TURUN YLIOPISTON JULKAISUJA
ANNALES UNIVERSITATIS TURKUENSIS

SARJA - SER. D OSA - TOM. 909

MEDICA - ODONTOLOGICA

GRANULATION TISSUE FORMATION

The effect of hydroxyapatite coating of cellulose on cellular differentiation

by

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UNIVERSITY OF TURKU
Turku 2010

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ISBN 978-951-29-4342-5 (PRINT)
ISBN 978-951-29-4343-2 (PDF)
ISSN 0355-9483
Painosalama Oy, Turku, Finland 2010

To Timo and Frans

ABSTRACT

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Cellulose was coated with a silica-rich hydroxyapatite layer resembling the mineral composition of bone in search for a possible bone filler material. The hydroxyapatite-coated cellulose did not, however, promote bone repair but instead favored inflammation and fibroplasia. When implanted subcutaneously, these sponges rapidly generated a highly vascular granulation tissue. Further investigation revealed that hydroxyapatite-coated cellulose attracted not only inflammatory cells but also stem cells of both hematopoietic and mesenchymal origin.

In the bone marrow, the hematopoietic stem cells reside near the endosteal surface of bone, where the calcium concentration is more than 20-fold of that observed in serum due to bone remodeling by osteoclasts. The hematopoietic stem cells are known to attach to their niche via calcium sensing receptors. The presence and release of calcium ions from the hydroxyapatite layer of the coated sponges might offer an explanation for more abundant accumulation of hematopoietic stem cells to the hydroxyapatite coated implants. Indeed, calcium sensing receptor-positive cells were found especially near the apatite-coated cellulose fibers in the implants. Further analyses indicated that the hematopoietic stem cells were able to differentiate into hemoglobin expressing cells. The presence of erythroid cell markers in the sponges suggests that granulation tissue is capable of extramedullary erythropoiesis. These cell-guiding properties of HA coated cellulose might be utilized in impaired wound healing situations.

Key words: wound healing, cellulose, hydroxyapatite coating, stem cells, cell guiding

TIIVISTELMÄ

Miretta Tommila

HAAVAN JYVÄISKUDOKSEN MUODOSTUMINEN – Hydroksiapatiittipinnoitetun selluloosasienen vaikutus solujen erilaistumiseen paranemisprosessin aikana

Biolääketieteen laitos, Lääketieteellinen biokemia ja genetiikka, Turun Yliopisto ja Anestesiologian, tehohoidon, ensihoidon ja kivunhoidon klinikka, Turun Yliopisto ja Turun Yliopistollinen keskussairaala.

Annales Universitatis Turkuensis, Ser. D, Medica – Odontologica, 2010, Turku, Finland

Etsittäessä uusia luun bioyhteensopivia täytemateriaaleja selluloosasieni päällystettiin luun koostumusta muistuttavalla runsaasti piitä sisältävällä hydroksiapatiittikerroksella. Vastoin odotuksia hydroksiapatiittipinnoitettu selluloosa ei parantanut luun kasvua, vaan päinvastoin ylläpiti tulehdusta ja sidekudossolujen hakeutumista vamma-alueelle. Ihon alle implantoituna sama sienimateriaali edisti merkittävästi haavan verekkään jyväiskudoksen kasvua. Tämän löydöksen perusteella hydroksiapatiittipinnoitetun selluloosasienen vaikutusta haavan soluihin paranemisprosessin aikana tutkittiin tarkemmin ja havaittiin, että tulehdussolujen lisäksi sieniin kertyi tavallista enemmän sekä hematopoieettisia että mesenkymaalisia kantasoluja.

