brittle material with very poor mechanical properties, leading to difficulties with its processing for BTE applications. To overcome these shortcomings, a bioactive composite material comprising of a biodegradable polymeric phase (PCL) and a bioactive inorganic phase (TCP) has been developed. This construct takes advantage of the flexibility and processability of the bulk PCL polymer phase, combined with TCP bioactive phase, allowing the scaffold to bond and integrate with bone spontaneously and possess both osteoconductive and osteoinductive properties. Furthermore, the acidic release from the polymer can be compensated by the alkaline calcium phosphate (Blaker et al., 2003; Maquet et al., 2004) . (Figure 3)

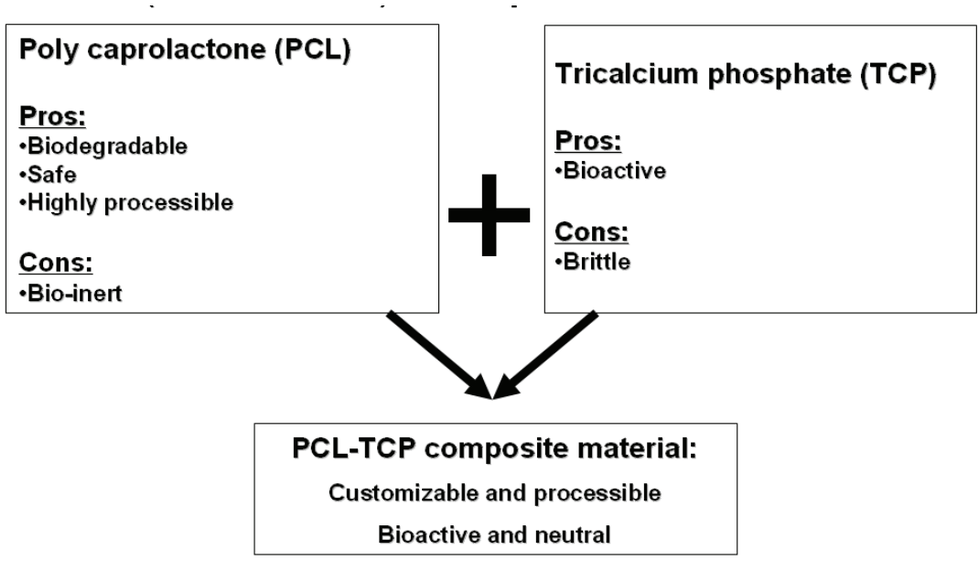


Fig. 3. PCL-TCP composite material, incorporates the strength of PCL and TCP while neutralizes their individual drawbacks

FDM, as a solvent-free rapid prototyping technique, was utilized to fabricate the PCL-TCP composite material into 3D scaffolds (Hutmacher et al., 2001; Zein et al., 2002) in three steps. Firstly, the PCL-TCP filament were fabricated by physically blending the PCL pellets with TCP granules in a desired ratio (e.g. 80% PCL to 20% TCP), melting in a high temperature (190˚C) and extruding through spinnerets with a die exit diameter of 1.63 mm. Secondly, the size and shape of the scaffolds were designed through computer assisted design (CAD) systems, for example, patient-specific scaffolds can be created based on the 3D computed tomography(CT) images, then these CAD models were imported into the Stratasys QuickSlice software and sliced into the horizontal layers. The deposition path were created and downloaded to the FDM machine, the 3D Modeler RP system from Stratasys Inc. (Eden Prairie, MN). Lastly, PCL-TCP filament prepared were fed into the FDM head, melted down in the liquefier and extrude out through the nozzle tip and deposit on a platform layer by layer according to the preprogrammed deposition path.

We have fabricated PCL-TCP scaffolds to achieve a honeycomb architecture, with a fully interconnected matrix, and mechanical properties close to cancellous bone. This should allow rapid vascularization to occur and maintain the defect space and the structural integrity of tissue engineered bone grafts in load-bearing applications (Hutmacher et al., 2001; Schantz et al., 2002) (Figure 4). In addition, use of the highly reproducible and computer-controlled FDM technique allows the fabrication of tissue-engineered grafts that have been designed on the basis of computed tomography (CT) scans of individual defect sites(Hutmacher, 2000; Endres et al., 2003) . PCL-TCP scaffolds have a favourable profile for achieving effective cellular adhesion, proliferation and differentiation in vitro (Rai et al., 2004; Zhang et al., 2009; Zhang et al., 2009) , and serve as an osteoconductive matrix for new bone regeneration in both critical-sized rat femoral (Rai et al., 2007) and canine mandibular defects (Rai et al., 2007).

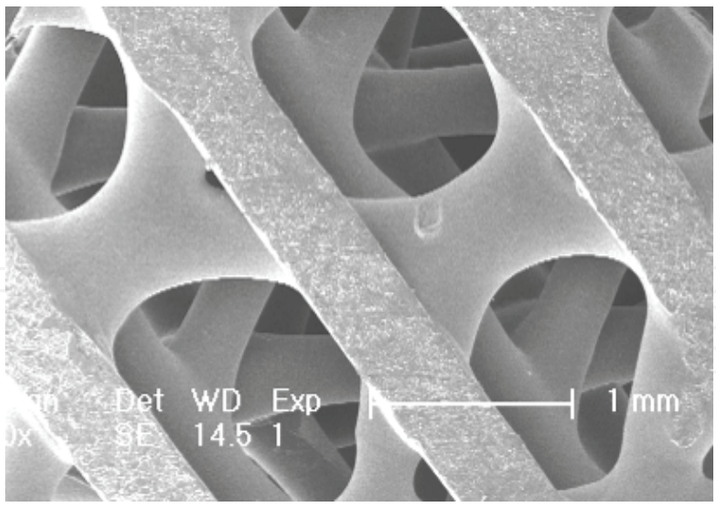


Fig. 4. Scanning Electronic Microscopy (SEM) image of PCL-TCP scaffolds

4. Human fetal mesenchymal stem cells for BTE application

4.1 Available cellular sources for cell based BTE

Cells are the central players for most biological process in human body. As the critical component in the BTE strategy, they play the most essential role and are directly involved in bone regeneration. The selection of cell sources will eventually determine the success of any BTE strategy. An ideal cellular source for cell-based BTE approach should include the following characteristics: (1) no immunogenicity, (2) no tumorigenicity, (3) immediate availability, (4) availability in pertinent quantities, (5) rapid cell proliferation rate, (6) predictable and consistent osteogenic potential as well as; (7) controlled integration into the surrounding tissues (Logeart-Avramoglou et al., 2005) . Different cell sources have been exploited for BTE applications, and can be divided into three categories with the respect to their differentiation status: fresh bone marrow, differentiated osteoblasts and mesenchymal stem cells (Bruder and Fox, 1999; Pioletti et al., 2006) (Table 2).

