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EFFECTS OF SILICA BASED BIOMATERIALS ON BONE MARROW DERIVED CELLS

Material aspects of bone regeneration

by

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ABSTRACT

Timothy Wilson: EFFECTS OF SILICA BASED BIOMATERIALS ON BONE MARROW DERIVED CELLS—Material aspects of bone regeneration
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Silica based biomaterials, such as melt-derived bioactive glasses and sol-gel glasses, have been used for a long time in bone healing applications because of their ability to form hydroxyapatite and to stimulate stem cell proliferation and differentiation. In this study, bone marrow derived cells were cultured with bioactive glass and sol-gel silica, and seeded into porous polymer composite scaffolds that were then implanted femorally and subcutaneously in rats to monitor their migration inside host tissue. Bone marrow derived cells were also injected intraperitoneally.

Transplanted cells migrated to various tissues inside the host, including the lung, liver spleen, thymus and bone marrow. The method of transplantation affected the time frame of cell migration, with intraperitoneal injection being the fastest and femoral implantation the slowest, but not the target tissues of migration. Transplanted donor cells had a limited lifetime in the host and were later eliminated from all tested tissues. Bioactive glass, however, affected the implanted cells negatively. When it was present in the scaffold no donor cells were found in any of the tested host tissues. Bioactive glass S53P4 was found to support both osteoblastic and osteoclastic phenotype of bone marrow derived cells, but it was resistant to the resorbing effect of osteoclastic bone marrow derived cells, showing that bioactive glass is rather dissolved through physicochemical reactions than resorbed by cells. Fast-dissolving silica sol gel in microparticulate form was found to increase collagen formation by bone marrow derived cells, while slow dissolving silica microparticles enhanced their proliferation, suggesting that the dissolution rate of silica controls the response of bone marrow derived cells.

Keywords: Silica, biomaterial, sol-gel, bioactive glass, bone marrow derived cell, bone, stem cell migration.

TIIVISTELMÄ

Timothy Wilson: PIIPOHJAISTEN BIOMATERIAALIEN VAIKUTUS LUUYDINPERÄISIIN SOLUIHIN—Materiaalinäkökantoja luun paranemiseen
Biolääketieteen laitos, Lääketieteellinen biokemia ja genetiikka, Turun Yliopisto, ja Turun kliininen biomateriaalikeskus
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Piipohjaisia biomateriaaleja, kuten sulatepohjaisia bioaktiivisia laseja ja sooli-geeli laseja, on jo pitkään käytetty luunparanemisapplikaatioina niiden hydroksiapatiittiä muodostavan ja luun kantasoluja proliferaatiota ja erilaistumista edistävien ominaisuuksien ansiosta. Tässä tutkimuksessa luuydinperäisiä soluja kasvatettiin bioaktiivisen lasin ja piidioksidin kanssa, ja niitä siirrostettiin huokoisiin polymeeritukirakenteisiin ja implantoitiin rottien reisiluihin sekä nahan alle, jotta voitaisiin seurata niiden liikkumista vastaanottajan elimistössä. Luuydinpohjaisia soluja myöskin injektoitiin rottien vatsaonteloon.

Siirretyt solut kulkeutuivat lukuisiin kudoksiin vastaanottajan elimistössä, mukaan lukien keuhkoihin, maksaan, pernaan, kateenkorvaan ja luuytimeen. Transplatointimenetelmä vaikutti solujen siirtymisen nopeuteen, siten että vatsaonteloon injektoidut solut kulkeutuivat kudoksiin nopeiten ja reisiluuhun implantoidut hitaiten, mutta se ei vaikuttanut siihen mihin kudoksiin solut kulkeutuivat. Siirrettyjen solujen elinaika vastaanottajan elimistössä oli rajallinen. Bioaktiivinen lasi vaikutti implantoituihin soluihin negatiivisesti. Kun sitä oli mukana tukirakenteissa, ei luovuttajasoluja löydetty vastaanottajan kudoksista. Bioaktiivinen lasi S53P4 tuki luuydinperäisten solujen sekä osteoblastista että osteoklastista fenotyyppiä, mutta se ei resorboitunut osteoklastisten solujen toimesta, osoittaen että bioaktiivinen lasi liukenee lähinnä fysikokemiallisten reaktioden kautta eikä solujen toiminnan seurauksena. Nopeasti liukenevan mikropartikkelimuotoisen sooli-geeli pii lisäsi luuydinperäisten solujen kollageenin muodostusta, kun taas hitaasti liukeneneva pii lisäsi niiden proliferaatiota, viitaten siihen että piin liukoisuus vaikuttaa luuydinperäisten solujen vasteeseen.

Avainsanat: Pii, biomateriaali, sooli-geeli, bioaktiivinen lasi, luuydinperäinen solu, luu, kantasolumigraatio

CONTENTS

ABBREVIATIONS

α-mem	Alfa-modified medium
ALP	Alkaline phosphatase
BAG	Bioactive Glass
$baSiO_2$	PLGA-silica sol-gel composite
BMP	Bone morphogenetic protein
BMSC	Bone marrow stromal cell
BSP	Bone sialoprotein
$CaPsiO_2$	Calcium phosphate doped PLGA-silica composite
CFU-F	Colony-forming unit fibroblast
CSF-1	Colony-stimulating factor 1
DMP	Dentin matrix protein
DSPP	Dentin sialophosphoprotein
ECM	Extracellular matrix
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
Gla	Gamma-carboxyglutamic acid
HAp	Hydroxyapatite
HSC	Hematopoietic stem cell
IGF	Insulin-like growth factor
Ihh	Indian hedgehog
MEPE	Matrix extracellular protein
MPG	Matrix gla protein
MSC	Mesenchymal stem cell
IP	Intraperitoneal
OCN	Osteocalcin
OSX	Osterix
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PLGA	Poly(lactide-co-glycolide)
PTHrP	parathyroid hormone-related peptide
RANK	Receptor Activator of Nuclear Factor κ B
RANKL	Receptor Activator for Nuclear Factor κ B Ligand
RGD	Arginine-glycine-aspartic acid tripeptide
SBF	Simulated body fluid
SEM	Scanning electron microscopy
SRY	Sex determining region of the Y-chromosome
TEOS	Tetraethylorthosilicate
TGF-β	Transforming growth factor beta
TNF	Tumor necrosis factor
TRACP	Tartrate resistant acid phosphatase

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, which are referred to in the text by their Roman numeral:

I Wilson T, Meretoja V, Malin M, Tirri T, Närhi T, Penttinen R, Ekholm E. In vivo evaluation of subcutaneously implanted silica based scaffolds with osteogenically differentiated rat bone marrow stromal cells. *Submitted.*

II Wilson T*, Stark C*, Holmbom J, Rosling A, Kuusilehto A, Tirri T, Penttinen R, Ekholm E. Fate of mesenchymal stem cells after intraperitoneal infusion or implantation into femoral bone defects in the host animal. (2010) *Journal of Tissue Engineering. Article ID 345806, 9 pages. Doi:10.4061/2010/345806.*
*** equal contribution**

III Wilson T, Parikka V, Holmbom J, Ylänen H, Penttinen R. Intact surface of bioactive glass S53P4 is resistant to osteoclastic activity. (2006) *J Biomed Mater Res A 77:67-74*.

IV Wilson T, Viitala R, Puska M, Jokinen M, Penttinen R. Macrophage induced effect of particulate silica on rat mesenchymal stem cells in vitro. (2009) *Key Engineering Materials Vols. 396:123-126*.

In addition, some additional results are published.

1 INTRODUCTION

Bone is a unique tissue in that unlike any other tissue type in the body it can regenerate without leaving a fibrous scar. However, when a bone defect is too large, for example as a result of bone tumor removal or trauma, it is a critical size defect, which the body can no longer heal. It has been estimated that the number of these cases is up to half a million each year in the US alone, and biomaterials have been suggested as one course of therapy to address this issue.

In order to function as a successful biomaterial, the material has to promote the healing capabilities of bone tissue. Bone is a dynamic tissue that is constantly remodeled in the body by osteoclasts that resorb damaged and unneeded bone and osteoblasts that synthesize new bone extracellular matrix and mineralize it. To promote the formation of new bone, a biomaterial must not only activate osteoblasts to make new bone but also osteoclasts to remodel it into functional, load bearing bone.

Silica based biomaterials such as bioactive glass and sol-gel glasses are known for their beneficial effects on bone healing. Although the exact mechanism are not completely understood, in addition for providing calcium and phosphate ions for the formation of hydroxyapatite, the primary mineral of bone, bioactive glasses increase the proliferation and differentiation of osteoblasts. These cell types originate in the bone marrow from a cell populations know as bone marrow stromal cells. Their differentiation is vital for the regeneration of all mesenchymal tissues including bone. Bioactive glasses have also been reported to support the differentiation of osteoclasts. For these reasons silica based biomaterials have been suggested to be used as bone filling materials or as part of tissue engineering scaffolds in bone repair. In tissue engineering bone marrow stromal cells are extracted form the bone marrow, expanded, differentiated, seeded into scaffolds and then implanted into the defect site.

In this thesis the effects of silica based biomaterials on bone marrow stromal cell proliferation, differentiation and migration in host tissue were examined from the point of view of bone tissue regeneration.

2 REVIEW OF LITERATURE

2.1 Bone

Bone is a complex and dynamic living tissue that engages in continuous dynamic remodeling—breaking down old bone and replacing it with new bone. It functions as mechanical support and assistance for movement in vertebrates, and as protection for many internal organs against injury, the brain for example. In addition to these mechanical qualities, bone also serves as the organ of blood cell production, i.e. hematopoiesis, and according to newest studies also as the source of bone marrow stromal cells (BMSCs), which can differentiate into mesenchymal lineages and repair injuries, for example in bone or cartilage (Caplan 1991). Bone also functions in mineral homeostasis and stores several vital minerals, especially calcium and phosphorus, which it can release into the bloodstream to maintain critical mineral balances.

2.1.1 Structure

Anatomically bone can be divided into two types, flat bones such as the skull, ilium, scapula and mandible, and long bones such as the femur and the humerus. The two different types of bone are formed through two distinct types of bone formation. Flat bones are formed through intramembranous ossification or direct osteogenesis where bone is formed on a fibrous membrane directly from condensed bone marrow stromal cells, while long bones are formed through endochondral ossification where the primitive cells are transformed into cartilage, which is then later on gradually replaced by bone.

Long bones contain a cylindrical shaft called the diaphysis, and wider expansions at either end called the epiphyses. Between these two parts is an area called the metaphysis, which in growing bones is separated by the growth plate. The diaphysis is hollow, and this medullar cavity contains the bone marrow where hematopoiesis occurs and it is also where bone marrow stromal cells reside.

Morphologically bone can be divided into two types, cortical or compact bone and trabecular or cancellous or spongy bone. Cortical bone comprises 80% of the mass of the skeleton while trabecular bone comprises only 20%. Both types of bone are made up of the same cells and extracellular matrix components, but they are organized differently. Cortical bone tissue contains few spaces and it forms the external layer of all bones and makes up the bulk of the diaphyses of long bones. Trabecular bone consists of a loosely organized porous matrix of interconnecting columns, and the spaces between these columns are filled with bone marrow. Cortical bone tissue provides protection and load-bearing capabilities to bone while trabecular bone is responsible for the metabolic functions of bone (Baron 2003, Mundy *et al*. 2003).

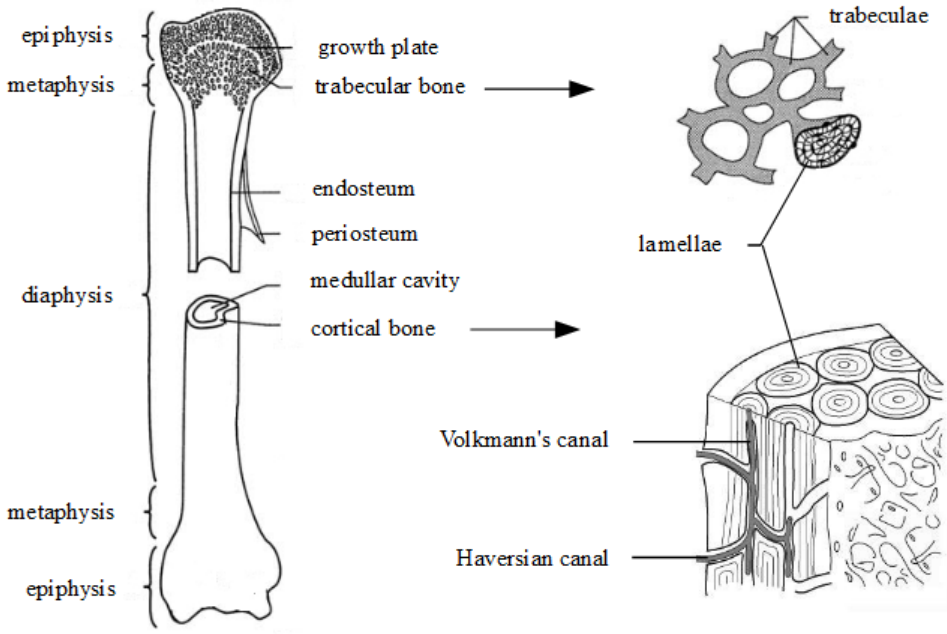

Figure 1. Structure of a long bone. Blood vessels and nerves perforate cortical bone through canal called Haversian and Volkmann's canals.

2.1.2 Extracellular matrix

Bone tissue consists of cells and extracellular matrix (ECM) like all connective tissues, and in bone the amount of ECM is especially abundant. The ECM is composed on an organic phase of collagens and other proteins, which provide elasticity and tensile strength, and an inorganic phase of minerals, predominantly calcium and phosphate, which give bone hardness and load-bearing capabilities. The organic matrix accounts for 20-40% of bone mass of which 95% is composed of structural collagens. The remaining part consists of proteoglycans and other non-collagenous proteins. The inorganic phase accounts for 50-70% of bone mass and cells a mere 1%. The rest is water, around 5-10%, and lipids, 3% (Robey and Boskey 2008).

2.1.2.1 Organic phase

Type I collagen is the most abundant and important protein of the organic phase of bone. This triple helical protein composed of two α1 chains (also called COL1A1) and one α2 chain (COL2A1) comprises 85-90% of the organic matrix. Collagens are rope like proteins that have a unique repeating polypeptide structure of (Gly-X-Y)n where X and Y often represent proline and hydroxyproline, respectively. Once outside the cell the triplex undergoes covalent cross-linking within and between other

collagen molecules to form fibrils. Type I collagen gives bone tensile strength, and also contains peptide motifs which are integrin binding sites for osteoblasts (McCann *et al*. 1997), and a structural template for mineral deposition (Landis *et al*. 1996; Landis 1999). The vitality of type I collagen for healthy bone formation is demonstrated by many single base mutations that disrupt the assembly of collagens and lead to bone defects commonly lumped together as the genetic disease osteogenesis imperfecta (Pace *et al*. 2002; Primorac *et al*. 2001). There are also trace amounts of type III, V, XI and XIII in bone (Gehron Robey 1989; Gentili and Cancedda 2009). Type III collagen has been detected in developing and remodeling bone (Keene *et al*. 1993) and in blood vessels and the bone marrow during fracture healing (Hiltunen *et al*. 1993), and type V collagen has been shown to have a role in the diameter control of type I collagen fibrils (Wenstrup *et al*. 2004).