Hematopoieettiset kantasolut sijaitsevat luuytimessä lähellä luun sisäpintaa. Luun hydroksiapatiitista vapautuu kalsium-ioneja luun jatkuvan fysiologisen uudismuodostuksen ja hajottamisen yhteydessä. Kantasolut etsiytyvät luuytimeen kalsiumia aistivien reseptorien välityksellä. Koska luun pintakerrosta muistuttavasta hydroksiapatiittipinnoitteesta vapautuu kalsiumia, tämän ajateltiin toimivan selityksenä sille, että hematopoieettiset kantasolut hakeutuvat runsaslukuisesti juuri hydroksiapatiittipinnoitettuihin selluloosasieniin. Tämän hypoteesin mukaisesti hydroksiapatiittipinnoitettujen selluloosapalkkien läheisyydestä löydettiin suuria määriä kalsiumreseptoreja sisältäviä soluja. Jatkotutkimuksissa todettiin lisäksi, että hematopoieettiset kantasolut pystyivät sienissä erilaistumaan hemoglobiinia tuottaviksi soluiksi. Havaittujen punasolulinjan merkkiaineiden perusteella näyttäisikin siltä, että haavan paranemiskudoksessa tapahtuu paranemisen aikana ekstramedullaarista erytropoieesia. Nämä soluja ohjaavat vaikutukset saattavat olla hyödyllisiä vaikeasti paranevien haavojen hoidossa.

Avainsanat: haavan paraneminen, selluloosa, hydroksiapatiitti-pinnoite, kantasolut, solujen ohjaus

TABLE OF CONTENTS

ABBREVIATIONS

ADAM	A disintegrin and metalloproteinase family
ADAMTS	A disintegrin-like and metalloproteinase with thrombospondin type I motifs
ALAS2	Erythoidspecific isoform of 5-aminolevulinate synthase
BMP	Bone morphogenetic protein
CaR	Calcium-sensing G-protein-coupled receptor
Cbfa1	Core binding factor alpha 1
CD	Cluster of differentiation
CXCR4	Chemokine cxc-motif receptor 4
DAPI	4´,6-diamidino-2-phenylindole
ECM	Extracellular matrix
EGF	Epidermal growth factor
EPC	Endothelial progenitor cells
FACIT	Fibril-associated collagens with interrupted triple helices
FDA	US Food and Drug Administration
FGF	Fibroblast growth factor
GAG	Glycosaminoglycan
GM-CSF	Granulocyte macrophage colony-stimulating factor
HA	Hydroxyapatite
Hb	Hemoglobin
HSC	Hematopoietic stem cells
IGF	Insulin-like growth factor
IL	Interleukin
MHC II	Major histocompatibility complex class II
MMP	Matrix metalloproteinase
MSC	Mesenchymal stem cells
NO	Nitric oxide
iNOS	Inducible nitric oxide synthase
PCNA	Proliferating cell nuclear antigen
PDGF	Platelet derived growth factor
RT-PCR	Reverse transcriptase polymerase chain reaction
SBF	Simulated body fluid
SDF-1α	Stromal-derived factor-1 alpha
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by the Roman numerals I-IV. These original works have been reproduced with the permission of the copyright holders.

I Erika Ekholm, Miretta Tommila, Ari-Pekka Forsback, Matis Märtson, Johanna Holmbom, Virpi Ääritalo, Christa Finnberg, Asko Kuusilehto, Jukka Salonen, Antti Yli-Urpo, Risto Penttinen. Hydroxyapatite coating of cellulose sponge does not improve its osteogenic potency in rat bone. Acta Biomaterialia 2005; 1: 535-544.

II Miretta Tommila, Jutta Jokinen, Timothy Wilson, A-P Forsback, Pekka Saukko, Risto Penttinen, Erika Ekholm. Bioactive glass-derived hydroxyapatite coating promotes granulation tissue growth in subcutaneous cellulose implants in rats. Acta Biomaterialia 2008; 4: 354-361.

III Miretta Tommila, Anne Jokilammi, Perttu Terho, Timothy Wilson, Risto Penttinen, Erika Ekholm. Hydroxyapatite coating of cellulose sponges attracts bone marrow-derived stem cells in rat subcutaneous tissue. The Journal of Royal Society Interface 2009; 6: 873-880.