Fresh bone marrow (BM) has been introduced to heal nonunion bone defects some three decades ago (Connolly et al., 1989; Connolly et al., 1991; Caplan, 2005). BM are readily harvested and contain the osteogenic progenitors, which can enhance bone regeneration. As they are autologous tissues, immunorejection is not an issue, nor are they subject to the regulation by the Food and Drug Administration (FDA) (Bruder and Fox, 1999) . However, their effectiveness is highly dependent on the number of the osteogenic progenitors, which represent approximately 0.001% of the nucleated cell in the healthy bone marrow at best (Bruder et al., 1997) and decreases dramatically with aging or disease process (Pioletti et al., 2006). Thus, this approach may be least applicable in those situations where it is most needed, as fractures tend to occur more frequently in an old age group. Moreover, the use of unmatched allogenic BM is not suitable, as it is beset by problems of either graft rejection or graft-versus-host syndrome (Weissman, 2000).

Osteoblasts participate in bone formation processes via the synthesis and secretion of collagen fibres to form unmineralized bone matrix (osteoid), and have been implicated in osteoid calcification. The use of osteoblasts for the cellular source for BTE applications has demonstrated enhancement in the rate and extent of bone regeneration compared with the

use of undifferentiated bone marrow (Okumura et al., 1997; Bruder and Fox, 1999; Montjovent et al., 2004). However, autologous isolation of the osteoblasts is considerably more difficult and complex than fresh bone marrow or mesenchymal stem cells. In addition, they posses a limited capacity for proliferation, and thus do not support in the generation of significant cell numbers for clinical use (Bruder and Fox, 1999; Montjovent et al., 2004; Pioletti et al., 2006).

Mesenchymal stem cells (MSC) are also known as marrow stromal cell or colony forming unit – fibroblast (CFU-F) or more recently named as multipotent mesenchymal stromal cells by International Society for Cellular Therapy (ISCT) (Dominici et al., 2006) . They were first identified and isolated from adult bone marrow (Friedenstein et al., 1966; Friedenstein et al., 1968) and demonstrated a series of favorable advantages for BTE application over other cell sources. Firstly, MSC are easily isolated through plastic adhesion method or simple antibody selection techniques (Simmons and Torok-Storb, 1991) . Secondly, the osteogenic differentiation pathway is well defined and MSC have been shown to generate greater amount of bone tissue than fresh bone marrow in preclinical studies (Kahn et al., 1995; Inoue et al., 1997; Pioletti et al., 2006). Thirdly, both undifferentiated and differentiated MSC have been reported as non-immunogenic, and are thus suitable for allogeneic applications (Le Blanc et al., 2003) . Finaly, cryostorage does not affect the osteogenic potential of MSC, which greatly facilitates their storage (Bruder et al., 1997).

	Collection	Expansion	Storage	Allogenic applications	Osteogenic potential
Fresh bone marrow	+++	N.A.	N.A.	N.A.*	+
Osteoblast	+	+	+	N.A.*	+++
MSC	++	++	+++	++	++

N.A.*: allogenic applications can be achieved by proper HLA matching

Table 2. Comparison of different cellular source for BTE.

4.2 MSC biology

Although there are no specific cell markers to distinguish MSC from other cell types, MSC are generally defined by their capabilities to (1) adhere to the plastic surface of tissue culture flask; (2) express D105, CD73 and CD90, with absence of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA II; and (3) differentiate into the osteoblasts, adipocytes and chondroblasts under standard *in vitro* differentiating conditions (Dominici et al., 2006). MSC are rare cells, and exist at low frequencies in human adult BM, accounting for 0.00001% to 0.001% of mononuclear cells (MNC) in BM. They are non-haemopoietic and can be separated from the haemopoietic fraction in BM by their adherence to plastic and ability to grow from an initial heterogeneous population into a more homogenous spindle-shaped cell type. MSC have been demonstrated to contain a heterogeneous population of cells. Properties including colony size, proliferation rate, cellular morphology, and multipotency both *in vitro* and *in vivo* have been routinely observed to differ in single colony-derived MSC strains (Muraglia et al., 2000; Mauney et al., 2005) . Other techniques for isolation of MSC include immuno-depletion (Baddoo et al., 2003) or immuno-selection techniques with STRO-1 and CD 271 antibodies (Simmons and Torok-Storb, 1991; Jones and McGonagle, 2008). However, the resulting cultures are still heterogeneous, ranging from spindle shaped cells to broad stromal-like cells (Digirolamo et al., 1999; Colter et al., 2000; Colter et al., 2001)

This is also reflected in intra-sample and inter-species differences in the capacity of MSC to self-renew or form CFU-F in low-density cultures (Digirolamo et al., 1999; Phinney et al., 1999; Banfi et al., 2000; Javazon et al., 2001).

MSC are not inherently immunogenic, making them highly attractive for allogeneic transplantation strategies (Caplan, 2007). MSC are HLA Class I positive and Class II negative and do not express co-stimulatory molecules CD40, CD40L, CD80, or CD86 (Klyushnenkova et al., 2005) . This phenotype is widely regarded as non-immunogenic and suggests that MSC may be effective at inducing tolerance. In the mixed lymphocyte cultures (MLC) assay, MSC do not elicit a proliferation response of allogeneic lymphocytes (Bartholomew et al., 2002; Le Blanc et al., 2003). After interferon γ (INF-γ) induction of HLA II expression, MSC are still capable of escaping recognition by alloreactive T-cells (Le Blanc et al., 2003; Klyushnenkova et al., 2005). Similarly, after cellular differentiation into along the adipogenic, chondrogenic and osteogenic lineages, they remain non-immunogenic (Le Blanc, 2003). MSC have been found to escape lysis by cytotoxic T-cells and alloreactive killer inhibitory receptor mismatched natural killer cells (Le Blanc, 2003; Rasmusson et al., 2003; Gotherstrom, 2007) , making them uniquely suited for allogeneic transplantation applications.