Osteonectin is the most abundant non-collagenous bone ECM protein. It is a glycoprotein that has multiple collagen Ca^{2+} binding sites and has been shown to be a potential nucleator of hydroxyapatite (Maurer *et al*. 1996; Young *et al*. 1992). It also regulates cellular function and experiments in mice showed that a deletion of ostenectin cause osteopenia with defects in the function of both osteoblasts and osteoclasts (Delany *et al*. 2000).

Osteocalcin (OCN) and Matrix gla protein (MPG) are gamma-carboxyglutamic acid (Gla) containing proteins which have vitamin K dependent post-translational modifications. Osteocalcin is the second most abundant non-collagenous protein in bone ECM. It has a very narrow expression pattern being made only by osteoblasts and osteocytes, while matrix gla protein is also expressed in cartilage and arteries. They are both inhibitors of mineralization. OCN deficient mice have increased bone formation (Ducy *et al*. 1996) while MPG knock out mice had spontaneous calcification arteries and cartilage (Luo *et al*. 1997).

Biglycan and Decorin belong to the family of small leucine-rich proteoglycans. Proteoglycans are macromolecules that contain acidic polysaccharide chains, glycosaminoglycans, attached to a core protein. Both biglycan and decorin are heavily enriched in bone ECM, but their role in bone physiology is still being investigated. They influence bone cell differentiation and proliferation, and mineral deposition (Waddington *et al*. 2003) as well as cell behavior by blocking adhesion motifs of RGD-containing molecules and by binding TGF-β (Halpert *et al*. 1996, Hildebrand *et al*. 1994). Biglycan deficient mice develop lower peak bone mass in a phenotype resembling osteoporosis (Xu *et al*. 1998) that seems to be cellularly mediated by defective osteoblasts which are fewer in number and less responsive to TGF-β (Chen *et al* 2002). Decorin deficient mice have no gross bone phenotype, but decorin/biglycan double knockout mice have even more defective long bones than biglycan knockouts, suggesting that decorin can partly compensate for biglycan in its absence (Bi *et al*. 2005; Corsi *et al*. 2002).

Alkaline phosphatase (ALP) is produced by osteoblasts in phospholipid membrane-bound matrix vessels, and it is ubiquitous in bone, but also in liver, kidney, placental and intestinal tissues. ALP plays a direct role in the induction of hydroxyapatite deposition on ECM proteins (Anderson 1995; Beertsen and van den Bos 1992; Storrie and Stupp 2005) and aids in the hydrolyzation of organic phosphate esters producing an excess of free inorganic phosphate which initiates biomineralization (Beertsen and van den Bos 1992; Nuttelman *et al*. 2006). Although the mechanism of its action is not completely understood, ALP remains an excellent indicator of osteodifferentiation and mineralization.

RGD containing glycoproteins contain an arginine-glycine-aspartic acid (RDG) tripeptide which is a ligand motif for the integrin class cell-surface molecules. There are many proteins of this type in the bone matrix and they convey attachment points to cells. *Fibronectin* is one of the most abundant non-collagenous proteins in bone ECM and has been shown to regulate osteoblast differentiation (Moursi *et al*. 1996, 1997) and their subsequent survival (Globus *et al* 1998). It may also regulate mineralization (Couchourel *et al*. 1999; Daculsi *et al*. 1999) by binding to other matrix proteins and modifying their activities (Dallas *et al*. 2000; Merle *et al*. 1999). *Osteopontin* has been suggested to be required for the resorption of bone by increasing vascularization related to it. This conclusion has been reached through studies on knockout mice, where the bone phenotype is grossly normal, but resorption is reduced in cases that normally induce it, such as ovariectomy (Yoshitake *et al*. 1999), reduced mechanical stress (Ishijima *et al*. 2001), and continuous parathyroid hormone treatment (Ihara *et al*. 2001). *Bone sialoprotein (BSP)* is involved in regulating hydroxyapatite formation in bones and teeth (Fisher *et al*. 2001). It promotes the initial formation of mineral crystals and is considered an early marker of osteogenic differentiation (Chen *et al*. 1992, 1994; Kasugai *et al*. 1992; Sodek *et al.* 1992). BSP deficient mice show impaired bone growth and mineralization, with thin cortical bone and greater trabecular bone volume and very low bone formation, indicating reduced osteoclast activity and osteoblast mineralization (Malaval *et al*. 2008). *Dentin sialophosphoprotein (DSPP)* was first believed to be tooth-specific, but was later found to be expressed also in bone (Qin *et al*. 2002, 2003). DSPP knockout mice display defective bone mineralization, which is thought to be due to an effect on bone turnover (Verdelis *et al*. 2008). *Dentin matrix protein (DMP)* was also first identified in dentin, but later discovered in other tissues. In adult bone DMP is associated with the mineralization process (Feng *et al*. 2002). DMP deficient mice show no abnormal phenotype at birth, but they develop abnormalities in the growth plate and delayed mineralization as adults (Ye *et al*. 2005). Over expression of DMP, on the other hand, induces bone marrow stromal cells mineralization and differentiation (Narayanan *et al*. 2001). *Matrix extracellular protein (MEPE),* also called osteoblast/osteocyte factor 45 (OF45), is a highly serine/glycine rich protein which is expressed in mineralized tissues (Petersen *et al*. 2000). Studies done on MEPE deficient mice show increased bone mass as a result of increased osteoblast number and activity (Gowen *et al*. 2003). *Thrombospondin 2* is an abundant protein in

bone (Gokhale *et al*. 2001; Robey *et al*. 1989). It inhibits the number of bone cell precursors (Hankenson and Bornstein 2002) and thrombospondin 2 knockout mice have increased bone density and cortical thickness (Hankenson *et al*. 2000). *Vitronectin* is a multifunctional protein present in the ECM and blood, which binds glycosaminoglycans, collagen, plasminogen (Schvartz *et al*. 1999). In bone tissue it is known to induce osteoclast polarization (Takahashi *et al*. 2007).

2.1.2.2 Inorganic phase

The mineral phase of bone consists almost completely of calcium phosphate hydroxyapatite (HAp) [$Ca_5(PO_4)_3(OH)$] with some impurities such as carbonate, citrate, magnesium, fluoride and strontium (Cowin 2001; Leventouri 2006). This phase gives bone its rigidity, hardness and load-bearing capabilities, and facilitates bone to act as a reservoir for calcium and phosphate and other ions. The inorganic phase deposition begins immediately after the formation of the organic phase with the formation of a primary hydroxyapatatite crystals. The crystals then grow to needles of variable length and of widths of about 30 to 45 nm and thickness of approximately 5 nm, and their longest side lies parallel to the axis collagen fibers. After the primary crystal is formed, the crystal may branch into several dimensions, becoming larger and more perfect as the bone matures. Secondary mineralization also occurs when new crystals are formed on the old one. Bone mineral matrix formation is a slow process which takes several moths or even years to reach really high densities of bone (Boskey 2006, Robey and Boskey 2003).

2.1.3 Cells

Bone is constantly being built, resorbed and then rebuilt through a physiological process called remodeling that is carried out and carefully controlled by a variety of cell types. Osteoblasts synthesize and mineralize bone matrix, which is maintained by osteocytes, and as required, resorbed by osteoclasts.

2.1.3.1 Osteoblasts

Osteoblasts are cells of mesenchymal origin that are nearly indistinguishable from fibroblasts, except for a mineralized ECM, and the expression of certain bone specific genes, such as osteocalcin and the transcription factors Runx2 , Osterix (OSX), ATF4. Runx2, also known as Cbfa1, was the first osteoblast specific transcription factor to be identified (Ducy *et al*. 1997). It is a master gene of osteoblast differentiation, which is expressed in the cells of the mesenchymal condensations during embryonic development, and Runx2 deficient mice have a skeleton where osteoblast differentiation never occurs, and which is entirely made of cartilage (Komori *et al.* 1997; Otto *et al*. 1997). Osterix is a zinc-finger containing transcripton factor expressed in osteoblast progenitor cells of all skeletal elements, which is required for osteoblast differentiation (Nakashima *et al*. 2002). OSX is not expressed in Runx2-

deficient mice indicating that it acts downstream of Runx2. Contrary to Runx2 and OSX, ATF4 is broadly expressed, but accumulation of the protein occurs mainly in osteoblasts (Yang *et al*. 2004). It is required to maintain osteoblast phenotype, but also to provide efficient amino acid import into cells (Harding *et al*. 2003). This function is particularly important in cells like osteoblasts that secrete large amounts of protein, and helps them to synthesize collagen type I, the most abundant protein in bone ECM. In addition to these transcription factors, osteoblast differentiation is controlled by numerous secreted growth factors, such as bone morphogenetic proteins (BMPs), insulin-like growth factors (IGFs), transforming growth factor beta (TGF-β), members of the fibroblast growth factor (FGF) family, indian hedgehog (Ihh) and parathyroid hormone-related peptide (PTHrP) (Ducy *et al*. 2000).

The process of bone formation *in vivo* can be divided into three stages. In the first stage osteoprogenitor cells proliferate and start to express type I collagen and transiently osteopontin. During the second stage osteoblasts start to form bone matrix by synthesizing and secreting type I collagen. In the third stage mineralization begins as the osteoblasts start expressing osteocalcin, osteopontin and collagenase. The active matrix producing osteoblasts have a large nucleus, enlarged Golgi apparatus and extensive endoplasmic reticulum, which is typical for a cell actively engaged in secretion. Active osteoblasts are also highly enriched in ALP and secrete type I collagen and other matrix proteins (Lian *et al*. 2003).

2.1.3.2 Osteocytes

Osteocytes are terminally differentiated osteoblasts, which have been incorporated into newly composed bone matrix. It has been estimated that around 10-20% of osteoblasts differentiate this way into osteocytes (Aubin and Turksen 1996). They lie within lacunae within mineralized bone and have extensive filipodial processes that lie within canaliculi in bone tissue (Bonewald 1999). Osteocytes maintain connection with each other and bone lining cells on the bone surface through multiple filipodial cellular processes that are created before and during matrix synthesis (Palumbo 1986). The transformation of a motile osteoblast into an entrapped osteocyte takes about three days, and the lifespan of mature osteocytes in bone that is not turned over can be decades (Frost 1963; Marotti *et al*. 1990). However, empty lacunae observed in aging bone suggests that they may undergo apoptosis (Noble *et al*. 1997), which is probably caused by the disruption of intercellular gap junctions (Xing and Boyce 2005). Once entrapped within the matrix, osteocytes are metabolically and electrically linked through gap junctions composed primarily of connexin 43, and these gap junctions are required for osteocyte maturation activity and survival (Plotkin *et al*. 2002). The canalicular system is also vital for osteocyte survival as the route of metabolic traffic (Lian *et al*. 2003).

The function of osteocytes is still relatively poorly known, but they are the most abundant cells in bone and they are actively involved in maintaining the bone matrix, and osteocyte death is eventually followed by matrix resorption (Junqueira *et al*. 1995). It has been suggested that osteocytes transmit mechanical signals within bone. They make up a synctial connection with osteoblast and bone lining cells which is capable of mechanosensation (Aarden *et al*. 1994; Burger and Klein-Nulend 1995). Transmission of mechanical signals to the osteocyte cytoskeleton through cell surface receptors occurs both directly through direct contact with the solid matrix structure of bone, but also through fluid pressure and shear stresses imparted by fluids moving through the lacunocanalicular system due to flow load-induced flow (Knothe Tate 2003). Osteocytes have also been suggested to regulate mineral homeostasis through a process called osteocytic osteolysis (Noble 2008).

2.1.3.3 Osteoclasts

Osteoclasts are multinucleated cells that are specialized in the removal of bone tissue. They derive from hematopoietic stem cells and progress through the colony-forming unit for granulocytes and macrophages (CFU-GM) and the CFU for macrophages (CFU-M) to the preosteoclast and finally to the mature multinucleated osteoclast (Teitelbaum and Ross 2003; Greenfield and Rubin 2005). Osteoclasts are only found in bone tissue where they differentiate with the help of stromal cells (Takahashi *et al*. 1988). Close contact with stromal cells in bone tissue is required for the production of two hematopoietic factors, the TNF-related cytokine RANKL and the polypeptide growth factor CSF-1 that are both necessary and sufficient for osteoclastogenesis (Lacey *et al*. 1998; Yasuda *et al*. 1998), and for the subsequent activation of RANK on the surface of hematopoietic precursor cells (Nakagawa *et al*. 1998; Hsu *et al*. 1999). These two factors are required to induce the genes that typify the osteoclats lineage, including tartrate resistant acid phosphatase (TRACP) and cathepsin K.

In order to resorb bone, the osteoclast attaches to its surface and begins to assume a polarized morphology. An actin ring that creates a tight junction between the cell and the underlying bone forms and the osteoclast's cell membrane inside this sealing zone becomes ruffled (Lakkakorpi *et al*. 1989). This vacuole is then acidified by the export of hydrogen ions by the ATP6i complex (Li *et al*. 1999), which dissolves the solid hydroxyapatite. The organic phase is then degraded mainly by the secretion of cathepsin K (Gowen *et al*. 1999), although other proteinases, such cathepsin D, B and L (Drake *et al*. 1996) and matrix metalloproteinases (Everts *et al*. 2006), are also present. TRACP is also expressed strongly during osteoclast differentiation and is used as a cellular marker for osteoclasts. TRACP functions as an acid phosphatase and it is capable of generating reacting oxygen species, which have been shown to facilitate collagen degradation, and it may thus be important in the final degradation of resorption products (Väänänen and Zhao 2008). After matrix degradation the

degradation products are removed from the resorption lacuna by transcytosis from the ruffled border to a functional secretory domain, where they are liberated into the extracellular space (Nesbitt and Horton 1997; Salo *et al*. 1997).

2.1.3.4 Bone lining cells

Bone lining cells are thin, elongated cells that cover the surface of bone when it is not under remodeling. The retraction of these cells from the bone surface is a mandatory step before osteoclastic bone resorption (Zambonin-Zallone *et al*. 1984). It has been proposed that bone lining cells are a subpopulation of osteoblasts, as bone lining cells, like osteoblasts, can enwrap and resorb collagen (Everts *et al*. 2002), which predisposes bone to resorption by increasing mineral exposure (Chambers and Fuller 1985). It has also been proposed that bone lining cells clean osteoclast resorption pits after matrix degradation and deposits deposit a thin line of collagen to form a cement line which demarcates sites of new bone formation, giving them a role in the regulation of bone remodeling (Everts *et al*. 2002).

2.1.4 Remodeling

Bone undergoes continual growth, modeling and remodeling during life. Modeling is a process by which bones change their overall shape in response to physiological or mechanical forces, leading to a gradual adjustment of the skeleton to the forces it encounters. This process is described by Wolff's law, according to Wolff who in 1892 discovered the skeleton's ability to adapt to changes (Wolff 1892). Bone modeling is less frequent in healthy adults than bone remodeling (Kobayashi *et al*. 2003). Modeling differs from remodeling in that during modeling bone formation is not as tightly coupled to resorption as in remodeling.

Bone remodeling is the process by which bone is constantly renewed to repair accrued microdamage and to maintain bone strength and to regulate mineral homeostasis. During remodeling small areas of bones tissue are removed and then replaced by new matrix, which is subsequently mineralized. It has been estimated that approximately 10-15% of bone surface is at any time undergoing remodeling (Kanis *et al*. 1995). The remodeling process advances in a cycle with four distinct phases: activation, resorption, reversal and formation and mineralization (Figure 2). The time when there is no remodeling going on is called the resting phase.