IV Miretta Tommila, Christoffer Stark, Anne Jokilammi, Ville Peltonen, Risto Penttinen, Erika Ekholm (2010). Hemoglobin expression in rat experimental granulation tissue. Resubmitted.

1. INTRODUCTION

Impaired bone repair and wound healing are both clinically remarkable problems. In orthopedic and reconstructive surgery, autologous bone transplantation is still the gold standard of treatment. The supply of living bone is, however, limited and bone from bone banks may carry a risk of contagious diseases. Thus, synthetic and/or biotechnically processed bone substitutes are needed to fulfill clinical demands. Another important subject is chronic cutaneous ulcers, which are very common among elderly people. Despite their generality, efficient treatment methods for persistent ulcers are still insufficient.

Cellulose is a biopolymer, which is inexpensive to produce, can be manufactured in porous form, and trimmed into desirable shape. Subcutaneously implanted cellulose sponges induce a rapid growth of granulation tissue, and due to this property cellulose has been used for experimental and clinical wound healing studies for several decades. As bone implants in rats, cellulose has been shown to be osteoconductive, but not osteoinductive (Märtson, 1999). In order to promote the osteostimulating property of cellulose, it was coated with a silica-rich hydroxyapatite (HA) layer that mimics the natural surface of bone. The HA coating was formed by incubating cellulose sponges with a special bioactive glass in simulated body fluid ((SBF); for SBF constituents see study I). This bioactive glass possesses a high osteoconductivity, but is difficult to handle because of its brittleness and fragility. The aim of this combination was to unite the beneficial features of both materials and to primarily create a functional bone substitute material. Since the bone implantation study revealed unexpectedly an enormous ability of the HA-coated cellulose to induce granulation tissue, the coated cellulose was tested subcutaneously as well for its possible suitability as a treatment method for chronic ulcers and other poorly healing wounds.

2. REVIEW OF THE LITERATURE

2.1 CONNECTIVE TISSUE

The anatomical structures of all mammals are classified into connective, epithelial, nerve and muscle tissues. Connective tissue is a form of fibrous tissue, derived from mesodermal origin, which provides structural and metabolic support for other tissues and organs of the body. Each connective tissue is composed of two major components; cells and extracellular matrix (ECM).

2.1.1 Different types of connective tissue

The most common form of connective tissue is loose connective tissue, which is found almost everywhere in the body, as it provides support for structures passing through it, such as blood, lymph vessels and nerves. It also serves to bind together other tissues, including organs and their components. Basically, loose connective tissue fills many free spaces in the body.

Dense connective tissues are completely dominated by fibers. In dense irregular connective tissue, found for example in skin, the fibers do not show a clear orientation within the tissue but instead form a densely woven three-dimensional network. The fibers in regular dense connective tissue run parallel to each other as in tendons and ligaments. Adipose tissue, blood, cartilage and bone are specialized forms of connective tissue. Blood serves as a vascular tissue, whereas the two latter ones are specific rigid forms of specialized supporting tissue, being the major components of the skeleton.

2.1.1.1 Cartilage

Cartilage is a type of dense connective tissue. It is composed of specialized cells called chondrocytes that produce a large amount of ECM. The three major types of cartilage are hyaline cartilage, fibrocartilage and elastic cartilage, each of these types having a varying composition of ECM. Cartilage has diverse roles in the body, and is found on most articular surfaces, in intervertebral discs and rib cage, in respiratory tract, in external ear, in epiglottis and in larynx. In adult mammals, cartilage has a limited distribution whereas in immature individuals, cartilage forms a template for most of the developing bony skeleton. Unlike other connective tissues, cartilage is avascular and its nutrition depends on diffusion from surrounding tissues. Therefore, cartilage repair is slow and often incomplete.