In addition to being non-immunogenic, MSC have been shown to possess immunomodulatory properties both *in vitro* and *in vivo*. *In vitro*, they exhibit immunosuppressive effects and inhibit T-cell alloreactivity (Pittenger et al., 1999; Bartholomew et al., 2002; Krampera et al., 2003; Maitra et al., 2004; Beyth et al., 2005). This suppression effect of MSC is dose dependent and not only influence T cell but also affect the differentiation and maturation of dendritic cells from monocytes (Aggarwal and Pittenger, 2005). *In vivo*, infusion of ex vivo-expanded MSC has been shown to prolong the survival of allogeneic major histocompatibility mismatched skin allografts in immunocompetent out-bred baboons (Bartholomew et al., 2002). Further evidence was provided by Djouad et al, who found that subcutaneously injected melanoma cells in allogeneic immunocompetent mice formed tumors only when co-injected with MSC, suggesting that the presence of MSC prevented immune rejection of the tumor cells (Djouad et al., 2003). More recently MSC have been used to treat graft-versus-host disease (GvHD) after allogeneic hematopoietic stem cell transplantation (Le Blanc et al., 2008). The mechanism governing those effects is not yet understood. However, it is quite likely that the paracrine effect of MSC will play a significant role in it, for example, it was shown that MSC may strongly inhibit T-cell recognition and expansion via inhibiting TNF-α and INF-γ production and, thus, increasing IL-10 levels. (Aggarwal and Pittenger, 2005; Beyth et al., 2005; Caplan, 2007)

MSC are currently defined by their trilineage differentiation capacity (osteogenic, adipogenic and chondrogenic differentiation). In addition, they have been shown to be able to differentiate to muscle, marrow stroma and other connective tissues (Caplan, 1991; Caplan, 2005; Caplan, 2007). Several reports have now expanded repertoire to neuroectodermal lineages such as neurons (Woodbury et al., 2000; Zhao et al., 2002) , hepatocyte (Ong et al., 2006; Banas et al., 2007; Sgodda et al., 2007) and endothelial cell (Oswald et al., 2004; Gang et al., 2006) . A subset of MSC, termed Multipotent adult progenitor cells (MAPC), have been isolated from a number of species. MAPCs have greater differentiation potential than MSC, and have been shown to be pluripotent (Reyes et al., 2001; Jiang et al., 2002). However they are difficult to isolate and have not been reliably reproduced by other laboratories.

The osteogenic differentiation pathway is the default pathway for MSC, which is retained regardless of cell passage number until senescence (Digirolamo et al., 1999). MSC are heterogenous in nature, and consist of subpopulations of cells with tri-potent (osteogenic, adipogenic and chondrogenic), bi-potent (osteogenic and chondrogenic) and uni-potent (osteogenic) differentiation capacities as demonstrated through clonal analysis (Muraglia et al., 2000). Clones have been found to progressively lose their adipogenic and chondrogenic differentiation potential at increasing cell doublings, although osteogenic differentiation ability is retained till senescence (Digirolamo et al., 1999; Muraglia et al., 2000) .

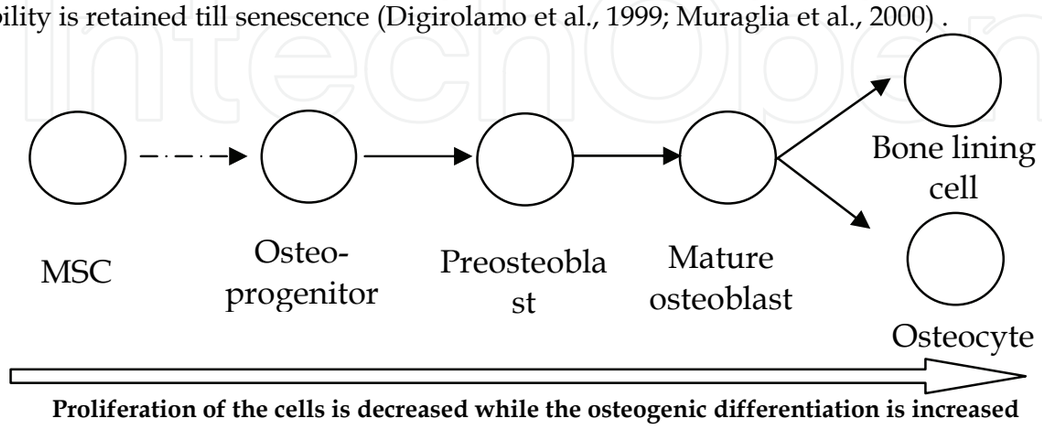


Fig. 5. Osteogenic differentiation pathway of MSC

Under the osteogenic differentiation pathway, MSC can be fully differentiated into osteoblasts, bone lining cells and osteocytes, which is a highly regulated and multi-step process with a sophisticated hierarchy as illustrated in Figure 5. MSC is likely to occupy the top position in this hierarchy, followed by osteoprogenitors, which are osteogenic committed progenitor cells with limited self-renewal and restricted differentiation capacity, but still maintaining substantial proliferation capacity. Further downstream are the preosteoblasts with more limited proliferation capacity. Mature osteoblasts are the terminally differentiated and functional cells, which will finally become the bone lining cells or osteocytes. With the progress of the osteogenic differentiation, proliferation of the cells is decreased while the osteogenic differentiation is increased. (Aubin, 1998; Aubin, 2001; Bilezikian, 2002)