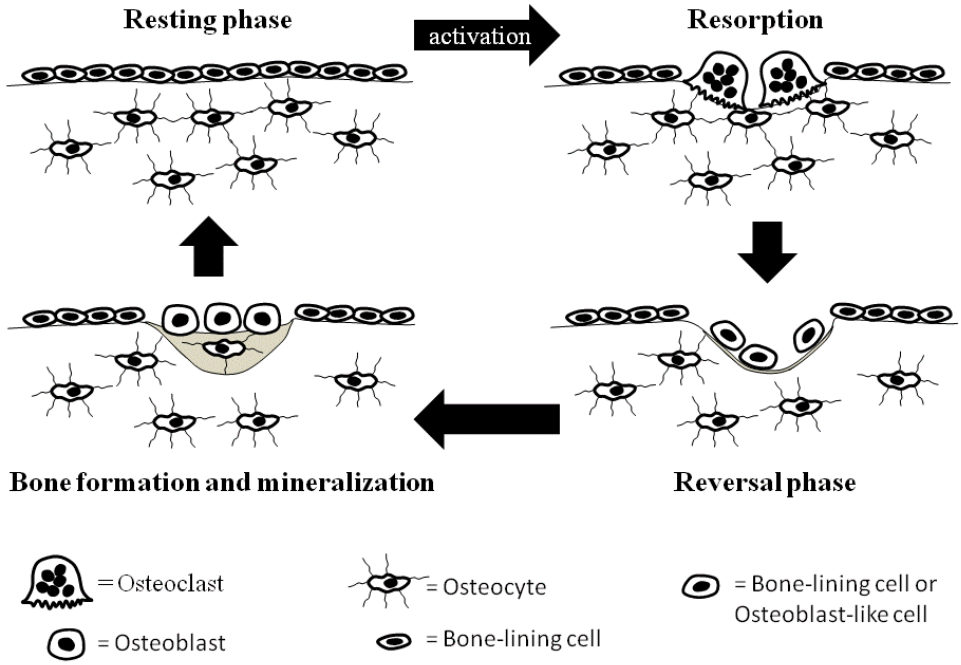

Figure 2. Bone remodeling cycle. Old bone is resorbed and new bone is formed in a tightly controlled sequence of events.

During the *activation phase* mononuclear monocyte-macrophage osteoclast precursors are activated and recruited form the circulation (Roodman 1999). Bone lining cells retract from the surface (Zambonin-Zallone *et al*. 1984) and multiple mononuclear cells fuse to form multinuclear maturing osteoclasts, which then bind to the surface of the bone. The *resorption phase* starts when osteoclasts have attached to the bone, polarized and formed a ruffled border. Bone resorption has been detailed earlier (see 2.1.3.3 Osteoclasts). This phase takes about two to four weeks to complete, after which osteoclasts go into apoptosis and bone resorption is terminated (Eriksen 1986; Reddy 2004). During the *reversal phase* bone resorption transitions to bone formation. Osteoblast-like cells (Mulari *et al*. 2004) or bone lining cells (Everts *et al*. 2002) appear on the surface of the resorbed bone and finalize the resorption phase and prepare the surface for osteoblasts. A cement line, a specialized matrix rich in osteopontin and other phosphoproteins is formed to demarcate new and old bone (McKee and Nanci 1996). In the *bone formation and mineralization phase* osteoblast precursors proliferate and differentiate at the remodeling site and start synthesizing new collagen matrix. They also initiate matrix mineralization by releasing small matrix vehicles which contain calcium and phosphate and alkaline phosphatase (Anderson 2003). Bone formation takes approximately four to six months to complete, after which 50-70% of osteoblasts undergo apoptosis, with the rest

becoming osteocytes that are buried within the matrix or bone lining cells. The new bone then enters the *resting phase* which can continue for years until a new remodeling cycle is initiated.

2.1.5 Healing

Bone is a unique tissue, which can regenerate itself completely after injury (Sommerfeldt and Rubin 2001). The processes of bone healing are similar to the healing of other tissue types, but unlike bone, other tissue types can only heal themselves by the formation of a scar. Bone, on the other hand, after healing and remodeling will retain its structural and functional properties (Glowacki 1998). The process of bone healing follows sequential phases of hematoma formation, acute inflammation, callus formation, mineralization and remodeling (Hollinger and Wong 1996). Initially a hematoma forms on the injury site and acts as a source of platelets and hematopoietic cells, such as neutrophils, monocytes and macrophages, which initiate the inflammatory cascade by secreting growth factors such as platelet derived growth factor (PDGF), TGF-β, TNF-α and others, which have a role in chemotaxis, angiogenesis and mesenchymal cell regulation (Buckwalter *et al*. 1996; Gurtner *et al*. 2008). Callus formation begins within the first week after angiogenesis and mesenchymal cell recruitment. Mesenchymal cells differentiate into chondroblasts and start making a cartilagenous callus which bridges the site of injury. This cartilage then calcifies through a process that mimics endochondral ossification. In the remodeling phase the callus is gradually remodeled and the pre-existing structure of the tissue is restored (Einhorn 1998).

There are several conditions, however, in which bone does not heal through a cartilagenous template. In mechanically stable defects, such as bone tumor removal or rigidly fixed fractures, new bone formation occurs primarily through intramembranous ossification (Karp *et al*. 1992). There are limitations to the maximum size of a defect if it is to be healed. When the size of the defect exceeds certain limits it never heals completely. This type of defect is often called a critical size defect.

2.2 Bone marrow derived cells

Bone marrow derived cells can be divided into two distinct multipotent stem cell populations, bone marrow stromal cells (BMSCs) and hematopoietic stem cells (HSCs), that are found in the bone marrow and can proliferate and differentiate into variety of adult mesenchymal cell types.

The idea that non-hematopoietic cells residing in the bone marrow could be a source of fibroblasts that contribute to wound healing is an old one (Cohnheim 1867), but only at first in the 1970s the work of Friedenstein *et al*. (Friedenstein *et al*. 1970) demonstrated that the guinea pig bone marrow had an adherent fibroblast-like cell population that could form colonies on plastic *in vitro*. These cells were named

colony-forming unit fibroblasts (CFU-Fs), and they were found to be able to both form bone and reconstitute a hematopoietic microenvironment (Friedenstein *et al*. 1974). CFU-Fs were subsequently isolated from the human bone marrow (Castro-Malaspina *et al*. 1980), and it was then shown that single bone marrow stromal cell clones could be subpassaged and differentiated *in vitro* into a variety of mesenchymal lineages such as osteoblasts, chondrocytes and adipocytes (Pittinger *et al*. 1999; Caplan 2007; Bianco *et al*. 2008). Reflecting the capability of CFU-Fs to differentiate into other cells of mesenchymal lineage they had been renamed mesenchymal stem cells by Caplan (Caplan 1991).

2.2.1 Origin

The classic model suggests that BMSCs are a distinct population of mesodermal origin cells that reside in the adult bone marrow, and coexist there with HSCs. The model holds that HSCs give rise to hematopoietic cells and osteoclasts, while BMSCs differentiate into mesenhymal lineages like chondrocytes, adipocytes and osteoblasts. The distinction between these lineages have been elegantly shown by sex-mismatched allogenic bone marrow transplantation where stromal cells remain host original (Simmons *et al*. 1987). More recent studies have challenged this clear distinction, reporting that in some cases HSCs can differentiate into mesenchymal cell types, at least in vitro (Olmsted-Davis *et al*. 2003; Ogawa *et al*. 2006; Rogers *et al*. 2007), while others have been unable to confirm a common lineage (Koide *et al*. 2007).

Although numerous cell-surface markers for isolated and cultured BMSCs have been identified over the years, the work for characterizing BMSCs *in vivo* is still very much incomplete (Bianco *et al*. 2008; Deschaseaux *et al*. 2010), and in the absence of clear and unique markers the localization of BMSCs is difficult. Recently, however, new studies have been coming out that point to pericytes and the perivascular niche (Bianco *et al*. 2008; Crisan *et al*. 2008) or the periosteal compartment (Zhang *et al*. 2008) as the origin of BMSCs. This field is still new and it remains to be seen where new studies take us. An overview of different lineages of cells originating in the bone marrow is given below in Figure 3.

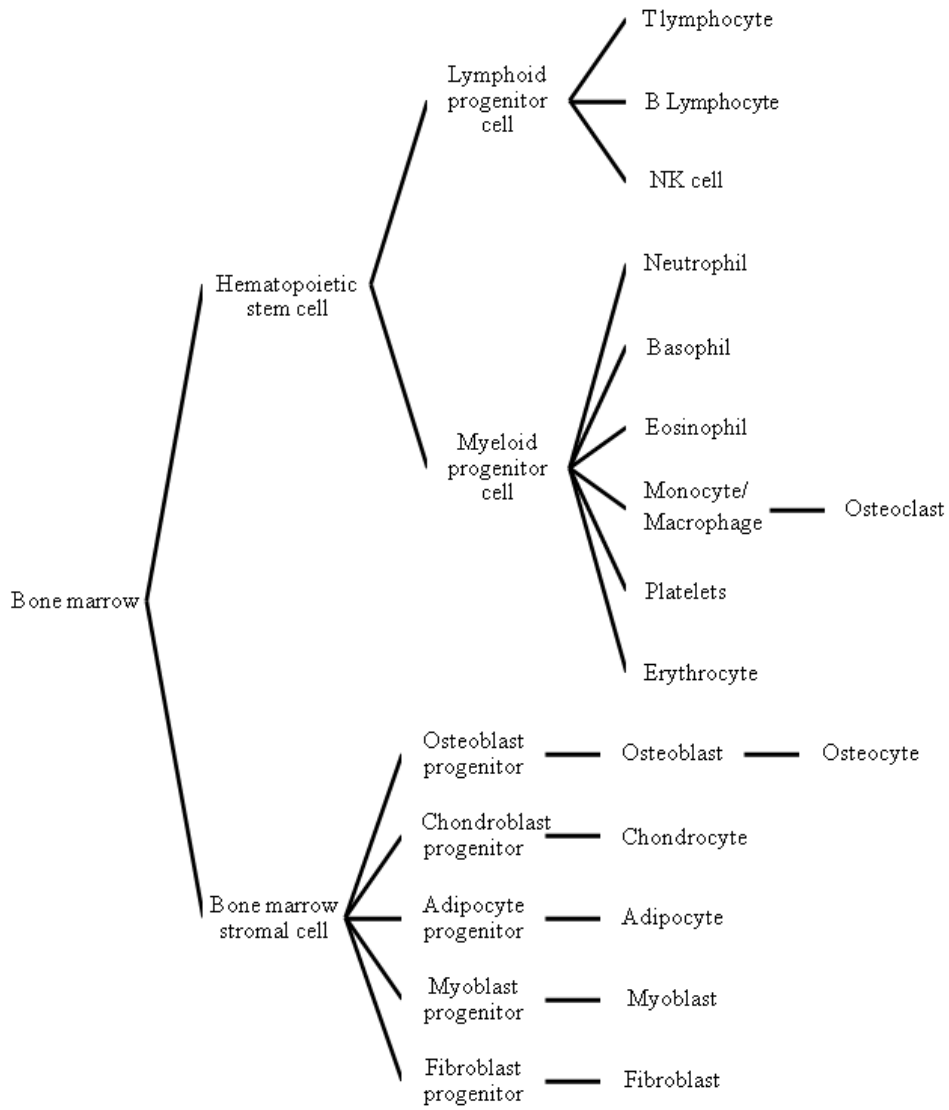

Figure 3. Schematic illustrating the differentiation of hematopoietic and bone marrow stromal cells. Bone marrow contains stem cells that gives rise to a variety of hematopoietic and mesenchymal lineages.

2.2.2 Isolation and characterization

Most of the current knowledge of the characteristics of BMSCs is based on *in vitro* studies done on isolated cultured cells. Initially the method used to isolate them was based on their property to adhere on plastic, as described by Friedenstein *et al*. (1970) However, it was discovered that only some of the clones isolated according to this method were osteogenic, which indicated that these cultures were quite heterogeneous (Kuznetsov *et al*. 1997).

Numerous studies have focused on flow cytometric analysis of specific cell-surface markers to categorize these adherent cells, and many surface antigens for BMSCs have been identified, including CD90, CD106, CD29, CD166, CD44, CD73, and CD105 (Chamberlain *et al*. 2007; Phinney and Prockop 2007). The International Society for Cellular Therapy (ISCT) has announced the criteria to be followed in order to define bone marrow cells as BMSCs. According to these criteria, BMSCs are required to be positive for CD73, CD90, and CD105 and show virtually no expression (<2%) of CD45, CD34, CD14, CD19, HLA-DR, in addition to displaying properties such as adherence to plastic and multilineage differentiation potential (Dominici *et al*. 2006).

Having said this, none of the different ways to characterize BMSCs have gained a position above the others, and thus in the field of BMSC research the studied cell populations tend to be rather heterogeneous. Even the nomenclature used is sometimes confusing, as names like mesenchymal stem cell, multipotent stromal cell, bone-marrow derived stromal cell and the like are used to described closely related types of cell populations.

2.2.3 Bone marrow stromal cells in bone tissue engineering

Bone marrow stromal cells have the capacity to differentiate into cells of all mesenchymal lineages and to repair damage to all tissues of mesenchymal origin. Since their discovery, it has also been proposed that the regenerative potential of BMSCs could be enhanced by harvesting them and expanding and differentiating them *in vitro* before reintroducing them into the body, usually seeded into a scaffold (Langer and Vacanti 1993).

Bone is a tissue that can usually heal and remodel itself without leaving a scar (Sommerfeldt and Rubin 2001), but there are limits to it's regenerative properties. When a sufficiently large defect is created in bone tissue, for example as a result of trauma or bone tumor removal it does not heal. There are up to half a million cases of critical size defect each year in the US (Castellani *et al*. 2009). BMSCs have been proposed as a method of treating these defects through tissue engineering. In tissue engineering these primitive cells are seeded into scaffolds and implanted within the scaffold to the defect site. There are different methods employed in using BMSCs in scaffolds. They can be seeded into scaffolds, and after a short incubation to insure cell

attachment, implanted to the target site (Dennis *et al*. 1998; Solchaga *et al*. 2000), or the cell scaffold composite can be incubated in a differentiation medium for 7-14 days to induce BMSC progression towards the osteogenic lineage (Ohgushi *et al*. 1993, 2005). Numerous studies have shown that these strategies promote bone formation in animals (Holy *et al*. 2003; Ishaug-Riley *et al*. 1997; Kadiyala *et al*. 1997; Mendes *et al*. 2003; Petite *et al*. 2000) and even in humans (Kon *et al*. 2000; Quarto *et al*. 2001) using various scaffold materials.

A suitable scaffold material for bone tissue engineering must have certain essential properties. Ideally, they should be biocompatible (Yang *et al*. 2001), porous with interconnecting pores within the 200-900 nm range to allow tissue and cell ingrowth and the diffusion of nutrients and metabolic waste (Vunjak-Novakovic and Freed 1998); they should be osteoconductive and osteoinductive to promote bone binding and formation (Albrektsson and Johansson 2001); they should have the mechanical properties needed for load bearing at the defect site (Leong *et al*. 2003); and they should be biodegradable to allow total renewal of the defect site (Langer and Vacanti 1993). Several different types of materials have been used as scaffold materials. Ceramics, such as natural or synthetic hydroxyapatite and β-tricalciumphosphate have in general been found to be osteoconductive and osteoinductive, but they are too brittle to provide mechanical support and furthermore their dissolution rates are hard to predict (Dong *et al*. 2002; Grynpas *et al*. 2002; LeGeros 2002; Vacanti *et al*. 2001). The other material type studied for use in tissue engineering of bone is natural and synthetic biodegradable polymers. Natural biodegradable polymers, such as collagen, fibrinogen and hyaluronic acid are potentially bioactive and can interact with host tissue, while synthethic biodegradable polymers, such as poly(α-hydroxy acids) and poly(ε-caprolactone), have chemical versatility and processability which cannot be matched by other material types (Abukawa *et al*. 2003; Kweon *et al*. 2003; Yang *et al*. 2003).