2.1.1.2 Bone

Bone is a rigid form of connective tissue and exists in two main forms; woven and lamellar bone. The immature woven bone consists of randomly organized collagen,

whereas lamellar bone has a highly organized infrastructure of collagen. In addition, lamellar bone is divided morphologically into two subgroups, cortical (compact) and cancellous (spongy or trabecular) bone in relationship 80 % to 20 % of the total bony mass. Cortical bone is harder and covers the external surfaces of all bones, while cancellous bone mainly forms a porous internal matrix of interconnecting plates and columns (reviewed in Marks and Hermey, 1996).

Bone not only functions as a supporting or protective tissue, but also participates in the production of movement and sound transduction via the ossicles . Bone also takes part in metabolic actions, like mineral and fat storage, detoxification (Haussler *et al*., 2008), and maintenance of acid-base balance by buffering excess metabolic H+ with alkaline hydroxyapatite (Arnett, 2008). In addition, the red bone marrow is the site of hematopoiesis.

Osteoid tissue is characterized by an ECM in which the four bone cell types are embedded. Osteoprogenitor cells are immature stem cells that act as a reserve for the production of future bone-forming cells. Osteoblasts are fully differentiated cells that are able to make bone. Osteocytes are mature osteoblasts imbedded within the bone matrix and responsible for its maintenance. Osteoclasts are large, multinucleated cells, which constantly degrade bone. The bone tissue is, thus, in a continuous turnover state, and there is a strict balance between bone formation and resorption. The purpose of this remodeling is to maintain calcium homeostasis of the body, as well as to repair its micro-damages and to shape the skeleton during growth.

Bone matrix, unlike other connective tissues, has the unique ability to become mineralized. Mineralization is mainly caused by deposition of inorganic ions, especially calcium and phosphate. Spindle or plate-shaped crystals of hydroxyapatite ($(Ca_{10}(PO_4)_6(OH)_2$), are found on the collagen fibers and within or between them (Viguet-Carrin *et al*., 2006; Allori *et al*., 2008 Part II).

2.1.1.3 Skin

The skin is the largest organ of the body in vertebrates and provides protection against water loss, chemicals, sunlight and microorganisms. It is composed of three distinct layers. The two upper layers, epidermis and dermis, are separated by a basement membrane. The main cells in the epidermal epithelial sheet are keratinocytes, but it also contains melanocytes, dendritic (Langerhans) cells and Merkel cells (Proksch *et al*., 2008). The dermis contains fibroblasts, macrophages, mast cells, and endothelial cells (Proksch *et al*., 2008). The third innermost layer hypodermis, also called subcutaneous tissue, binds skin loosely to subjacent tissues. In rodents, this layer mainly consists of loose connective tissue that lies between the dermis of the skin and underlying fascia. In humans, hypodermis also contains more or less abundant amount of fat. Understandingly, the size of this layer varies throughout the body and individual. The subcutaneous adipose tissue insulates the body,

absorbs trauma, and serves as a reserve energy source. Thus, the subcutaneous layer is important in the regulation of temperature of the skin itself and the body.

2.1.2 Extracellular matrix components of connective tissue

Most of the cells in multicellular organisms are surrounded by an ECM, a complex mixture of proteins and polysaccharides. ECM offers structural support, but also regulates tissue development and regeneration via several mechanisms. The macromolecular constituents of ECM vary in different tissues.

Collagens are the major constituent of the ECM. They serve as fibrous protein building blocks, which provide strength, integrity and structure. To date, at least 28 different types of collagen are known (Kadler *et al*., 2007; Järveläinen *et al*., 2009). A specific gene codes for each collagen chain, but they all share a similar basic structure, which is made of three polypeptide chains. The amino acids of these chains constantly repeat the Gly-X-Y triplet. Although X and Y can be any amino acid residue, the most often represented are proline and hydroxyproline (van der Rest and Garrone, 1991). Each chain winds upon itself in a left-handed helix, and then three chains unite to form a right-handed coil called tropocollagen. These tropocollagen units are arranged in a highly regular fashion to form collagen filaments, which further combine to collagen fibrils. Finally, collagen fibers are formed from aggregated collagen fibrils (reviewed in Stadelmann *et al*., 1998).