4.3 Limitations of adult BM derived MSC and alternative MSC sources

The adult BM is the first tissue source where MSC are found and isolated, and are thus most investigated. They have been exploited as an autologous cell source for BTE applications, and demonstrated their effectiveness for fracture healing not only in animal but also in a number of reported human clinical trials (Quarto et al., 2001; Schimming and Schmelzeisen, 2004; Ueda et al., 2008; Yamada et al., 2008) . However, several barriers currently exist, limiting their further clinical applications. Firstly, adult BM-MSC are rare cells with a very low frequency, accounting for 0.00001% to 0.001% of mononuclear cells (MNC) in the adult bone marrow (BM). Furthermore, they have limited proliferative capacity, only undergoing an average approximately 38 ± 4 population doublings before reaching cellular senescence (Bruder et al., 1997). As a result, in order to obtain clinically significant cell numbers, a large amount of bone marrow aspiration is required initially. Secondly, for autologous usage, the

derivation and expansion of MSC require a long period (4-6 weeks) for *ex vivo* expansion, limiting their clinical use, especially where the cells are required acutely. Secondly, the frequency of MSC within bone marrow and the proliferative capacity decrease dramatically as a function of donor age (it can drop from 0.001-0.0004% before the ages of 30 to 0.00025-0.00005% after the ages of 50) (Caplan, 2007) , with systemic diseases adversely affecting their numbers and function (Suzuki et al., 2001) . Similarly, the osteogenic differentiation ability *in vitro* and *in vivo* has also been found to be inversely correlated with donor age (Mueller and Glowacki, 2001) and reduced in several disease phenotypes (Rodriguez et al., 2000).

More recently, MSC with osteogenic potential have been isolated from a diverse range of other tissue types and ontogeny: (1) **In postnatal tissue**, MSC have been isolated from tissues as diverse as periosteum(Nakahara et al., 1991) , trabecular bone (Sakaguchi et al., 2004) , synovial membrane (De et al., 2001) , adipose tissue (Zuk et al., 2001) and peripheral blood (Eghbali-Fatourechi et al., 2005). (2) **In perinatal tissues,** MSC have been found in the umbilical cord (Sarugaser et al., 2005) , umbilical cord blood (Bieback et al., 2004; Lee et al., 2004) , amniotic fluid (in't Anker, P; Scherjon, S A; Kleijburg-van, Keur C; Noort, W A; Claas, F H; Willemze, R; Fibbe, W E; Kanhai, 2003; De et al., 2007) . (3) **In the prenatal tissue**, human fetal MSC have been isolated and characterized from first-trimester fetal blood, liver, and bone marrow (Campagnoli et al., 2001) , second trimester bone marrow, spleen and lung (in't Anker P ; Noort, W A; Scherjon, S A; Kleijburg-van, Keur C; Kruisselbrink, A B; van, Bezooijen R; Beekhuizen, W; Willemze, R; Kanhai, H H; Fibbe, 2003) , fetal metanephros (Almeida-Porada et al., 2002) , dermis (Zhao et al., 2005) , pancreas (Hu et al., 2003) and thymus (Rzhaninova et al., 2005) . While investigations into their basic biology, immunogenicity and osteogenic potential have been reported, MSC have not been systematically compared for bone tissue engineering applications. Hence, it remains unclear how these novel prenatal, perinatal and postnatal MSC sources compare with their standard adult BM MSC counterparts for osteogenic differentiation and potential for tissue engineering.

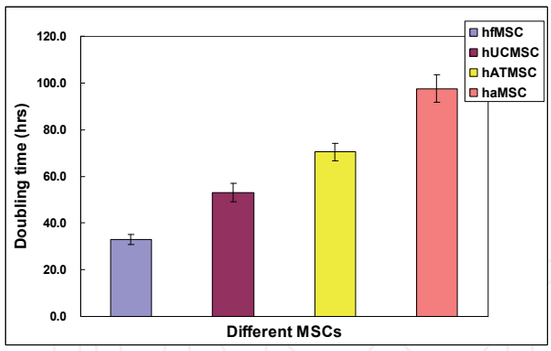
4.4 Human fetal BM derived MSC (hfMSC) as a promising cellular source for BTE

Recently, we reported a systematic investigation into four types of MSC from different ontological and anatomical origins in a direct head-to-head manner for BTE applications (Zhang et al., 2009). In this study, MSC were isolated from prenatal (human fetal bone marrow (hfMSC)), perinatal (human umbilical cord (hUCMSC)) and postnatal sources (human adult adipose tissue (hATMSC) and human adult bone marrow (haMSC)). In vitro comparative studies were performed in monolayer cultures and three dimensional bioactive scaffold cultures to investigate their proliferation capacity, osteogenic differentiation and mineralization, and in vivo ectopic bone formation.

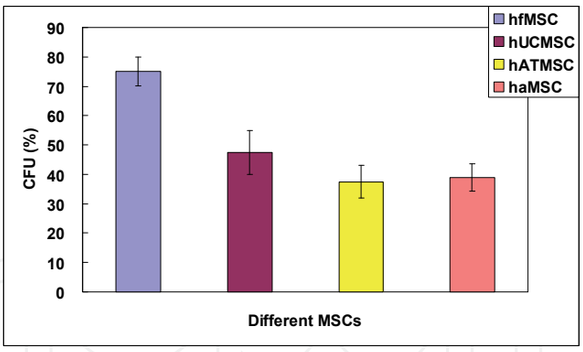
hfMSC, haMSC, hUCMSC and hATMSC adopted a similar spindle-shaped morphology when cultured in monolayers, and expressed a consistent MSC immunophenotype which was negative for haemopoietic (CD14, CD34, CD45) and endothelial markers (CD31, and vWF), and positive for mesenchymal markers (CD105 (SH2), CD73 (SH3, SH4)), intracellular markers (Vimentin and Laminin) and cell adhesion molecules (CD29, CD44, CD 106, CD 90). All the four MSC types expressed HLA-I but not HLA-II and possessed the typical trilinage differentition capacities. However, compared with other MSC, hfMSC expressed a lower

level of HLA-I and a higher level of Stro-1 with the implication of lower immunogenicity and higher osteogenic capacity.