2.2.4 Migration of implanted cells

It has been shown that BMSCs, when administered systemically, are able to migrate into different tissues. After transplantation into fetal sheep, transplanted BMSCs can be found in liver, bone marrow, spleen, thymus, adipose tissue, lung, articular cartilage, perivascular areas of the central nervous system, and cardiac and skeletal muscle tissue (Liechty *et al*. 2000). In adult animals systemic delivery of BMSCs has been studied as a remedy for various conditions, such as myocardial infarction and osteogenesis imperfecta. These studies commonly use intravenous or intra-arterial injection as the method of cell delivery and the follow up periods range from hours to a few weeks.

Systemic delivery of BMSCs to treat myocardial infarction have shown that transplanted BMSCs migrate to the injured cardiac muscle (Barbash *et al*. 2003), suggesting that injured tissue might express specific receptors or ligands that facilitate

the migration, adhesion and infiltration of BMSCs. A similar injury specific migration is seen in other studies in response to myocardial infarction (Abbott *et al*. 2004; Freyman *et al*. 2006) as well as cerebral ischemia (Chen *et al*. 2001), pulmonary fibrosis (Ortiz *et al*. 2003), nephropathy (Hauger *et al*. 2006) and osteogenesis imperfecta (Horwitz *et al*. 2002). Majority of systematically administrated BMSCs, however, end up in the lung, with smaller amount in the liver and spleen (Assis *et al*. 2009; Barbash *et al*. 2003; Gao *et al*. 2001), while some studies show BMSCs ending up in a variety of organs including the bladder, kidney, brain and skin (Devine *et al*. 2003; Assis *et al*. 2009; Pereira *et al*. 1995, 1998). The entrapment of BMSCs in the capillaries of the lung is most likely explained by the relatively large size of expanded BMSCs (Koç *et al*. 2000) and their expression of adhesion molecules (Dennis *et al*. 1992), which is corroborated by the fact that a vasodilator decreases the number of entrapped cells (Gao *et al*. 2001). Systemically administered BMSCs are also detected in mesenchymal tissues, such as bone, bone marrow and cartilage (Allers *et al*. 2004; Erices *et al*. 2003; Pereira *et al*. 1995, 1998; Wynn *et al*. 2004).

2.3 Silica based biomaterials

Silica, or SiO_2, has been used in the field of biomaterials science to produce bioactive glasses. Their high biocompatibility and the positive biological effects of their reaction products, especially silica species that have been shown to possess osteoinductive properties (Xynos *et al*. 2000a, 2000b), have kept bioactive glasses in the hub of biomaterials research for forty years. Bioactive glasses have been used both in solid and particulate form mostly in bone tissue reconstruction, such as ossicle replacement in the middle ear, filling of jaw defects, restoring eye orbit structures, bone tissue replacement in periodontal diseases, maxillofacial reconstructions and spinal fusion, but also for soft tissue augmentation (Wilson *et al*. 1993). When implanted into the body, bioactive glass bonds with the surrounding tissue without promoting inflammation or forming fibrous tissue (Hench 1991). Currently there is also great interest in using bioactive glasses in tissue engineering composites. Silica-based bioactive glasses can be divided into two main groups by their manufacturing process: melt-derived bioactive glasses and bioactive glasses prepared using the sol-gel method. The sol-gel method can also be used to produce various other types of matrices composed solely of silica, such as gels and xerogels.

2.3.1 Bioactive glass

Bioactive glass (BAG) was first introduced in 1969 by Hench *et al*.. They found that an implanted phosphosilicate glass did not become surrounded by a fibrous scar, but instead bonded with bone (Hench *et al*. 1971). This bone bonding melt-derived glass was trademarked 45S5® Bioglass (45% SiO_2, 24,5% Na_2O, 24,5% CaO and 6% P_2O_5 (wt%)), and it went on to generate a whole family of melt derived and later sol-gel derived glasses collectively known as bioactive glasses. Bioactive glass S53P4 was developed in Turku, Finland by Andersson and Karlsson (1991). Its constituents

are 53% SiO_2, 23% Na_2O, 20% CaO and 4% P_2O_5 (wt%). It has been used in clinical applications in Turku University Hospital (Aitasalo *et al*. 2001; Peltola *et al*. 2000, 2006; Stoor *et al*. 2010) as well as in other hospitals (Lindfors *et al*. 2010a, 2010b).

Glasses are prepared by cooling a mixture of raw materials from the liquid state (Hench and Wilson 1993). The basic constituents of glass are SiO_2, Na_2O and CaO, where silica works as a network former which is disrupted by Na^+ or Ca^{2+}, or in some cases other ions, working as network modifiers, creating a non-crystalline glass structure. The composition of bioactive glasses differs from traditional glasses which contain more than 25 wt% of SiO_2, less than 15 wt% of Na_2O and about 10% CaO. Bioactive glasses typically contain less than 60 wt% of SiO_2 and large amounts of alkali and alkaline earth oxides. If the percentage of SiO_2 is higher than 60%, the number of bridging oxygen atoms is so high that it dramatically reduces matrix dissolution and consequently bioactivity. On the other hand, if the SiO_2 content is below 40% there is no glass formation (Hench *et al*. 1991). The *in vivo* bioactivity of glasses with different Na_2O-CaO-SiO_2 constituents is summarized in Figure 4.

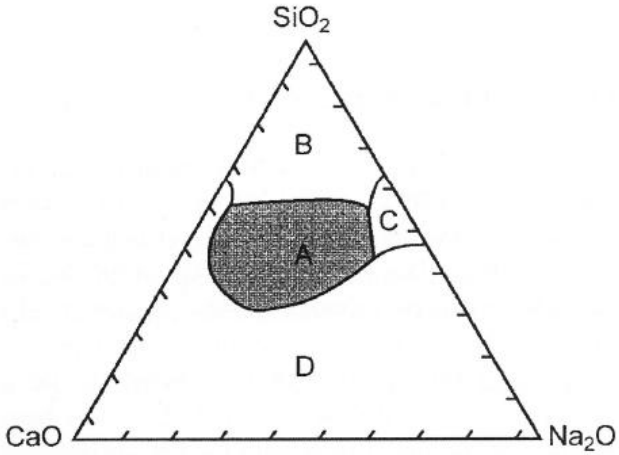

Figure 4. Ternary phase diagram showing the in vivo bioactivity of different bioactive glasses. All compositions have a constant P_2O_5 content of 6 wt%. A: Bone and tissue bonding; B: no bioactivity; C: fast dissolution or resorption; D: no glass formation (Hench et al. 1991).

From a biomaterials point of view, the bioactivity of bioactive glass is often defined by the formation of hydroxyapatite layer on top of the material and later on, the ability of the material to induce the formation of an interfacial bonding between the implant and bone. The formation of a hydroxyapatite layer happens in five stages which can be seen *in vitro*, while the seven latter stages which lead to bone bonding happen only *in vivo*.

The five reaction stages leading to the formation of a hydroxyapatite layer are (Hench 1998):

1. Ion exchange: Si-O-Na^+ + H^+ + OH^- → Si-OH + Na^+ + OH^-.
2. Loss of soluble $Si(OH)_4$ and formation of Si-OH (silanols): Si-O-Si + H_2O → Si-OH + OH-Si.
3. Condensation and repolymerization of a SiO_2-rich layer on the surface.
4. Migration of Ca^{2+} and PO_4^{3-} groups to the surface, forming an amorphous CaO-P_2O_5 film.
5. Crystallization of the amorphous CaO-P_2O_5 film by incorporation of OH^-, CO_3^{2-}.

The *in vivo* sequence leading to the formation of new tissues are (Pereira and Hench 2004):

6. Adsorption of biological moieties and growth factors on the hydroxycarbonate apatite layer.
7. Action of macrophages.
8. Attachment of stem cells.
9. Differentiation of stem cells.
10. Generation of matrix.
11. Crystallization of matrix.
12. Proliferation and growth of bone.

Through these twelve stages bioactive glass bonds to bone, is gradually absorbed and replaced by new bone tissue.

2.3.2 Sol-gel silica

A sol is a colloidal suspension of solid particles (1-1000 nm) in a liquid. It can aggregate into a particulate gel forming a sol-gel when a continuous skeleton of particles encloses the liquid phase. Many precursors can be used to form a sol-gel skeleton, including silicon alkoxide. Using silicon alkoxides as precursors, the sol-gel process has been used to produce bioactive glass (Li *et al*. 1991) and pure silica matrices with different morphologies such as monoliths (Kortesuo *et al*. 2001; Viitala *et al*. 2005b), fibers (Peltola *et al*. 2001) and microparticles (Kortesuo *et al.* 2002; Viitala *et al*. 2005a).

Silica sol-gels are typically made using tetraethylorthosilicate $Si(OC_2H_5)_4$ (TEOS) as the silicon alkoxide precursor. Silicon alkoxide works as a precursor for sol-gel reactions because they undergo hydrolysis and condensation reactions. They react with water resulting in the replacement of alkoxy groups with hydroxyl ions. After hydrolysis, two completely or partially hydrolyzed molecules link together in a condensation reaction and form Si-O-Si bonds. All these reactions are reversible. The structure and solubility of the formed sol-gel depends on many factors that influence the rate of these reactions, such as H_2O/alkoxide-ratio, pH, used solvents, reagent concentrations, aging time and temperature (Brinker and Scherer 1990). Figure 5 shows the hydrolysis and condensation reactions of silicon alkoxide.

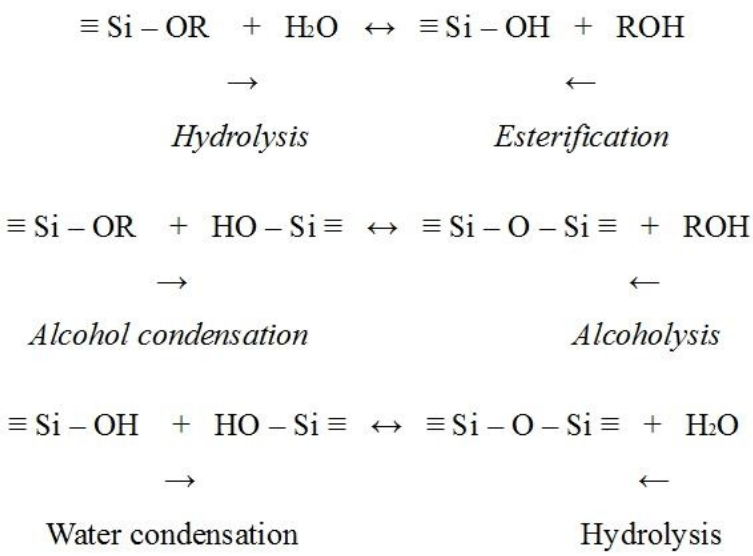

Figure 5. Schematic representation of the hydrolysis and condensation reactions of silicon alkoxide.

Once a sol-gel has formed, it is typically dried in order to give it mechanical strength and to produce monoliths or microparticles. Evaporation of liquid from the pores of the structure during drying produces capillary pressure that causes the gel to shrink. The gels can also be heat treated, which decreases the porosity and surface area of a silica xerogel (Ahola *et al*. 1999). In principle, heat treatment further condenses the matrix, lowering its dissolution rate (Brinker and Scherer 1990).

2.3.3 Dissolution of silica based matrices

As bioactive glasses and silica xerogels dissolve, they release their constitutive ions into the surrounding solution. Bioactive glasses are mainly made out of silicon, calcium, phosphorus and sodium oxides, and these are the ionic products they release during dissolution. Silica xerogels, on the other hand being made solely out of silicon oxides, release silicon ions.

2.3.3.1 Ionic products of bioactive glasses

As bioactive glass is resorbed it releases its ionic constituents into the body. Dissolving in an aqueous solution these constituents produce SiO_4^{4-}, Ca^{2+}, Na^+ and $H_2PO_4^-/HPO_4^{2-}$ ions. These ions are all physiological chemicals found in the body making it very difficult to study them *in vivo*. Studying the ionic products of bioactive glasses *in vitro* is also challenging as the conditions of *in vitro* tests have been shown

to affect the bioactivity and degradation of bioactive glasses (Ducheyne and Qui 1999; Radin *et al*. 1997). In addition to the composition of the bioactive glass, the temperature, pH, agitation rate, the type of medium used and the concentration of glass in it play a significant role in glass degradation results. Some universal aspects of bioactive glass degradation, however, can still be described.

Simulated body fluid (SBF) is a solution which contains all the essential inorganic constituents of human blood (Kokubo *et al*. 1992), and it was developed for *in vitro* studies of bioactivity and bioactive glass degradation, because it simulates conditions *in vivo*. Silicon concentration increases in SBF within a couple of hours after which silicon ion concentrations remain approximately constant (Xynos *et al*. 2000). The same effect is seen when bioactive glass is incubated in cell culture medium (Jones *et al*. 2001). The release of silicon ions is caused by the breakup of the silica network, and it is inversely dependent on the SiO_2 content of the glass—the higher the silica content, the slower the dissolution of the glass. The release of the other major constituents of bioactive glass, calcium, phosphorus, sodium, happens in another manner. They are not part of the silica network of the glass, but rather are trapped within in it, and therefore are leached out of the glass matrix. When put into solution sodium is released rapidly contributing to the increase in pH seen with bioactive glasses in a solution. Calcium and phosphorus are similarly leached from the matrix. As the alkali and alkaline earth is depleted form the surface of the glass, a silica-rich layer is formed, which decreases the leaching of ions. At his stage Ca^{2+} an PO_4^{3-} groups migrate to the silica-rich layer and starts forming a CaO-P_2O_5-rich layer which further decreases the leaching of ions and later crystallizes into a hydroxyl carbonate apatite layer (Hench and Anderson 1993).

2.3.3.2 Degradation of silica xerogel

Silica xerogel degrades in an aqueous solution by two mechanisms: Surface erosion and bulk erosion. Surface erosion occurs when the rate of erosion exceeds the rate of water permeation into the bulk of the material, causing the material to erode from the surface. Bulk erosion, on the other hand, occurs when water molecules permeate into the bulk of the matrix at a quicker rate than erosion. As a consequence molecules in the bulk of the matrix may be hydrolyzed and diffuse out of the matrix. The erosion of a silica xerogel matrix can also be an intermediate between bulk erosion and surface erosion (Langer and Peppas 1981). In both cases the degradation of the matrix happens through hydrolysis of siloxane bonds to $Si(OH)_4$ or SiO_4^{4-} ions (Brinker and Scherer 1990), which diffuse to the surrounding tissue.