Collagen has different amino acid compositions, which most probably reflect their specific functions in the body. Collagens are classified into fibrillar (types I, II, III, V), nonfibrillar (e.g. type IV), or so-called FACIT (fibril-associated collagens with interrupted triple helices collagen (Raghow, 1994; Kadler *et al*., 2007), and their various combinations. About 80-90 % of the collagen in the body consists of types I, II and III (reviewed in Kavitha and Thampan, 2008). The organic matrix of bone consists almost exclusively of type I collagen (two α1(I) chains and one α2(I) chain), which is involved in the mechanical properties of bone. Trace amounts of III, V, XI, and XIII may be present and modulate the fibril diameter (Gehron Robey, 1989; Viguet-Carrin *et al*., 2006). The most abundant types of collagen in skin are I and III, which mostly are responsible for its mechanical properties. The skin contains smaller amounts of collagen V, VI, and XII, which seem to have a supportive function. Collagens have also an important role in both wound and bone healing (reviewed in Kavitha and Thampan, 2008). The most common collagen types participating in wound and bone healing, and their distribution is shown in Table II.

Proteoglycans are a group of proteins, which have developed a number of different functions, including maintenance of tissue sturdiness and stiffness. Structurally, proteoglycans consist of a protein core covalently bound to long sulphated glycosaminoglycan (GAG) chains of variable lengths. The structures of the disaccharides of the GAG determinate the character of the proteglycan. On the basis of the constituting

disaccharide units, three different types of sulphated GAGs can be distinguished: 1) chondroitin/dermatan sulphate, 2) heparin sulphate/heparin and 3) keratan sulphate. Proteoglycans are enormously heterogeneous in structure and in function, and they are believed to be important mediators of ECM-cytokine interactions (Rapraeger *et al*., 1991; Yayon *et al*., 1991; reviewed in Schönherr and Hausser, 2000).

Decorin belongs to the largest group of the matrix-associated proteoglycans, the small leucine-rich repeat proteoglycans. Decorin received its name because it binds to the surface of collagen fibrils, thus "decorating" them (Fisher *et al*., 1989; reviewed in Schönherr and Hausser, 2000). With its glycosaminoglycan chain, decorin stabilizes the inter-fibrillar organization of ECM (Orgel *et al*., 2009), and regulates collagen fibrillogenesis (Zhang *et al*., 2006). In addition to binding different types of collagen, decorin can also interact with a variety of molecules. For example, decorin is linked to inflammatory induced angiogenesis (Nelimarkka *et al*., 2001), and it can modulate growth factor balance during chronic inflammation (Järveläinen *et al*., 2006).

Glycoproteins contain oligosaccharide chains covalently attached to their polypeptide chains. This group includes a variety of distinct constituents, and their expression depends on the tissue type. The prevalent ECM glycoproteins are fibronectin, laminin, thrombospondin, osteonectin, tenascin and entactin (Raghow 1994; Allori *et al*., 2008 Part II; reviewed in Järveläinen *et al*., 2009). Glycoproteins have several functions, but they are specifically involved in cell attachment. The signals between individual matrix molecules and cells are primarily mediated via specific cell-surface receptors called integrins. Integrins are transmembrane heterodimeric glycoproteins composed of one α and one β subunit (Hynes, 2002). To date, 18 α and 8 β subunits have been identified in mammals, making it possible to form at least 24 combinations of integrins, each able to specifically bind one or several ECM molecules (reviewed in Järveläinen *et al*., 2009).

In addition to structural components of ECM, quite recently the term "matricellular proteins" has been applied to a group of ECM molecules, including thrombospondin-1 and -2, SPARC (secretes protein, acidic and rich in cysteine), tenascin-C, and osteopontin (Reed *et al*., 1993; reviewed in Järveläinen *et al*., 2009). These molecules modulate cell-matrix interactions and cell functions e.g. in tissue repair (reviewed in Bornstein and Sage, 2002; reviewed in Kyriakides and Bornstein, 2003; Sage *et al*., 2003; Puolakkainen *et al*., 2005).