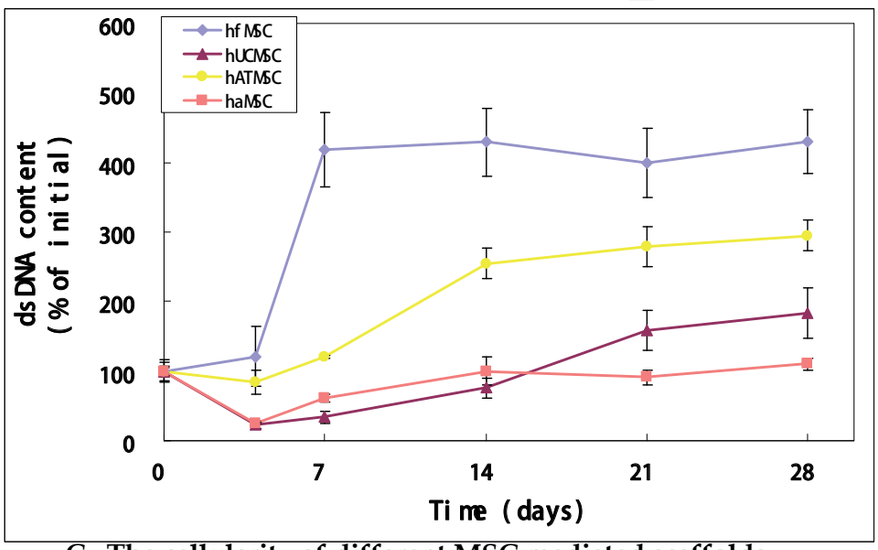
In two-dimensional (2D) monolayer cultures, hfMSC proliferated fastest (population doubling time 32.3±2.5 hours), followed by hUCMSC (54.7±4.3 hours), hATMSC (70.4±3.6 hours), with the slowest being haMSC (116.6±22.4 hours, n=3, $p<0.01$) (Figure 6A). In addition, hfMSC had significantly higher self-renewal ability, with 75.1± 5.0% of cells forming colonies, compared to 37.5-47.5% of other three MSC (Figure 6B). When cultured in 3D bioactive scaffold, hfMSC proliferated rapidly and reached confluence within the scaffold, taking up all available spaces by Day 7, while other MSC types achieved confluence only at the end of the 28 day experimental period as shown by a double-stranded DNA (dsDNA) quantification method (Figure 6C). Furthermore, hfMSC mediated scaffold constructs showed more robustly upregulated of key osteogenic genes, deposited significantly higher mineralizaton and demonstrated greater osteogenic capacity compared to other MSC scaffold constructs, as evaluated by a series of assay methods including von-Kossa staining, ALP activity, calcium deposition, calcium visualized on micro-CT and scanning electron-microscopy, and osteogenic gene expression (Figure 6D). Finally, subcutaneous implantation of these 3D constructs in immunodeficient NOD/SCID mice was performed to compare the osteogenic potential of MSC scaffolds in vivo. MSC scaffolds were cultured for two weeks in vitro as osteogenic pre-induction before implantation. Two months after implantation, all MSC scaffolds demonstrated neo-vascularization, with blood vessels infiltrating the scaffolds from the surrounding tissue macroscopically, and shared a similar chimerism rate of human cells in the murine tissue (60 to 67% chimerism) as demonstrated by human specific nuclear stain (lamins A/C), with infiltration of murine cells accounting for a third of the cellular population within the internal spaces of the scaffolds. Compared to other MSC scaffolds, hfMSC-mediated scaffolds generated more ectopic bone formation in vivo (1.8-13.3x, $p<0.01$) through micro-CT quantification (Zhang et al., 2009).



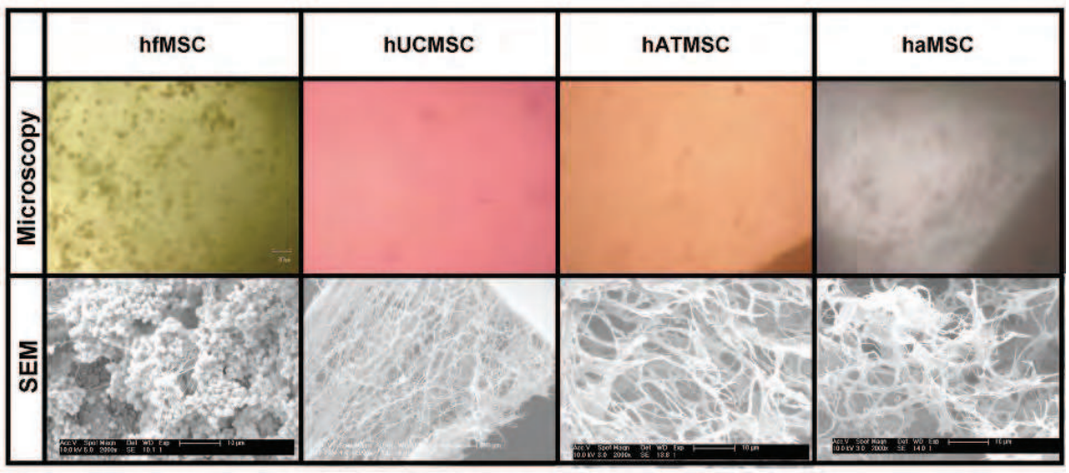
A. Doubling time of different MSCs



B. CFU of different MSCs



C. The cellularity of different MSC mediated scaffolds



D. Osteogenic differentiation of MSC mediated scaffolds

Fig. 6. Comparison of different MSC for BTE application. (A) hfMSC proliferated faster than other MSCs with the shortest doubling time, and had higher self-renewal capacity on CFU-F

assay (B-C) hfMSC expanded at a faster rate, reaching confluence by day 7 and have a higher final cellularity than other MSC mediated scaffolds. (D) hfMSC mediated scaffolds underwent more robust osteogenic differentiation and mineralization compared to others as seen through light microscopy and scanning electron microscopy (SEM).