2.3.4 Interaction with bone cells

Bioglass 45S5® was the first bioactive glass, and it obtained FDA approval in 1985 for middle ear prosthesis. Numerous studies *in vivo* (Hamadouche *et al*. 2001; Livingston *et al*. 2002) and *in vitro* (Bielby *et al*. 2004; Bosetti and Cannas 2005)

studies have shown that Bioglass 45S5® and other bioglass formulations with various SiO_2 concentrations stimulate bone formation. The molecular processes governing the cellular response to bioactive glasses have recently began to be unraveled, and it has been consequently shown that the release of ionic compounds are critical to bioactivity and cellular response (Tsigkou *et al*. 2007; Xynos *et al*. 2000a). The majority of studies done on gene activation by bioactive glasses and their ionic products have focused on bone formation (Christodoulou *et al*. 2005; Effah Kaufmann *et al*. 2000; Knabe *et al*. 2005; Tsigkou *et al*. 2009; Valerio *et al*. 2004). They show that bioactive glass promotes or even up-regulates several important bone related genes. The results from these studies are summarized below in Table 1.

Table 1. Gene expression in response to bioactive glass. + expressed, ++ upregulated expression. Abbreviations: FOB = fetal osteoblast, HOB = human osteoblast, ROB = rat osteoblast. References: 1 = Christodoulou et al. 2005; 2 = Tsigkou et al. 2009; 3 = Knabe et al. 2005; 4 = Effah Kaufmann et al. 2000; 5 = Valerio et al. 2004; 6 = Xynos et al. 2000a.

Gene	Function	Cell Type	Expression	Reference
Alkaline Phosphatase	Makes phosphate available for calcification.	FOB	+	1
		FOB	++	2
		HOB	+	3
		ROB	+	4
Bone sialoprotein	Promotes the initial formation of mineral crystals in bone.	FOB	++	2
		HOB	+	3
		ROB	+	4
Collagen I	The major organic component of bone matrix.	FOB	+	1
		HOB	++	3
		ROB	++	5
Osteopontin	Increases bone resorption	FOB	+	1
		HOB	++	3
		ROB	+	4
Osteocalcin	Inhibitor of mineralization, expressed by osteoblasts and osteoclasts	FOB	+	1
		HOB	++	3
		ROB	+	4
Osteonectin	Binds to collagen type I. Potential nucleator of hydroxyapatite.	FOB	+	1
		FOB	++	2
		HOB	++	3
		ROB	+	4
Cbfa1/Runx2	Master gene of bone formation.	FOB	+	1
IGF-II	Inducer of osteblast proliferation	HOB	++	6

The effects of bioactive glasses on other cell types have also been studied. BAG was found to enhance the expression of proteins involved in endochondral ossification in fetal rat chondrocytes (Asselin *et al*. 2004), and also to stimulate an inflammatory response through TNF-α secretion in mouse macrophages (Bosetti *et al*. 2002), and angiogenesis through VEGF secretion in human fibroblasts (Day 2005), which are both vital in the formation of new bone tissue.

BAG affects cellular function in many ways, such as surface chemistry and topography, rate and type of dissolution ions and shear stress at implant interfaces. For many years the emphasis of biomaterials research was concerned with the mechanisms of interfacial bonding to bone (Hench 2006). Later it was found that bioactive glass causes new bone formation outside the interface area (Wilson and Low 1992). The rate of bone regeneration was found to correlate with the release of silicon and calcium ions from the implanted BAG (Oonishi *et al*. 1999, 2000), and finally it was discovered that the ionic dissolution products of BAG influenced the differentiation of osteogenic cells (Xynos *et al*. 2000a; Hench *et al*. 2000), leading to the current hypothesis that the ionic dissolution products of BAGs stimulate osteogenic cells toward regeneration and new bone formation.

The dissolving of BAG releases its main constituents into the surrounding tissue as SiO_4^{4-} (Si^{4+}), Ca^{2+}, Na^{+} and PO_4^{3-} (P^{5+}) ions. The effects and importance of these various dissolution products remains unclear, because they are dissolved concurrently from bioactive glass. A summary of studies done on the ionic products of BAGs and their major constituents is given below in Table 2.

Table 2. Cellular effects of the ionic products of bioactive glasses. The element/s denote the type or types of ionic products studied. ↑ denotes expression, synthesis or activity has gone up in the presence of the ionic product/s. ↓ denotes expression, synthesis or activity has gone down. Alkalosis denotes the alkalinization of the surrounding medium caused by the ionic products of bioactive glasses.

Ionic Product/s	Effect	Reference
Silicon	Osteoblast proliferation ↑	Valerio *et al*. 2004
	Collagen type I synthesis in osteoblast-like cells ↑	Refitt *et al*. 2003
Si-Ca-P	Collagen formation by osteoblasts ↑	Valerio *et al*. 2004
	Mineralization, ALP activity of embryonic stem cells ↑	Bielby *et al*. 2005
Si-Ca-Na-P	ALP, osteonectin and bone sialoprotein expression, Collagen I and osteocalcin production in fetal osteoblasts ↑	Tsigkou *et al*. 2009
	IGF-II production by osteoblasts ↑	Xynos *et al*. 2000a
Calcium	Alkalosis, calcium ions for mineralization	Hench 1998
	ALP activity ↓	Xynos *et al*. 2000b
Phosphate	Phosphate ions for mineralization	Hench 1998
	Osteopontin expression ↑	Beck *et al*. 2000
	Osteoblast differentiation ↑	Beck *et al*. 2003
Sodium	Alkalosis	Hench 1998
Alkalosis	Conductive to Collagen I synthesis in osteoblast-like cells.	Bosetti *et al*. 2003
	Calcification of cartilage *in vivo* ↑	Cuervo *et al*. 1971
	Collagen I synthesis by osteoblasts ↑	Bushinsky 1996

Although the effects of the ionic constituents of bioactive glass are complex phenomenon resulting from the interaction of all the dissolved ions, some specific effects can be attributed to certain dissolved ions. Silicon ion dissolution is correlated with osteoblast proliferation and collagen type I formation (Refitt *et al*. 2003; Tsigkou *et al*. 2009; Valerio *et al*. 2004). It has also been shown to increase bone mineral density as a part of diet (Jugdaohsingh *et al*. 2005). While calcium ions by themselves reduce alkaline phosphatase activity (Xynos *et al*. 2000b), together with the release of phosphate ions they correlate with the expression of proteins commonly associated

with osteoblast differentiation and ECM formation, such as alkaline phosphatase (ALP), osteonectin, osteocalcin, bone sialoprotein and type I collagen (Bielby *et al*. 2005; Tsigkou *et al*. 2009; Valerio *et al*. 2004). Phosphate ions have been suggested to be vital for osteoblasts to form calcium phosphate deposition and matrix mineralization (Chung *et al*. 1992; Coelho and Fernandes 2000), and phosphate ion treatment correlates with the differentiation of osteoblasts and the mineralization of bone ECM (Beck *et al*. 2000, 2003; Bielby *et al*. 2003). In addition to providing calcium ions for calcium phosphate deposition, calcium ions also together with sodium ions participate in the alkalinization of the surrounding medium (Hench 1998). Alkalosis in its part enhances new bone formation, as it has been shown to facilitate type I collagen synthesis and cartilage calcification (Bosetti *et al* 2003; Bushinsky 1996; Cuervo *et al*. 1971).

3 AIMS OF THE PRESENT STUDY

The aim of the present study was to examine the effects of S53P4 bioactive glass and silica xerogel matrices on the behavior of rat bone marrow derived cells. The objective was to study the osteogenic properties of bone marrow derived cells and silica based biomaterials from the point of view of using them in such bone regeneration applications as bone tissue engineering and bone defect fillers. The following specific aims were set for this study.

1. To study the migration of implanted bone marrow stromal cells inside host tissue, and the effect of bioactive glass S53P4 and differentiation of the cells on migration.
2. To study the effect of soluble silicon and cell contacting silicon on the proliferation and extracellular matrix formation of bone marrow stromal cells.
3. To study the osteoblastic and osteoclastic differentiation of bone marrow derived cells when grown on bioactive glass S53P4.
4. To study whether the degradation of bioactive glass S53P4 is osteoclast mediated.

4 MATERIALS AND METHODS

4.1 Preparation of experimental materials

Three different types of silica-based or silica containing biomaterials were used in this thesis work: porous poly(lactide-co-glycolide)/BAG S53P4 composites for the migration studies (I; II), BAG S53P4 for the BMSC differentiation and osteoclast resorption studies (III), and sol-gel derived SiO2 microparticles for the macrophage induced ECM induction studies (IV). The materials are detailed below.

Table 3.List of all silica-based materials used in this thesis.

Material	Study
Porous silica composites	
poly(lactide-co-glycolide) 70/30	I
poly(lactide-co-glycolide) 70/30 + bioactive glass S53P4 (30 wt-%)	I
poly(lactide-co-glycolide) 70/30 + $baSiO_2$ (10 wt-%)	I
poly(lactide-co-glycolide) 70/30 + $CaPSiO_2$ (6 wt-%)	I
poly(lactide-co-glycolide) 90/10	II
poly(lactide-co-glycolide) 90/10 + Polar bioactive glass S53P4 (20 wt-%)	II
Solid bioactive glass	
Bioactive glass S53P4 plates	III
Silica microparticles	
Fast-dissolving silica microparticles heat treated at 170 ºC	IV
Slow-dissolving silica microparticles heat treated at 500 ºC	IV

4.1.1 Preparation of subcutaneous implants (I)

Macromers in 70/30 caprolactone/lactide ratio were blended with 2 wt-% of qamphorquinone cross-linking initiator (Fluka Chemica, Switzerland) to prepare porous Poly(lactide-co-glycolide) (PLGA) implants and composites with bioactive glass, sol-gel silica ($baSiO_2$) and calcium phosphate doped silica ($CaPSiO_2$). The synthesis of branched poly(ε-caprolactone/D,L-lactide) macromers with controlled monomer ratios and four methacrylate end groups is described in detail elsewhere (Helminen *et al*. 2002). For BAG composites, 30 wt-% of BAG S53P4 granules (Vivoxid, Finland), for $baSiO_2$ composites, 10 wt-% silica sol-gel, and for $CaPSiO_2$ composites, 6 wt-% of calcium doped silica sol-gel was added to the mixture. The composition of the BAG was 53 wt-% SiO2, 23 wt-% Na_2O, 20 wt-% CaO and 4 wt-% P_2O_5, and the granule size was <45 µm. The composition of the $CaPSiO_2$ granules

was $CaO-P_2O_5-SiO_2$ with a molar ratio of 35-5-60 and the granule size was <45 µm, and the $baSiO_2$ was pure silica with a granule size <90 µm. The preparation of these silica sol-gels is described in more detail elsewhere (Korventausta *et al*. 2003). The constituents were mixed in a Brabender W50EH batch mixer (Brabender, Germany), using 50 rpm at 50 °C for five minutes. In order to obtain a final scaffold structure with either 70 V-% or 80 V-% porosity, corresponding amounts of sieved $CaCl_2 \cdot 6H_2O$ crystals (400 – 500 µm; Riedel de Haën, Germany) were manually added to the mixture at room temperature. The mixture was then cast to a plate (2 mm thick), and cured in a Triad 2000 Light Curing System (350-550 nm; DeguDent, Germany) for thirty minutes on both sides. The plates were immersed in ethanol overnight to leach the salt particles. Porous scaffolds, 6 mm in diameter, were punched out from the leached plate, and porogen removal was ensured with three additional ethanol washes (two hours each). The scaffolds were dried and sterilized with gamma radiation (25 kGy) before use.

4.1.2 Preparation of porous PLGA and PLGA/BAG composites (II)

PLGA 90/10 and PLGA/BAG 90/10 (20 wt-%) composites were made by solvent casting using dichloromethane (DCM) as a solvent. Polar composites were made as follows. A uniform layer of BAG S53P4 (20 wt%) was applied on the bottom of a Teflon-coated form. The composition of the BAG was 53 wt-% SiO2, 23 wt-% Na_2O, 20 wt-% CaO and 4 wt-% P_2O_5, and the granule size was 45-90 µm. PLGA/DCM solution (25 w/v% (g/ml)) was poured onto the BAG layer. The solvent was first slowly evaporated (4 °C; 1 week) after which the forms were placed in a fume cupboard at room temperature (48 h). The prepared films were stored in a desiccator until further use. Films were processed into porous scaffolds by gas foaming with high-pressure CO_2 in an autoclave (50 bar) for 1 h after which the gas was rapidly released from the vessel (5 s). The porous composite films were stored under nitrogen in a desiccator (RT) prior to use. This method has been described in detail in previous work (Orava *et al*. 2007).

4.1.3 Preparation of glass plates for resorption studies (III)

Bioactive glass S53P4 was prepared as described earlier (Anderson *et al*. 1991). The glass was supplied as 12-mm thick rods, which were cut into 1.5-mm thick slices with a diamond saw, polished with ultra fine sandpapers, and the smoothness of the surface was checked with a microscope. The total reactive surface area including the top and the sides, but not the bottom as it sits against the plastic cell culture dish, was 216 $mm^{2.}$ The volume of cell culture medium to be used in the resorption study was 3 ml or 3000 mm^3, giving a SA/V ratio of 0,072. The procedure was carried out in absolute ethanol to protect the glass plates from moisture. The polished plates were stored in 96 % ethanol at room temperature before use.

4.1.4 Preparation of silica microparticles (IV)

The SiO_2 microparticles were prepared by the hydrolysis and polycondensation of tetraethoxysilane (TEOS, 98 %, Aldrich). An H_2O/TEOS mole ratio of 2, an EtOH/TEOS ratio of 1 was used, and the pH of 2.0 was used. Microparticles were prepared by spraying the silica sol with a mini spray dryer (B-191, Büchi Labortechnik AG, Switzerland). One group of microparticles was post heat treated at 170ºC for 1 h and another group at 500ºC for 1 h to obtain sterilized microparticles with different SiO_2 dissolution rates. The preparations were named CJ08_170 and CJ08_500, respectively.

4.1.4.1 SiO_2 dissolution (IV)

SiO_2 xerogel matrix dissolution of microparticles was measured in autoclaved 0.005 M TRIS (Trizma® pre-set Crystals, Sigma) solution buffered to pH 7.4, T = 37 ºC, with continuous shaking at 60 rpm in a water bath (HAAKE SWB25). The saturation level of SiO_2 at pH 7.4 is about 130-150 mg/l and therefore the SiO_2 concentration in the dissolution medium was kept below 30 mg/l to ensure the free dissolution of the SiO_2 matrix. 5 ml samples of dissolution medium were removed at 5, 10, 24, 52, 74 and 99 h for testing and the dissolution medium was increased with enough fresh TRIS in order to keep the increasing SiO_2 concentration below 30 mg/l. The Si concentration of the samples was then measured with a spectrophotometer (UV-1601, Shimadzu), by analyzing the molybdenum blue complex absorbance at 820 nm (Koch *et al*. 1998).

4.2 Cell seeding on scaffolds (I, II)

4.2.1 Subcutaneous implantation experiments (I)

BMSCs were isolated from male Sprague-Dawley rats and expansion cultured. After seven days of expansion culture, the adherent osteoblast like cell population was enzymatically detached (0.25% trypsin / 0.02% EDTA (Sigma)), and a stock of 3.8 x 10^6 cells/ml was prepared in osteogenic culture medium (alpha-MEM, antibiotics supplemented with 15% fetal bovine serum (Gibco), 50 µg/ml of ascorbic acid (Sigma), 8.5 mM Na-β- glycerophosphate (Merck, Darmstadt, Germany), and 10 nM dexamethasone (Sigma)). Each PLGA scaffold was subsequently soaked with a 50 µl drop of the cell suspension (190000 cells / scaffold) for 15 minutes, and 0.5 ml of culture medium was then carefully added to the scaffolds. After one hour of adhesion, further 1.5 ml of medium was added and the cell-scaffold constructs were cultured overnight. The following day scaffolds were placed into serum-free medium, and translocated to the animal facility. Substrates were kept at 37°C during surgery, and washed with physiological saline solution before implantation.