2.2 STEM CELLS

Stem cells are immature cells capable of self-renewing and differentiating into a variety of mature cell types under appropriate conditions. A characteristic feature is an asymmetric division in which one daughter cell is committed to differentiation whereas the other remains as a stem cell (Chan-Ling *et al*., 2006). Stem cells are found in the embryo and in

most adult tissues participating in endogenous tissue regeneration (Chan-Ling *et al*., 2006; Tárnok *et al*., 2010). The true totipotent stem cells are the cells of the early morula stage embryo. These cells are able to differentiate to all possible embryonic and extraembryonic cell types, and thus capable of forming the entire organism. During embryogenesis the differentiation capacity gradually diminishes. The cells of the blastocyst stage embryo are pluripotent and can differentiate into any specialized cells of the adult organisms, but lack the potential to form extraembryonic tissue. Some of the undifferentiated cells remain in adulthood, but have lost some of their differentiation capacity. Adult stem cells are able to differentiate into a number of cell types originating from different germ layers, which is referred to as stem cell plasticity (Ohishi and Schipani, 2010).

2.2.1 Adult bone marrow-derived stem cells

Adult bone marrow-derived stem cells are usually classified into two major groups; hematopoietic (HSC) and mesenchymal (MSC) stem cells. This division is based on their dissimilar differentiation potential. Their identification is based on detection of surface antigens. However, thus far no perfect markers have been identified for different stem cells, therefore the conclusions are made by testing the existence or lack of several antigens. The markers used vary between publications, and different species of animals, including humans, may also differ in their antigenic patterns. In addition, the line between immature and mature is artificial, and the concept of stem cells often includes progenitor cells with various levels of maturation.

The most common surface antigens for hematopoietic and mesenchymal stem cells are listed in the table below.

Table I. Identification of hematopoietic and mesenchymal stem cells based on the presence of certain cell surface antigens. (Tao and Ma, 2003; Le Blanc, 2006; Lee *et al*., 2009).

Surface antigen	HSC	MSC	Marker of
CD11a	+	-	lymphocytes
CD11b	+	-	granulocytes, monocytes
CD14	+	-	monocyte/macrophage
CD29	-	+	mesenchymal stem cells
CD31	(+)	-	endothelial cells
CD34	+	-	hematopoietic stem cells
CD44	+	+	hemato- and non-hematopoietic
CD45	+	-	pan-hematopoietic marker
CD73	-	+	mesenchymal stem cells
CD90	-	+	mesenchymal stem cells
CD105	-	+	mesenchymal stem cells
CD166	-	+	mesenchymal stem cells
c-kit (CD117)	+	(+)	general primitive cell marker
HLA-DR	+	-	hematopoietic stem cells
Sca-1	+	-	hematopoietic stem cells
Stro-1	-	+	mesenchymal stem cells

2.2.1 Hematopoietic stem cells and progenitors

Already in the 1950´s, bone marrow was found to function as a reservoir of hematopoietic stem cells, which continually replenish circulating blood cells during the entire human life span (Lorenz *et al*., 1951). This finding lead to syngeneic bone marrow transplant experiments in animals (Nowell *et al*., 1956), and eventually to the current therapies of hematological malignancies in humans.

HSCs are responsible for the generation of all mature blood cells. In adults, HSCs mainly reside in the bone marrow, where they sustain hematopoiesis throughout life. During the classical scheme of hematopoiesis, HSCs gradually lose one or more developmental options according to a certain hierarchy: HSCs can undergo either self-renewal or differentiation into multilineage committed progenitor cells, which are either common lymphoid or myeloid progenitors (Kaushansky, 2006). These cells then give rise to more differentiated precursors, which then produce erythrocytes, leukocytes and platelets. In human embryogenesis from week three to week six, the primary site of hematopoiesis is the yolk sac, followed by the aorto-gonado-mesonephric region, then fetal liver from week six to 22, and finally the bone marrow for the rest of life (Sequiera Lopez *et al*., 2003; Qiu *et al*., 2005).