Through this study, it was found that the ontological and anatomical origins of MSC have profound influences on the proliferative and osteogenic capacity of MSC. MSC from the ontologically more primitive source have higher proliferative capacities, while MSC from BM related origin showed more osteogenic potential than MSC from other origins. In summary, hfMSC had the most proliferative and osteogenic capacity of MSC sources, as well as being the least immunogenic, suggesting they are superior candidates for BTE applications.

4.5 hfMSC as an off-the-shelf cellular source for BTE application

hfMSC have different immunologic properties to haMSC, as illustrated in Table 3. hfMSC not only express significantly lower levels of HLA Class I than haMSC, they do express any intracellular HLA Class II, which are found haMSC. hfMSC require 7 days of interferon-gamma for the induction of HLA II expression, compared to 2 days for haMSC (Gotherstrom et al., 2003; Le Blanc, 2003) , alluding to their utility for allogeneic transplantation. In fact, hfMSC have been exploited for intrauterine cell therapy (Chan et al., 2007; Guillot et al., 2008; Kennea et al., 2009) , with the first successful clinical applications of hfMSC has been reported through allogeneic hfMSC transplantation in a fetus with osteogenesis imperfecta (Le Blanc et al., 2005).

Immunologic properties	Fetal MSC	Adult MSC
HLA Class I expression	0- (+ +)*	+ + +
Intracellular deposits of HLA Class II	0	+ + +
Induction time to epxress HLA Class II	+ + (7 days)	+ + + (2 days)

"+" means positive, and "0" means no expression. *: no expression or lower

Table 3. Immunologic properties of fetal and adult MSC

Aside from their lower immunogenicity, faster proliferation rate and more robust osteogenic potential, hfMSC possess other favorable advantages over other cellular source, making them become an ideal off-the-shelf cellular candidate for BTE application (Table 4). Firstly, fetal tissues have a lower risk of viral and bacterial contamination compared to adult tissues as the fetus resides in an immunoprivileged environment within the uterus. Secondly, hfMSC have a higher proliferative capacity, and can undergo over 70 population doublings without senescence, compared to haMSC which typically senesces after 38 population doublings (Bruder et al., 1997; Campagnoli et al., 2001; Chan et al., 2005) . Theoretically, an unlimited number of cells can be derived from one hfMSC donor. With a proliferation capacity of more than 70 population doublings, theoretically one single cell isolated at Passage 0 can be proliferated to 1.2×10^{21} (2^{70}) cells at Passage 70 and can be utilised more than 10^{12} patients (utilizing a dose of 1×10^{9} cells). "Single-cell banks" can be established using cells derived from a single cell colony with well-investigated biological properties and

greatly minimized variation; moreover, all the cells can be frozen down and made available off-the-shelf, eliminating the precious waiting time for patients. Lastly, the ethical conundrum faced with cells derived from embryonic stem cells are less of a problem with the use of fetal stem cells, considering that various human fetal cell types have been explored for therapeutic applications in the clinic already, such as the use of fetal neural progenitors for the treatment of Huntington's (Rosser and Dunnett, 2003) or Parkinson's disease (Clarkson, 2001) , and the use of human fetal liver cells to treat severe immunodeficiencies, haematological disorders and inborn errors of metabolism (Touraine et al., 1993) . More recently human fetal skin cells have been utilized for constructing a tissue engineered skin graft (Hohlfeld et al., 2005; Pioletti et al., 2006).

	Collection	Expansion	Storage	Allogenic applications	Osteogenic potential
Fresh bone marrow	+++	N.A.	N.A.	N.A.*	+
Differentiated osteoblast	+	+	+	N.A.*	+++
MSC from adult BM	++	++	+++	++	++
MSC from fetal BM	++	+++	+++	+++	+++

N.A.*: allogenic applications can be achieved by proper HLA matching

Table 4. Comparison of hfMSC with other cellular source for BTE.

5. Biaxial rotating bioreactors for BTE application

Bioreactors are the biomechanical devices with closely monitored and tightly controlled environmental and operating conditions. Bioreactors have been widely used in industries for fermentation processing, wastewater treatment, food processing and production of pharmaceuticals. Recently bioreactors have been introduced to tissue engineering field in order to mimic the native *in vivo* environment and provide cellular tissue engineered constructs with physiologically relevant stimuli that facilitate and orchestrate the conversion of a "collection of cells" into a specific tissue phenotype (Lanza et al., 2007). Specifically, they can increase the mass transport to mitigate the diffusion limitation of 3D scaffolds, providing adequate nutrient, oxygen and regulatory molecules to the cells while removing metabolites and CO_2 away. Bioreactors can provide proper physiological stimuli, especially the mechanical cues to trigger the mechanotransduction signaling pathway for the differentiation of cellular constructs, and can be used to enable efficient and homogenous cellular seeding in the complex 3D scaffolds. (Martin et al., 2004; Bilodeau and Mantovani, 2006; Chen and Hu, 2006)

Currently, several types of bioreactors such as spinner flasks, perfusion bioreactors and rotating wall vessel (RWV) bioreactors have been investigated for BTE applications. However, various limitations have been reported with the use of these bioreactors. Spinner flasks can improve fluid flow, which can lead to the enhanced cell proliferation, distribution and osteogenic differentiation (Mygind et al., 2007; Song et al., 2008; Stiehler et al., 2008), but the turbulence generated by stirring can be detrimental for seeded cells and newly laid

down ECM (Sikavitsas et al., 2002; Chen and Hu, 2006). Perfusion bioreactors have been shown to enhance MSC proliferation and osteogenic differentiation in scaffold constructs (Sikavitsas et al., 2003; van et al., 2003; Gomes et al., 2006), however, they have been beset by non-homogenous cellular distributions, with cells at the frontal zones being washed away by the oncoming perfusion flow (Singh et al., 2007). Rotating wall vessel (RWV) bioreactors (Molnar et al., 1997; Granet et al., 1998; Yu et al., 2004), which generates low shear forces and three dimensional high mass transfer capacity, are prone to problems of non-homogenous cellular growth and ECM deposition (Goldstein et al., 2001; Sikavitsas et al., 2002; Chen and Hu, 2006). Moreover, the free floating culture of RWV bioreactors usually lead to the collision between the scaffolds and the bioreactor walls, which can induce cellular damage and disrupt cellular attachment and matrix deposition on the scaffolds (Goldstein et al., 2001; Sikavitsas et al., 2002; Chen and Hu, 2006).