4.2.2 Femoral scaffolds (II)

Freshly isolated BMSCs were expanded for one week and then seeded and differentiated in porous scaffolds for two weeks. Prior to cell seeding, the polymer discs 10 mm in diameter were sterilized in 70% ethanol for 1 h and then washed twice in PBS. The discs were then incubated over night in α-MEM without serum at 37ºC and put into 24 well plates.

For femoral implant experiment I, the PLGA scaffolds were then seeded with 50000 expanded BMSCs and differentiated using three protocols. In group I, the seeded cells were kept in expansion medium, i.e. α-MEM with antibiotics and 20% FCS for two weeks. In group II, the cells were grown in proliferation medium for one week and then given osteogenic medium (α-MEM, antibiotics, FCS, ascorbic acid, β-glycerophosphate and dexamethasone) during the second week. In group III, the cells were given osteogenic medium for two weeks with dexamethasone present only the first week. For femoral implant experiment II, the cells were seeded into porous PLGA scaffolds and polar porous PLGA BAG S53P4 composites, and given osteogenic medium for two weeks with dexamethasone present only the first week. All cultures were done in a humidified incubator at 37ºC and 5 % CO_2.

After two weeks some of the seeded implants were either stained for ALP using p-nitrophenol as a substrate to show the presence of live cells inside the scaffolds (Sigma; Kit 86-r) and implanted into rat femoral bone defects according to the following procedure.

4.3 Implantation procedure (I, II)

The animal used in the following experiments were housed individually in their cages with free access to food pellets (RM1, Special Diet Services Ltd, Witham, Essex, UK) and drinking water. The animal facilities are managed according to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes and inspected for GLP Compliance. The study design was approved by the Animals Ethical Committee of the State Provincial Office of Southern Finland. In all cases, the animals were killed by cervical dislocation under CO_2 anesthesia.

The migration of cells inside the host was studied using sex specific SRY sensitive PCR, which detects DNA from only male cells. BMSCs were extracted from male Sprague-Dawley rats and later implanted into their female siblings. A flow-chart illustrating study set-up is given below in Figure 6. For the subcutaneous experiments, syngeneic Fisher 344 rats were used.

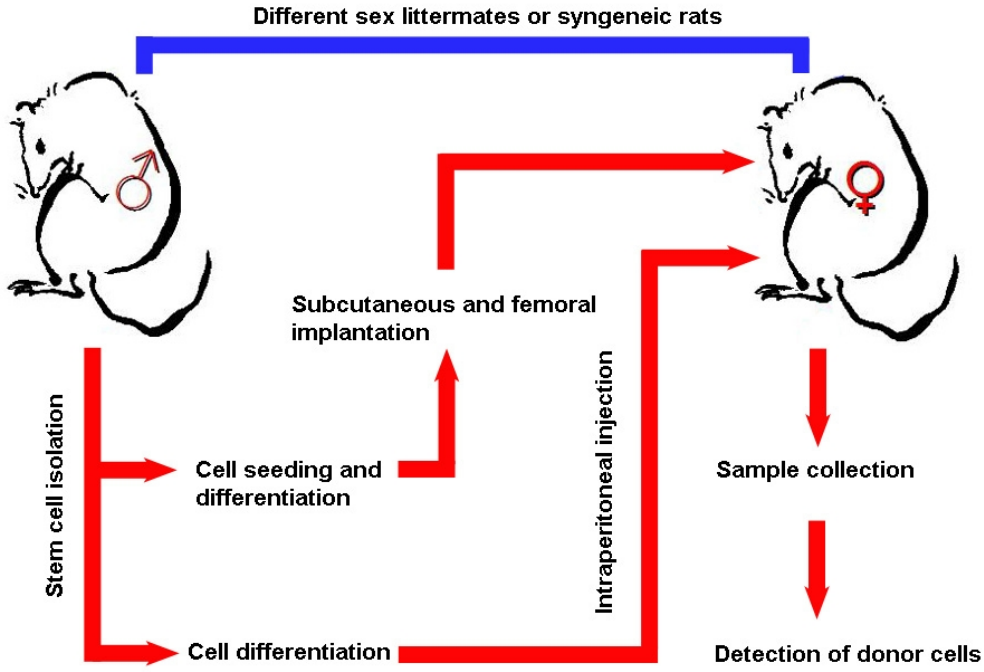

Figure 6. Schematic representation of the intraperitoneal and femoral implantation migration experiments.

4.3.1 Subcutaneous implantation (I)

Cultured cell-scaffold constructs were implanted in 12 nine-week-old female (135 to 155 g) syngeneic Fisher 344 rats. Subcutaneous implantation was performed through longitudinal incisions in the midline of the dorsal skin under general anesthesia induced by subcutaneous injection of 0,15-0,2 ml/100 g rat weight Hypnorm (Jansen Pharmaceuticals, Belgium):Dormicum (Roche, Switzerland):sterile water (1:2:2). Individual implant beds were prepared by blunt dissection. Each animal received four implants—a plain 70/30 PLGA implant, a BAG composite, a baSiO_2 composite and a $CaPSiO_2$ composite. Skin wounds were closed in two layers with individual resorbable sutures (Ethicon Monocryl 5-0, Johnson & Johnson Intl, Belgium). The animals were sacrificed after one, four and twelve weeks respectively, and the implants and the organ samples from the lungs, spleen, liver, thymus and bone marrow were retrieved, and fixed in 4% buffered formalin at 8 °C for two weeks.

4.3.2 Intraperitoneal implantation (II)

For the intraperitoneal experiment, 18 female Sprague Dawley rats weighing about 200 g were used. These eighteen animals were divided into six different groups, which each had a unique combination of BMSC differentiation protocol and implantation time. 1 ml of the cell suspension (100000 cells/ml) was injected intraperitoneally into the right lower abdomen of the rats.

The rats were killed 1 or 2 weeks after injection and the samples of lung, spleen, liver, thymus and bone marrow from the femur were collected for DNA extraction. There was one group of three animals of each differentiation protocol for both time points. The different protocols and the roman numerals designating the different groups are given below in table format.

Table 4. Differentiation protocols used in the intraperitoneal experiment.

Intraperitoneal	MSC differentiation	Implantation time
Group I, IV	expansion culture only	I-III one week, IV-VI two weeks
Group II, V	1 week in α-MEM + 1 week in osteogenic media	
Group III, VI	2 weeks in osteogenic media, 2nd week w/o dexa	

4.3.3 Femoral implantation experiments (II)

For the femoral implantation study, 15 one-year old Sprague-Dawley rats weighing at least 300 g were used. General anesthesia was induced by subcutaneous injection of 0,15-0,2 ml/100g rat weight Hypnorm (Jansen Pharmaceuticals, Belgium):Dormicum (Roche, Switzerland):sterile water (1:1:2). Bone defects of 2.4 x 3 x 8 mm were made with a dental drill (rose bur) in the anterolateral part of subtrochanteric femur. Each femoral implant (2.5 x 3 x 8 mm) was inserted into the left or right femur of the rats. The polar glass composite material was inserted with the BAG containing side to the dorsolateral side. After the operation 100 µl of 0,3 mg/ml buprenorphin (Temgesic®, Leiras Finland) was injected subcutaneously to relieve postoperative pain. Further details of the surgical procedure can be found elsewhere (Ekholm *et al*. 2005).

After four weeks the animals were killed by cervical dislocation under CO_2 anesthesia and the implants and the organ samples (i.e. opposite femur and pieces of the spleen, liver, kidney, lung, heart, thymus and the skin from the defect area, and blood) were collected for DNA analyses. The sample from the heart was taken from the apex in order to avoid blood contamination.

A table of the differentiation protocols and the material used is given below.

Table 5. Differentiation protocols and materials used in the implant experiments.

Implant I	Material	MSC differentiation
Group I	PLGA	2 weeks in plain medium
Group II	PLGA	1 week in α-MEM + 1 week in osteogenic media
Group III	PLGA	2 weeks in osteogenic media, 2nd week w/o dexa
Implant II		
Group I	PLGA	2 weeks in osteogenic media, 2nd week w/o dexa
Group II	PLGA + BAG	2 weeks in osteogenic media, 2nd week w/o dexa

4.4 Macrophage isolation and culture (IV)

Macrophages were isolated from two month old Sprague Dawley rats by lavaging their peritoneal cavities. The animals were killed by cervical dislocation under CO_2 anesthesia and then 5 ml of physiological saline containing heparin (5 I.U./ml) was injected in their peritoneal cavities using a 20 g needle. The abdomens of the rats were massaged for five minutes to detach as many macrophages as possible and the the saline then collected by opening the peritoneal cavity with a scalpel and by drawing the saline into a syringe. All samples showing a red tinge, characteristic for blood cell contamination, were discarded. The cells were used fresh straight after isolation.

The SiO_2 microparticle experiment was conducted in two separate phases. First, SiO_2 microparticles were cultured with rat peritoneal macrophages to induce the secretion of growth factors, and the medium was removed for later use. Second, the growth factor rich medium was used as an additive in BMSC cultures in which the proliferation of cells and their secretion of collagen into the medium was measured. SiO_2 microparticles were cultured with BMSCs as controls to study whether their effect on BMSCs was direct or macrophage induced. The set up of the experiment is illustrated in Figure 7 below.

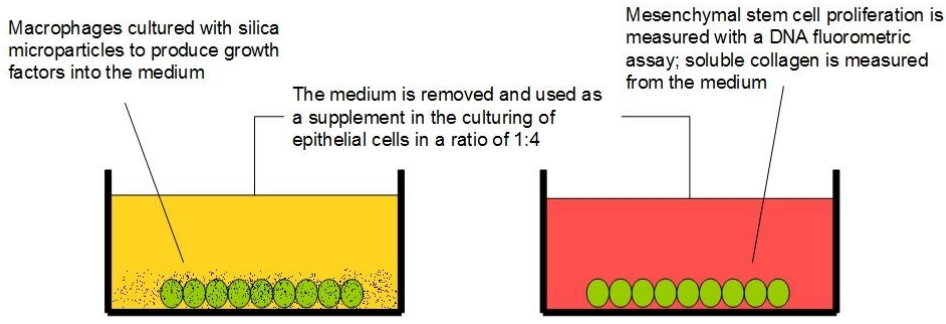

Figure 7. A schematic showing the basic set up of the microparticle culture experiment.

In the first phase, 50000 macrophages from rat peritoneal cavities were seeded on the 24-well plates (Nunc, Roskilde) and cultured for five days in 1 ml of RPMI medium (Gibco, USA) in a humidified incubator at 37 ºC and 5 % CO_2 without changing the media to maximize the concentration of growth factors secreted by the macrophages. One third of the wells contained fast dissolving CJ08_170 microparticles, one third slow dissolving CJ08_500 microparticles and on third contained only macrophages. After the fifth day, the macrophage treated medium was removed with a syringe and sterile filtered Millex® syringe filter (Millipore, USA) and used fresh as an additive in BMSC cultures.

In the second phase, 25000 BMSCs that had been isolated from six week old male rats' femurs and expansion cultured for a week were plated onto 24 well cell plates (Nunc, Roskilde) and cultivated in 1 ml of serum-starved (0,5 % FCS) α-mem containing 50 µg/ml β-aminopropionitrile and 50µg/ml ascorbic acid to inhibit the precipitation of secreted collagens. Macrophage treated medium was used as an additive in the cultures in a ratio of 1:4. The amount of soluble collagens was assayed from the culture media on days one and five using the Sircol assay (Biocolor, Belfast, N. Ireland) and the proliferation of BMSCs using Hoechst 33258-based fluorescent spectrometry of cellular DNA (Rago *et al*. 1990).

4.5 General laboratory methods

The general laboratory methods used in this thesis are summarized in table 5.

Table 6. General laboratory methods.

Method	**Described in**
Isolation of bone marrow stromal cells	I, II
Isolation of bone marrow derived cells	III
Preparation of tissue sections	I, II
Light Microscopy	I
Isolation of DNA	I, II
PCR	I, II
Tartrate resistant acid phosphatase	III
Alkaline phosphatase staining	II, III
Hoechst staining	IV
Sircol assay	IV
DNA fluorometric staining	IV
Scanning electron microscopy	IV

5 RESULTS

5.1 Implantation of BMSCs (I, II)

Subcutaneous implants and various tissues (see Table 5) were collected at one, four and twelve weeks postoperatively. Tissues from intraperitoneally implanted rats were collected one and two weeks after cell infusion with three different cell types. Femoral implants and tissues were collected at four weeks postoperatively. All tissue types and time points are illustrated in Table 6.

5.1.1 Subcutaneous implants (I)

There were no obvious structural differences between the polymer scaffolds and the composite scaffolds containing BAG, silica sol-gel and calcium phosphate silica sol-gel. The scaffolds were encompassed in a thin fibrous capsule, and were well integrated into the surrounding tissue. No multinucleated giant cells were observed. After one week loose immature fibroconnective tissue with capillaries filled the periphery of the implant, while the innermost 30% of the scaffold cross-section was characterized by numerous empty pores with clusters of erythrocytes. Occasionally different types of cluster were also seen, likely originating from the implanted BMSCs, although this could not be confirmed. There were no notable differences between the scaffold types at this time point. At week four, tissue ingrowth was observed throughout the scaffolds. There was well organized connective tissue with good vascularization, and some fat tissue was seen in the periphery of the scaffolds. Also, several loci with immature mineralizing tissue was observed. The scaffolds containing BAG and calcium phosphate silica sol-gel had more mineralizing tissue than polymer scaffolds or scaffolds with silica sol-gel. At week twelve, the amount of fat was slightly increased but the appearance of vascularized fibroconnective tissue was similar to that of four weeks. More bone formation was again observed in the scaffolds containing BAG and calcium phosphate silica sol-gel, but the amount of mineralized tissue had not increased from four weeks.

5.1.2 Femoral implants (unpublished)

Micrographs taken from the defect area (Figure 8) at week four show the formation of new bone around both implants with and without BAG. The growth of new bone was stronger around the implant with BAG (Figure 8A), but the same reaction was not seen within the implant. The implants without BAG (Figure 8B) had islets of newly formed bone, while the BAG containing implants are filled by a large number of cells. These possibly inflammatory cells were not found in plain 90/10 PLGA implants.

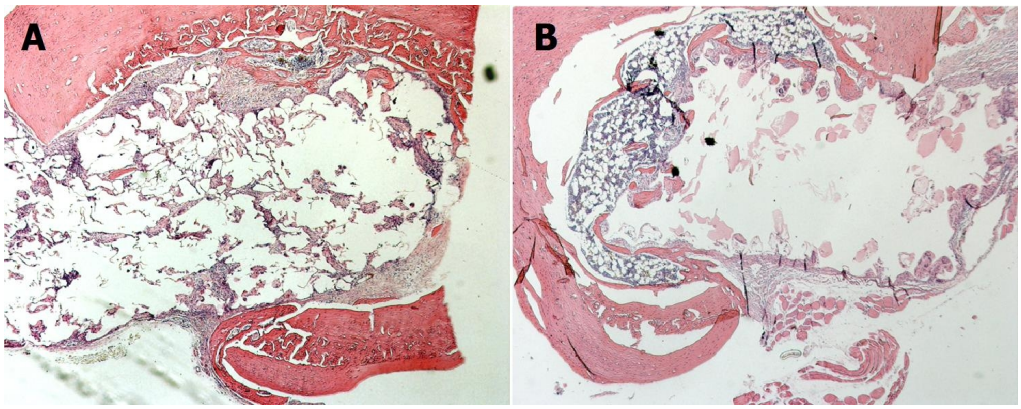

Figure 8. x100 magnification of the femoral defect area. A = implant with BAG has numerous cells and new bone formation around the edges. B = implant without BAG is almost completely devoid of cells and has only islets of mineralized tissue.