In recent years, knowledge on the details of hematopoiesis has undergone a rapid change. In embryonic yolk sac blood islands, hematopoietic and endothelial cells emerge simultaneously and in close association with each other from mesodermal aggregates (Palis and Yoder, 2001). In the current view, cells at the periphery of these blood islands develop into endothelial cells, while inner cells form primitive erythrocytes (Jaffredo *et al*., 2005). The close developmental link between these two cell types has lead to the hypothesis of the hemangioblast, a common progenitor of endothelial and hematopoietic cells. An alternative explanation for embryogenic hematopoiesis is the existence of hemogenic endothelium. In this concept, definitive hematopoietic cells are generated through an endothelial intermediate that has the potential to give rise to hematopoietic cells (Jaffredo *et al*., 2005). The hematopoietic capacity of an embryo is more widespread than earlier has been thought, and multiple regions of the embryo are capable of forming blood both before and during organogenesis (Sequiera Lopez *et al*., 2003).

Adult HSCs are considered a heterogenic cell population that consists of stem cells committed to hematopoiesis and endothelial progenitor cells (Asahara *et al*., 1999). Accumulating evidence indicates that hematopoietic cells with hemangioblast activity persist into adulthood (Park *et al*., 2005). Bone marrow-derived HSCs or their progeny may contribute to the maintenance and repair of both the hematopoietic and vascular systems during adult life (Bailey and Fleming, 2003). A large number of signaling molecules (such as BMP4, Flk-1, c-kit, Notch, and Hedgehog) and transcription factors (such as Brachyury, Scl, Lmo2, GATA-2, Runx1, CBFβ, c-Myb, and Stat5a) are involved in the hematopoietic stem cell differentiation (Jaffredo *et al*., 2005; Park *et al*., 2005). The multipotent HSCs

can transdifferentiate, at least *in vitro*, into non-hematopoietic cell types such as epithelium (Borue *et al*., 2004), myocardium (Balsam *et al*., 2004; Orlic, 2003), liver (Petersen *et al*., 1999), osteoblasts (Matsumoto *et al*., 2008), and brain (Bonilla *et al*., 2005).