Consequently, we designed and developed an biaxial rotating bioreactor (Figure 7) to address the deficiencies found in current bioreactor designs. Firstly, a perfusion system was included to allow the media circulation between the vessel and reservoir, allowing maximal mass transfer with consequential low shear stress, and minimizing the washout problem associated with RWV bioreactors. Secondly, the improvement of biaxial rotation movement upon uni-axial rotating, leads to more homogenous cellular and ECM distribution of the scaffold, as previously predicted from in-silico simulation (Singh et al., 2005). Lastly, cellular scaffolds were secured by pins and were not kept in free suspension, avoiding the risk of scaffold collisions with the vessel walls.

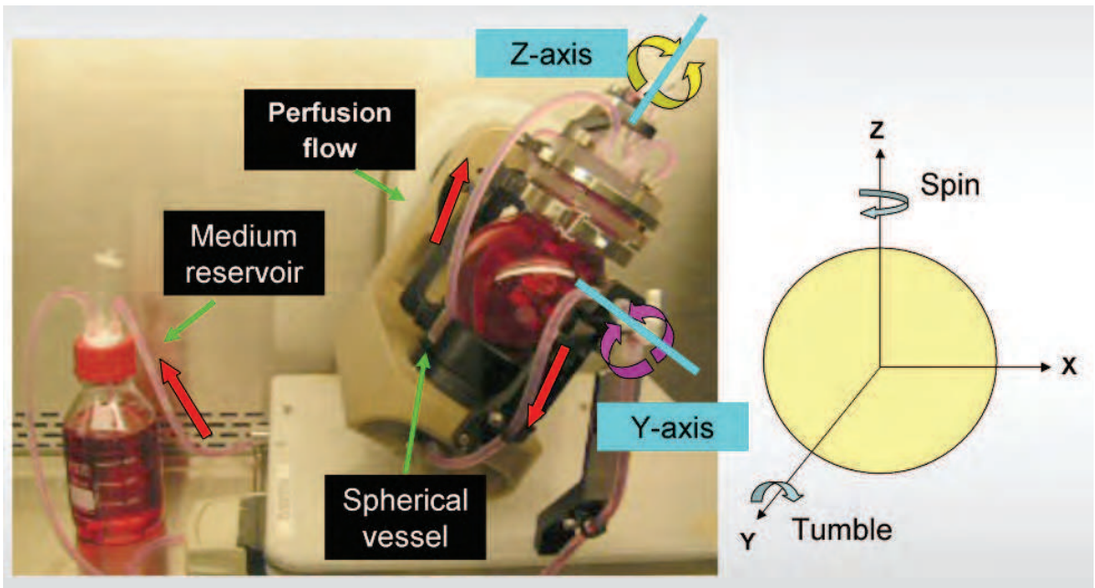
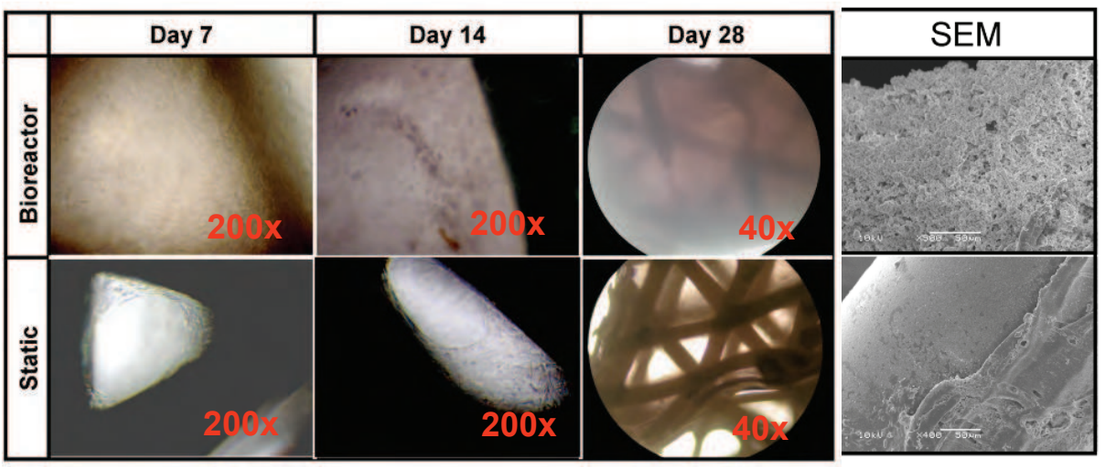


Fig. 7. The working mechanism of biaxial rotating bioreactor. This biaxial bioreactor can rotate in two perpendicular axes simultaneously with an inbuilt perfusion system.

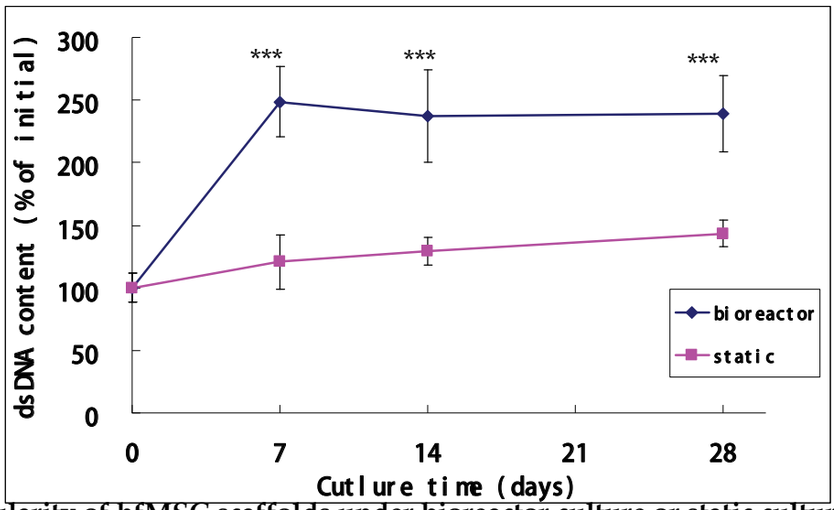
In a recent study, we investigated the performance of this biaxial rotating bioreactor for generation of highly osteogenic bone graft using hfMSC mediated polycaprolactone-tricalcium phosphate (PCL-TCP) scaffolds. hfMSC-scaffolds were cultured and matured in either bioreactor or static cultures, and evaluated for their cellular viability, proliferation