5.1.3 BMSC migration inside the host (I, II)

Subcutaneously implanted osteogenically differentiated BMSCs were found only in thymus of one animal out of four at week one, whereas at four weeks the lung, spleen and liver of all animals, and the thymus and bone marrow of one animal each contained donor cells. At the end of the experiment at twelve weeks, no cells were found in any of the tested tissues.

Intraperitoneally injected BMSCs were detected in the lung, thymus and spleen of all animals at both one and two weeks regardless of the cell culturing protocol. The liver of most animals was positive for donor cell DNA, except for one animal in differentiation groups III and V, respectively. Donor cells were also found in the bone marrow of all animals except for one in group I and one in group III.

Femorally implanted BMSCs had migrated to all studied tissues except the kidney at least in one animal/differentiation group at four weeks. The spleen and skin tested positive for all animals, as did the implants. BAG affected the implanted BMSCs negatively; no donor cell specific DNA was found in any of the tested tissues.

A master chart showing the results of all the experiments is given below.

Table 7. Distribution of donor bone marrow stromal cells inside the host. IP=intraperitoneal injection; SC=subcutaneous implantation; FM=femoral implantation. Numerals after methods denote the differentiation protocol used for the implanted cells. 1=expansion cultured BMSCs; 2=BMSCs osteogenically differentiated for one week; 3= BMSCs osteogenically differentiated for two weeks. + denotes at least one positive parallel sample, ++ denotes all parallel samples positive, - denotes no positive samples.

Time point	1 w				2 w			4 w				12 w
Lung	-	++	++	++	++	++	++	++	+	-	+	-
Bone marrow	-	+	+	+	+	+	+	+	+	+	+	-
Thymus	+	++	++	++	++	++	++	+	+	++	+	-
Liver	-	++	+	++	++	+	++	++	+	+	+	-
Spleen	-	++	++	++	++	++	+	++	++	++	++	-
Heart									+	+	+	
Kidney									-	-	-	
Skin									++	++	++	
Blood									+	+	+	
Implant									++	++	++	
Method	SC 2	IP 1	IP 2	IP 3	IP 1	IP 2	IP3	SC 2	FM1	FM2	FM3	SC 2
n	4	3	3	3	3	3	3	4	3	3	3	4

5.2 Bone marrow derived cell cultures on bioactive glass slides (III)

Bone marrow derived cells were isolated from long bones of newborn rats. The cells were cultured on BAG slides or on plastic and studied 1-5 days after plating.

5.2.1 Cell viability and proliferation on bioactive glass

Bone marrow derived cell nuclei were visualized with DNA-binding fluorochrome Hoechst 33258, photographed with x100 magnification and counted at days one through five. Cells with round healthy nuclei were deemed viable and cells with condensed pycnotic nuclei, apoptotic. Osteoclasts with any pycnotic nuclei were counted as apoptotic. Examples of the photographs used for cell viability measurements are given below in Figure 9.

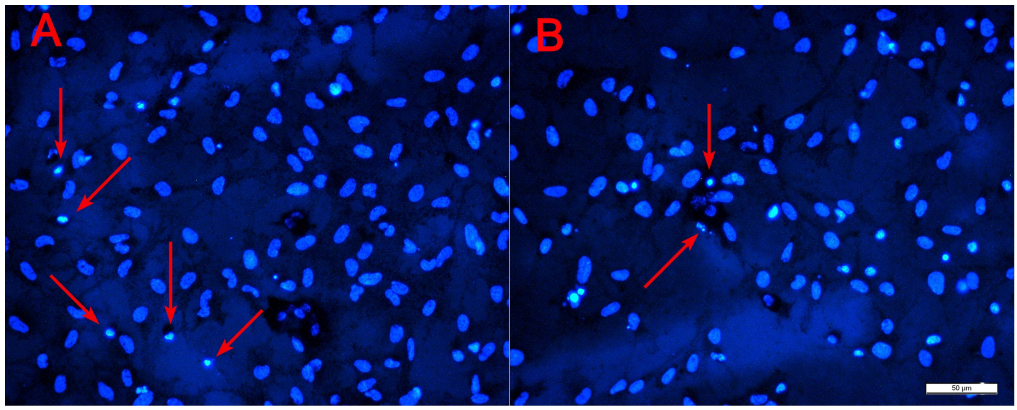

Figure 9. Pycnotic osteoblast (A) and osteoclast (B) nuclei visualized by hoechst staining. x100 magnification.

Cell numbers on BAG S53P4 and on cell culture plastic grew steadily through days one to five. No statistically significant differences were seen in the number of viable cells on either material. The number of apoptotic cells was also similar between these two groups at every time point except on day one. At that time the number of apoptotic cells was almost three times higher on BAG than on plastic. Although the difference in viable cells between BAG and plastic at day one was not statistically significant, there were less viable cells on BAG. After the first day, however, the numbers of viable cells on BAG recovered, and their proliferation resembled the proliferation of cells on plastic.

5.2.2 Osteoblastic phenotype of the cells

Bone marrow derived cells grown on BAG S53P4 and on cell culture plastic were stained with ALP to visualize the extent of their osteoblastic phenotype. Both types of materials showed osteoblastic cells on day one with roughly half of the cells staining positive, but the progression of the phenotype differed greatly depending on the material they were grown on. The cells grown on BAG became increasingly osteoblastic during the culture and by the fifth day almost all cells stained positive for alkaline phosphatase whereas the cells cultured on plastic behaved oppositely. On plastic, they started to lose their osteoblastic phenotype, becoming more fibroblastic, with only a fifth of the cells staining positive for ALP at day five.

5.2.3 Osteoclastic phenotype of the cells

Bone marrow derived cell cultures on BAG S53P4 and cell culture plastic were stained for tartrate resistant acid phosphatase (TRACP) to visualize the osteoclastic phenotype of bone marrow derived cells in the culture. TRACP positive cells were found both on BAG and plastic. On glass osteoclastic cells and their precursors attached firmly, enlarged and looked healthy and viable, became multinuclear but did

not proliferate in cultures. Some osteoclastic cells were connected by cytoplasmic extensions of variable lengths. On BAG osteoclastic cells maintained their phenotype all the way through to day five. On plastic, however, these cells suffered, became rounded, and detached from the culture vessel, and many of them started to die on the second and third days of culture.

5.2.4 Degradation of bioactive glass in cell culture

To clarify the role of osteoclastic cells in the degradation of BAG S53P4, the surface of the glass was studied in a bone marrow derived cell culture using conventional and electron micrographs. Microscopic examination of the surface of the BAG slides showed rapid erosion as rounded pits emerged from day one and progressed to cracks and elongated valley-like formations on days two to five. These erosions were not, however, associated with osteoclastic TRACP positive cells. Pits were found universally along the surface of the slide, and in a few cases where an osteoclastic cell had migrated, it revealed a smooth unblemished patch of glass, which was often the only in its kind on the whole slide. Electron micrographs confirmed this finding. Pits and erosions were found unassociated with osteoclastic multinuclear cells, and in one micrograph where the edge of the cell had detached and folded, a smooth surface was once again found. It can therefore be said that the degradation of the glass S53P4 in the bone marrow derived cell culture was due to dissolution by the cell culture medium, and not to lytic capabilities of the osteoclastic cells. Micrographs showing the smooth glass surface under the osteoclastic cells are given below in Figure 10.

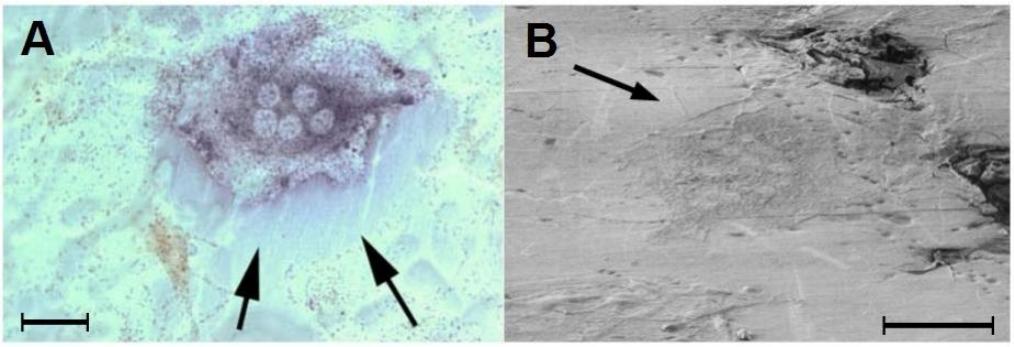

Figure 10. Pits and erosions on bioactive glass surface shown on conventional micrograph on day three (A) and electron micrograph on day two (B). Smooth glass surface revealed from under osteoclastic cells marked with arrows. Scale bar = 25 µm (A) and 50 µm (B).

5.3 Macrophage induced effect of particulate silica on BMSCs (IV)

The effect of the medium from rat peritoneal macrophages cultured in the presence of silica microparticles was studied on serum starved BMSCs culture for 1-5 days on plastic.

5.3.1 Scanning electron microscopy imaging of microparticles

Scanning electron microscopy (SEM) images confirmed that heat treatment did not change the shape or size of the microparticles. The size distribution was between 2 and 15 μm after heat treatment at 170 and 500 ºC. The microparticles were spherical in both groups, but showed a slightly crumpled surface morphology probably due to shrinkage of the matrix.

5.3.2 SiO_2 dissolution from microparticles

Heat treatment dramatically lowered the rate of SiO_2 dissolution from the microparticles. In the group treated at 170 ºC (CJ08_170) for one hour the microparticles had dissolved completely after four days in sink conditions, with the amount of dissolved silica plateauing at 70% of the theoretical mass of tested silica microparticles. In the group heat treated at 500 ºC (CJ08_500) SiO_2 dissolution was almost nonexistent. After four days in 0.005 M TRIS under 1% of silica had dissolved from the microparticles. This difference was statistically significant, and a typical reaction to the condensation of the matrix which occurs in high temperatures.

5.3.3 SiO_2 microparticle induced BMSC proliferation

BMSCs cultured in serum-starved control media with microparticles did not proliferate in the course of five days. The presence of microparticles, whether heat treated at 170 or 500 ºC, did not affect the proliferation of cells. In the cell cultures with added macrophage medium, however, the situation was opposite. Medium taken from macrophages cultured with both types of microparticles, and also without microparticles, showed a statistically significant induction of stem cell proliferation in serum-starved conditions. Medium from macrophages cultured with slow-dissolving silica microparticles showed the biggest increase in cell numbers with 41.1% more BMSCs at the five day time-point than the day one starting point. Medium from macrophages cultured with fast-dissolving microparticles increased the cell numbers by 22.7%, making it the second most proliferation inductive group, whereas medium from macrophages grown without the influence of any microparticles at all showed an increase of 12.8% in cell numbers, the lowest significantly different increase in induction.

5.3.4 Effect of macrophage treated medium on collagen secretion

At day one, macrophage treated medium induced the secretion of collagen into the culture medium by BMSCs. The induction was strongest in the BMSCs treated with medium from macrophages grown with fast-dissolving microparticles (CJ08_170) and weakest in the cells treated with medium from macrophages grown with slow-dissolving microparticles (CJ08_500), with the BMSCs treated with medium from macrophages grown without the influence of microparticles inducing a level of collagen secretion between these two groups. The levels of soluble collage, however, were low and the differences between the groups not statistically significant.

The differences in collagen secretion of macrophage medium treated BMSCs compared to non-treated controls after five days of culture mirrored the differences seen after the first day. They were, however, larger and also statistically significant, except in the case of BMSCs treated with medium from macrophages grown with slow-dissolving (CJ08-500) microparticles. The amount of collagen secreted by BMSCs treated with medium from macrophages grown on fast-dissolving (CJ08-170) microparticles was significant compared to its control and eleven-fold stronger compared to the slow-dissolving microparticle group. Collagen secretion from BMSCs treated with macrophages that had not been influenced by microparticle was also significant, with an increase of five and a half folds greater compared to the slow-dissolving microparticle group, and almost half when compared to the fast-dissolving microparticle group.

6 DISCUSSION

6.1 *In vivo* evaluation of implants

Previous reports have shown an increase in *in vitro* osteogenicity in composite scaffolds with BAG (Yao *et al*. 2005). The findings of this study, where subcutaneously implanted scaffolds containing BAG and calcium phosphate silica sol-gel had more mineralizing tissue at week four than polymer scaffolds or scaffolds with silica sol-gel, are in line with these, but also suggest that calcium and phosphate increase the osteopromotive capabilities of silica. The amount of mineralized tissue, however, did not increase after the fourth week. This is to be expected as the expression proteins associated with bone formation, such as collagen type I, II and X, aggrecan and decorin, are known to peak around 3-5 weeks after trauma (Ekholm *et al*. 1995). This result may also be augmented by the migration of BMSCs out of the scaffold into other tissues.

More mineralized tissue was seen in femorally implanted BAG containing PLGA scaffolds compared to plain polymer scaffolds, which contained only islets of mineralized tissue and very few cells. The BAG containing scaffolds, on the other hand, had a large number of cells. As these cells did not test positive as Y chromosome specific donor cells, it can be speculated that they are inflammatory cells. There is also a possibility that the BAG interfered with the extraction of DNA from the implants, giving a false negative result.

6.2 Migration of bone marrow stromal cells

It has been well documented that a small amount of BMSCs continually circulate in the bloodstream (Roufosse *et al*. 2004), and that they mobilize into peripheral blood in response to trauma, such as burns and skeletal muscle injuries (Mansilla *et al*. 2006; Ramirez *et al*. 2006). Many studies have also shown that BMSCs migrate inside the host when injected systemically (Barbash *et al*. 2003; Hara *et al*. 2008) or intraperitoneally (Gao *et al*. 2001; Gordon *et al*. 2008) and that they engraft to sites of tissue injury and inflammation (Pereira *et al*. 1995). In this study the distribution of subcutaneously, intraperitoneally and femorally implanted BMSCs into various target tissues was evaluated using the widely accepted method of detecting Y-chromosome specific sequences originating from male donor cells in female host tissue using PCR amplification (Suttorp *et al*. 1993; Tashiro *et al*. 1994). Female tissues are negative for the Y chromosome except in some cases if they have given birth to male offspring (Nguyen *et al*. 2006), and the recipient rats in this study had not been pregnant.

Several studies show that systematically transplanted BMSCs migrate most readily into the lungs (Assis *et al*. 2009; Basbash *et al*. 2003; Gao *et al*. 2001; Pereira *et al*. 1995, 1998). This accumulation in the lungs is attributed to the comparatively large size of bone marrow derived stromal cells (Koc *et al*. 2000) and their expression of

adhesion molecules (Dennis *et al*. 1992), which cause them to get trapped in the capillaries of the lungs. This tendency is of clinical significance as respiratory failure is one of the most common complications after bone marrow transplantation (Bojko and Notterman 1999; Leveneu *et al*. 1999). In this study, BMSCs migrating from all three implantation sites got trapped in the lungs, but there was less lung entrapment of femorally implanted cells, probably due to the slower release rate of cells escaping from the implants compared to intraperitoneally injected cells.