and osteogenic differentiation *in vitro*, and after transplantation into immunodeficient mice. Compared with traditional static culture, biaxial bioreactor culture enhanced cellular proliferation, with hfMSC-scaffolds reached cellular confluence earlier (Day 7 vs Day 28) with greater cellularity (2x, $p<0.01$) (Figure 8). Over 28 days of *in vitro* culture, the biaxial bioreactor enabled the maintenance of high cellular viability throughout the scaffolds, including the core, 2 mm from the surface of the scaffold. Thus the use of this bioreactor enabled a ten-fold improvement of mass transfer of nutrients, generally taken to be around 150-200 nm. In contrast, the static culture only allowed the cellular survival at a surface region of the thick scaffolds, with massive cell necrosis within the core of the scaffolds.

Biaxial bioreactor cultured-cellular scaffolds were associated with greater osteogenic induction as indicated by *in vitro* assays such as higher ALP expression (1.5x $p<0.01$), more calcium deposition (5.5x, $p<0.001$) and bony nodule formation observed under scanning electron microscopy (SEM) (Figure 8A), and the *in vivo* ectopic bone formation in immunodeficient mice (3.2x, $p<0.001$) compared with static-cultured scaffolds.



A. Prolfieration and differentiaiton of hfMSC scaffolds under bioreactor culture or static culture



B. Cellularity of hfMSC scaffolds under bioreactor culture or static culture

Fig. 8. Comparison of the biaxial bioreactor culture and static culture. (A) Biaxial bioreactor cultured hfMSC scaffolds proliferated faster and underwent more robust osteogenic differentiation compared to static cultured ones, and (B) resulted in significantly higher cellularity in the cellular scaffolds (*** $p<0.001$).

6. Conclusion –Generating effective TE bone grafts

By combining an inter-disciplinary approach in scaffold technology, bioreactor development and stem cell biology, we have generated an effective bone graft through the seeding of highly proliferative and osteogenic hfMSC onto the osteoconductive PCL-TCP scaffold matrix, and maturing the hfMSC mediated PCL-TCP scaffold under biaxial rotating bioreactor (Figure 9). Our ongoing animal experiment showed that this hfMSC mediated TE bone graft can be used to heal critical sized femoral defect in a rat model (unpublished observations), paving the way for clinical trials to be initiated. This strategy presents a

promising solution to fulfill the increasing need for bone grafts and provides effective TE bone grafts, which are available off-the-shelf like allografts and synthetic grafts and can stimulate rapid healing like autografts.

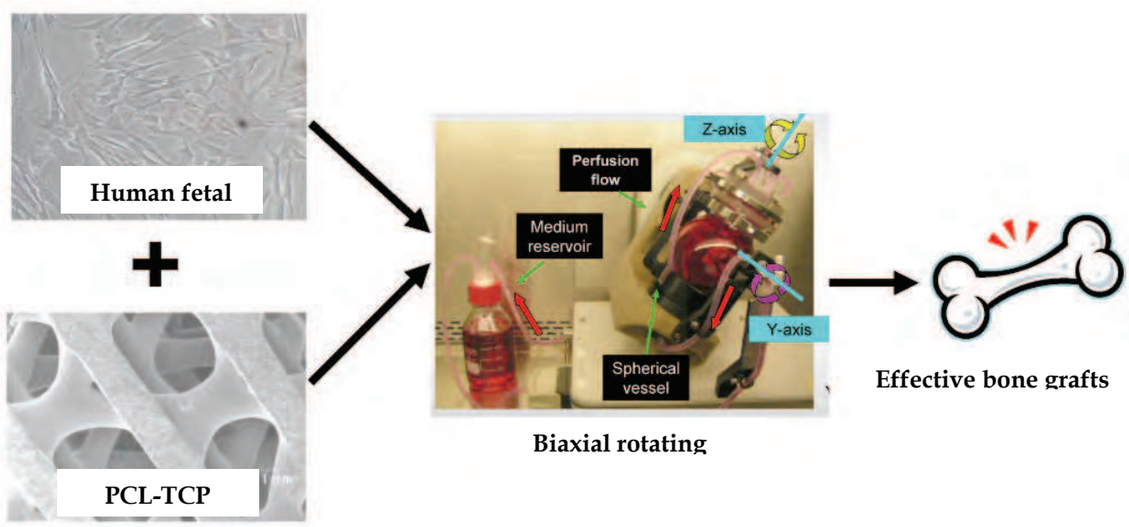


Fig. 9. Generation effective bone grafts with the combinational use of hfMSC, PCL-TCP scaffold and biaxial rotating bioreactor.

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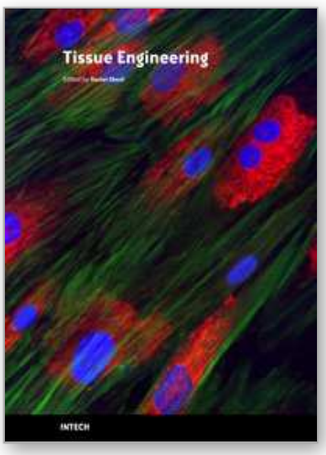

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The Tissue Engineering approach has major advantages over traditional organ transplantation and circumvents the problem of organ shortage. Tissues that closely match the patient's needs can be reconstructed from readily available biopsies and subsequently be implanted with minimal or no immunogenicity. This eventually conquers several limitations encountered in tissue transplantation approaches. This book serves as a good starting point for anyone interested in the application of Tissue Engineering. It offers a colorful mix of topics, which explain the obstacles and possible solutions for TE applications.

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