A similar result as in the lungs was also seen in the migration of BMSCs into the spleen and liver. Systematically transplanted bone marrow stromal donor cells have been reported in many to be found in the spleen (Assis *et al*., 2009; Barbash *et al*., 2003; Gao *et al*., 2001), and the liver (Assis *et al*. 2009; Barbash *et al*. 2003). It has been suggested that they are similar kind of size dependent barrier for transplanted cells as the lungs. Our finding, where donor cells are found in the spleens of all animals and the livers of almost all of them after intraperitoneal implantation, and where the migration pattern of BMSCs to the spleen and liver from subcutaneous implants is similar to the migration into the lungs, gives credibility to this conclusion.

Several studies report that systematically administered BMSCs are capable of homing to the bone marrow (Allers *et al*. 2004; Erices *et al*. 2003) and the thymus (Li *et al*. 2000, von Lüttichau *et al*. 2005). Both are lymphopoietic organs important in the generation of T lymphocytes. These cells are formed in the bone marrow and migrate to the thymus to undergo maturation. Although thymus is the primary site where T cell maturation occurs, the bone marrow can provide a suitable support for T cell maturation if the thymus is missing (Dejbakhsh-Jones *et al*. 1995). In this study, transplanted BMSCs seem to favor homing to the thymus instead of the bone marrow, especially after intraperitoneal injection. Adhesion molecules, such as chemokines receptors, appear to have an important role in tissue specific homing of bone marrow derived progenitor cells (von Lüttichau *et al.* 2005, Wynn *et al.* 2004; reviewed in Fox *et al.* 2007) that do not get trapped in the size dependent barriers. Since the transplanted cells circulate around in the host body passing both tissues innumerable times it can be speculated that the receptors expressed by these cells promote active homing to the thymus in preference to bone marrow. The homing ability of the cells to the bone marrow might be reduced due to culturing of the cells prior implantation (Rombouts and Ploemacher 2003).

Osteogenically differentitated cells seem to retain their homing capacity into both thymus and bone marrow when administered intraperitoneally or implanted into a femoral defect. However, following subcutaneous implantation, differentiated BMSCs were not found in the bone marrow at all at one week and only one rat out of four had transplanted cells in its thymus at the same time point. At four weeks, one animal had donor cell in thymus and another one in the bone marrow. Either the subcutaneously implanted donor cells had been destroyed before four weeks or they lack active adhesion molecules for these organs.

Transplanted BMSCs are also detected in high self-renewal tissues, like small intestine, salivary gland and skin (von Lüttichau *et al.* 2005) including wounded skin (Mansilla *et al.* 2006). Therefore, it was not a surprise that strong signals were detected in all skin samples taken from the wound area after femoral implantation. Numerous studies show that BMSCs may contribute to the regeneration of skin (Kataoka *et al.* 2003; Nakagawa *et al.* 2005), and that cells from the bone marrow contribute to a significant amount of structural, i.e. non anti-inflammatory, cells in it (Fatkhe *et al.* 2004; Deng *et al.* 2005; Brittan *et al.* 2005).

It is well known that transplanted BMSCs migrate into ischemic heart tissue (Abbott *et al*. 2004; Freyman *et al*. 2006). Interactions between coronary artery epithelium and BMSCs have been detected in ischemic hearts and in animals pretreated with inflammatory cytokines (Segers *et al*. 2006), and intravenous BMSC injection has been shown to increase left ventricular ejection fraction of infarcted pig hearts (Price *et al*. 2006). Intravenously infused BMSCs also home to intact myocardium, although to a much lesser extent (Barbash *et al*. 2003). In this study, however, femorally implanted BMSCs readily migrated into healthy heart tissue. This is a novel observation, and it is not currently clear why this happens.

Positive signals were detected in almost all tested tissues after femoral implantation, including in the blood of some animals. Bone is a highly vascularized tissue and it seems that this facilitates the capability of implanted BMSCs to enter the bloodstream and thereupon almost any organ in the body. There is, however, fairly limited knowledge on the time frame of BMSC migration. Most studies on systematically transplanted BMSCs have had a short follow-up period (Assis *et al*. 2009; Barbash *et al*. 2003; Gao *et al*. 2001), or if the follow up period has been longer, the host animals have been total body irradiated (Pereira *et al*. 1995, 1998). Here, the access of implanted cells to the circulatory system seemed to be a major factor in the release rate of implanted BMSC. Intraperitoneally injected cells gained instant access via the lymphatic system and were detected at week one in all target tissues. Donor cells after subcutaneous implantation, where the formation of new blood vessels take days and even weeks, however, were detected at first at week four to any greater extent. The time frame of transplanted BMSCs in healthy hosts is still largely in the dark, but the results of our study suggest that although BMSCs readily migrate to many tissues, they don't permanently engraft onto them when there is no injury for them to repair.

BAG S53P4 affected the outcome of the seeded cells, but their fate can only be speculated. As no traces of donor cells were detected in these implants, it can be assumed that all cells had either been destroyed or migrated from the implants. Furthermore, as no donor cells were observed in any of the other tested tissues, the cells seem to have been removed from the host before the tissue samples were taken for DNA analyses four weeks after implantation, or more unlikely accumulated in

different organs than the ones tested. This negative effect of BAG on BMSCs *in vivo* could possibly be due to high local alkalinity of dissolving bioactive glass (Sepulveda *et al*. 2002).

6.3 Differentiation of bone marrow derived cells on bioactive glass

The ability of bone tissue to repair itself is unrivaled by any other tissue type in the body thanks to the capability of bone marrow cell to differentiate in osteoblasts and osteoclasts (Laczka-Osyczka *et al*. 1998). Osteoblasts are required to regenerate new mineralized bone tissue while osteoclasts are needed for remodeling of the newly formed bone. An effective bone biomaterial must posses the ability to promote the differentiation of both cell types.

BAG is known to stimulate to differentiation of bone marrow derived cells into osteoblastic cells *in vitro*. Studies suggest that the promotion of the osteoblastic phenotype is both solution mediated (Bielby *et al*. 2005; Bosetti *et al*. 2003; Tsigkou *et al*. 2009) and surface mediated (Dieudonné *et al*. 2002; Effah Kaufmann *et al*. 2000; Knabe *et al*. 2005). In this study bone marrow derived cells were grown both on BAG S53P4 and on plastic on the same culture dish, resulting in a population of cells affected by both the surface and solution mediated effects of BAG, and a population of cells where only the solution mediated effects of bioactive glass is seen. While ALP positive cells were seen in the population growing on plastic their numbers dwindled as the number of ALP positive cells grew in the population grown on BAG. This suggests that the surface mediated effect of BAG is important in the osteoblastic differentiation of bone marrow derived cells.

Osteoclast precursors of the monocyte lineage are present in the adherent population of cultured bone marrow derived cells (Miyamoto *et al*. 2000). BAG promotes osteoclastic differentiation of bone marrow derived cells and several studies have shown the growth of multinucleated TRACP positive cells on bioactive glass surfaces (Bosetti and Cannas 2005; Karpov *et al*. 2006; Laczka-Osyczka *et al*. 1998). In these studies the cell cultures also contain osteoblastic cells, which are thought to express proteins, such as osteopontin and RANKL, which has been shown to stimulate osteoclast differentiation (Aitken *et al*. 2004; Gori *et al*. 2000). The results of this study are in accordance to previous literature for osteoclastic cells growing on BAG. However, when grown on plastic, osteoclastic cells suffered and started to die even though there were osteoblastic cells in the same culture. This implies that in addition to expression of osteoclastic differentiation promoting proteins secreted by osteoblasts, direct contact with BAG is also beneficial for osteoclast differentiation.

6.4 Degradation of bioactive glass

It is well known that BAGs are dissolved in an aqueous solution. However, the same reactions that cause these glasses to dissolve cover the material with calcium phosphate layer that resembles natural bone apatite (Hench *et al*. 1991). Thus, BAGs

could be subject to osteoclastic degradation like bone. The resorption of calcium based biomaterials has been studied extensively. Calcium phosphate cement (Schilling *et al*. 2004) and calcium carbonate (Doi *et al*. 1999; Leeuwenburgh *et al*. 2001; Redey *et al*. 1999) have been shown to be resorbed by osteoclasts while hydroxyapatite is not (Doi *et al*. 1999; Redey *et al*. 1999). A similar result is seen in this study where the surface of BAG S53P4 was not resorbed by osteoclasts.

The sequence of events leading to resorption by osteoclasts is called the resorption cycle (Väänänen 1996). Before resorption can occur, the osteoclasts must attach to the surface of the material it is trying to resorb through the sealing zone (Väänänen and Horton 1995). The sealing zone is required to keep the low local pH in the resorption lacuna which enables the resorption of the substrate (Väänänen 1990). This study showed that TRACP expressing osteoclastic cells attach to the surface of BAG, but still do not resorb it. This is in congruence with studies done on hydroxyapatite (Doi *et al*. 1999 Redey *et al*. 1999) where attachment and actin ring formation is seen without the resorption of the substrate. This is probably due to the fact that apatite surfaces dissolve very slowly even in acidic conditions (Lelievre and Bernache-Assolant 1992). EDXA analysis confirmed that the formation of a hydroxyapatite layer at the end of our study, but as the cells were seeded immediately in the beginning of the experiment, it is unlikely that hydroxyapatite could have protected the surface of the glass from degradation.

The results of this study suggest that either the sealing zone between an osteoclastic cell and BAG S53P4 is imperfect or that HCl secreted into the resorption lacuna cannot dissolve this glass. This could be explained by the liberation of alkaline ions from the BAG matrix during its dissolution. Although the glass remained smooth and uneroded under the osteoclastic cells, rapid erosion was seen elsewhere on the glass surface. This is a characteristic physicochemical reaction for BAGs in aqueous solutions, and similar results have been reached in other studies (Itälä *et al* 2003; Mortin and Shelton 2003). Taken together the results suggest that the degradation of BAGs occur by non-osteoclastic reactions.

6.5 Interaction of silica xerogels with macrophages

BMSCs were cultured in serum-starved culture medium supplemented by the medium of macrophages cultured with silica xerogel microparticles. In cell culturing serum is a universal nutrient for almost all mammalian cell lines, and provides a variety of components such as carbohydrates, lipids, hormones, binding proteins, attachment factors (Paul 1975). It was therefore hypothesized that without the introduction of growth factors from any other source the BMSCs could not proliferate or synthesize type I collagen into the cell culture medium. It is well known that the activation of numerous white blood cells, including macrophages, by crystalline silica produces cytokines that regulate the proliferation, chemotactism and secretory

activity of fibroblasts in pulmonary fibroblasts (Holian *et al*. 1994; Olbruck *et al*. 1998; Piguet *et al*. 1990). In this study, we tested whether amorphous sol-gel based silica xerogel in the form of microparticles also provokes this reaction.

BMSC cultures were supplemented with medium from macrophages cultured with slow and fast dissolving silica microparticles as well as without any silica as a control. All formulations increased the proliferation of BMSCs—an effect not seen without macrophage supplementation—but the proliferative effect was much stronger in cells supplemented with medium from macrophages grown with slowly dissolving silica microparticles. Peritoneal macrophages release cytokines after phagocytosis of crystalline silica that influence the proliferation and collagen synthesis of fibroblasts (Heppelston and Styles 1967). A specific cytokine mediating this effect was discovered and named alveolar-macrophage-derived-growth-factor (Bittermann *et al*. 1982), and later identified as insulin-like growth factor-1 (IGF-1) (Rom *et al*. 1988). The release of IGF-1 from macrophages after phagocytosis has been shown to increase proliferation of fibroblasts when cultured in serum-free conditions (Olbruck *et al*. 1998; Seemayer *et al*. 1997). Expression of IGF-2, a close relative of IGF-1, has been shown to be induced by the dissolution products of BAG (Xynos *et al*. 2000), but in this study dissolved silica did not enhance BMSC proliferation without macrophage mediation indicating that it is caused by cytokines produced by macrophages after phagocytosis. The fact that BMSC proliferation was the strongest in cultures supplemented with medium from macrophages cultured with slowly dissolving silica microparticles suggests that they are phagocytosed while the fast dissolving microparticles are not due to their fast dissolution rate. The proliferation seen in response to culture medium from macrophages culture with fast dissolving microparticles and control macrophages with no silica based conditioning is most likely due to a base level secretion of cytokines such as IGF-1.

Macrophages also secrete cytokines that induce collagen synthesis and ECM formation in response to silica. Several studies have shown that the secretion of TGF-β (Olbruck *et al*. 1998) and TNF-α (Bosetti *et al*. 2002; Piguet *et al*. 1990) is increased after contact with crystalline silica. These two cytokines have been show to induce collagen synthesis (Piguet *et al*. 1990; Reed *et al*. 1994), and to by prevented by anti-TGF-β (Olbruck *et al*. 1998) and anti-TNF-α antibodies (Piguet *et al*. 1990). TNF-α secretion has also been shown to be increased by being conditioned with amorphous silica in the form of BAG (Bosetti *et al*. 2002), making TNF-α the likely candidate of increasing collagen synthesis in BMSCs supplemented with silica conditioned macrophage medium, as seen in this study. Contrary to the proliferation study, collagen synthesis in this study was increased by conditioning with fast dissolving silica which is most probably not phagocytosed. Studies have shown that there is no relationship between particle phagocytosis and TNF-α release (Bosetti *et al*. 2002; Nakashima *et al*. 1999). This suggests that the increased synthesis of collagen mediated by macrophages is caused by dissolved silica ions.

7 CONCLUSIONS

Implanted BMSCs stay viable inside host tissue for at least four weeks and can be found to have migrated to most tissues inside the host. While the types of tissues to which the cells migrate to is not affected by the method of implantation, the speed at which they reach these tissues clearly is. Intraperitoneal implantation provides the fastest route for introducing stem cells into host tissues, compared to subcutaneous and femoral implantation. It is, however, unclear whether the speed of migration will also quicken the elimination of most of the implanted cells inside the host. This seems to be an expected outcome, since at twelve weeks after subcutaneous implantation, no cell can be found within host tissue. In spite of these challenges, the results of this study provide ideas for possible new routes for the therapeutic use of BMSCs and the complexities of using BMSCs in tissue engineering.

BAG S53P4 supports both the osteoblastic and osteoclastic phenotype of bone marrow derived cells. As bone marrow derived cells are the primary cell type for repairing bone defects, BAG is highly suitable for use in biomaterials aiming to enhance the healing of bone tissue. BAG, however, is not actively resorbed by osteoclastic cells, but rather by dissolution into the surrounding water based medium. For this reason, BAG S53P4 is perhaps best used as an additive in a bone regenerating biomaterial, unless the target tissue has extensive fluid exchange or the biomaterial not is intended to be resorbed from the site in the first place.

Silica microparticles affect the proliferation of BMSCs and their efficiency of producing collagen depending on the release rate of silica from the microparticles. Dissolved silica molecules cause an increase in the production of collagen, while non-dissolving silica microparticles seem to enhance the proliferation of bone marrow stromal cells. Proliferation of BMSCs and their efficiency at producing ECM are two key components of expedited bone defect healing. This effect, however, is mediated by macrophages that ingest micro-scale particles of silica making it a challenge to design proliferation enhancing silica based biomaterials.

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