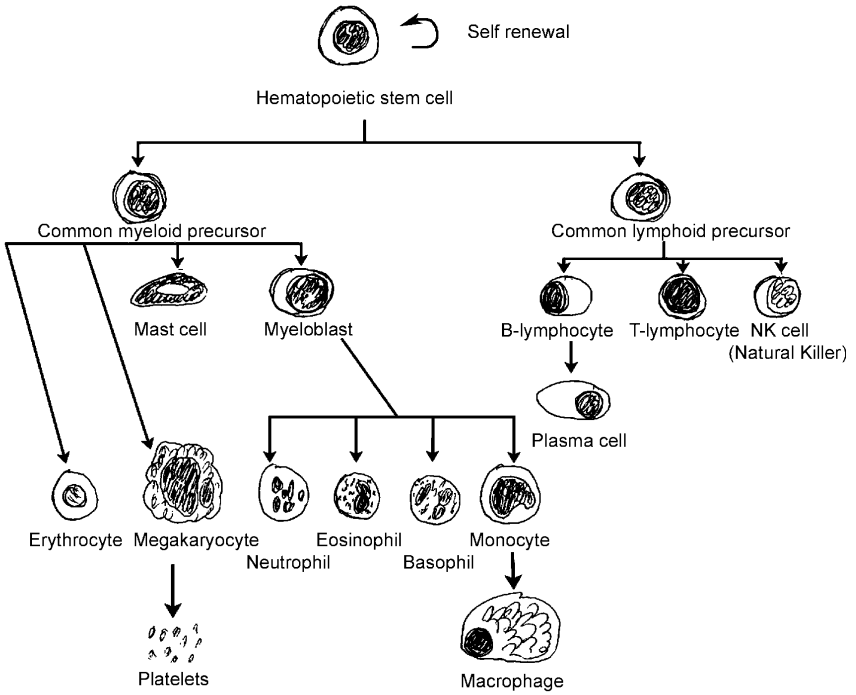

Figure 1. A general model of hematopoiesis. Hematopoietic stem cells differentiate to hematopoietic precursors, and further to mature cells representing erythroid, megakaryocytic, myeloid, macrophage, and lymphoid lineages.

2.2.1.2 Hematopoietic stem cell niche and calcium sensing receptor

Stem cells require a specific microenvironment for self-replication, and for this reason stem cells gather to certain anatomic locations called niches during vertebrate development (Ballen, 2007). The word niche descends from French; its literal meaning is a doghouse. The niche harbors stem cells and regulates their growth proliferation and differentiation (Ballen, 2007). In adult individuals, stem cell niches are found in several different tissues, such as skin (Crigler *et al*., 2007), skeletal muscle (Pallafacchina *et al*., 2010), heart (Gherghiceanu and Popescu, 2010), kidney (Park *et al*., 2010), and brains (Liu and Martin, 2006). The HSC niche is located in the bone marrow and functions as a dynamic system, where stem cells and their surrounding are in an active interaction (Ballen, 2007).

In mammalian bone marrow, HSCs are located close to the endosteal surfaces, with more differentiated cells closer to the axis of the bone (Lord, 1990). This organized structure

of the marrow suggests a relationship between HSCs, osteoblasts and osteogenic cells lining the endosteal surface (Ballen, 2007). The interaction between HSCs and the niche is mediated by two important receptors, namely osteopontin and a calcium-sensing G-protein-coupled receptor, CaR. Osteopontin is a sialoprotein, whose production varies with the activity of osteoblasts. One of its functions is to limit stem cell numbers (Nilsson *et al*., 2005). CaR is expressed by HSCs, and acts as a regulatory component of the HSC niche. This receptor seems to retain HSCs in close physical nearness of the endosteal surface, where the concentration of ionic calcium is high (Adams *et al*., 2006).

HSCs can also be found in extramedullary tissues, such as spleen and liver, despite the absence of bone or endosteum, implying that other cells than osteoblasts and osteoclasts can create an environment capable of sustaining adult HSCs. It is suggested that HSCs reside in extramedullary tissues in so called perivascular niches (Kiel and Morrison, 2008). This implies that there might be other adhesive proteins besides Ca-receptors that retain HSCs in their sites.

2.2.1.3 Mesenchymal stem cells

Mesenchymal stem cells or multipotent stromal cells isolated from the bone marrow of adult organisms were initially characterized as plastic adherent, fibroblastoid cells with the capacity to generate heterotopic osseous tissue when transplanted *in vivo* (Friedenstein *et al*., 1968). MSCs comprise less than 0,1% of all bone marrow cells, but can be isolated from whole bone marrow aspirates by their ability to adhere to plastic and form colonies (Lee *et al*., 2009).

Consequently, in 2006, the International Society of Cellular Therapy defined MSCs by three criteria: i) MSCs must be adherent to plastic under standard tissue culture conditions; ii) MSCs must express certain cell surface markers such as CD105, CD90 and CD73, but must not express other markers including CD45, CD34, CD14 or CD11b; and iii) MSCs must have the capacity to differentiate into mesenchymal lineages including osteoblasts, adipocytes and chondroblasts under *in vitro* conditions (Dominici *et al*., 2006).

In bone marrow, MSCs are presumed to reside at perivascular sites, lining blood vessels (da Silva Meirelles *et al*., 2006; reviewed in Kolf al., 2007) and function as support cells for HSCs (Mendes *et al*., 2007). It is obvious that MSCs are far more versatile than acting only as a supportive scaffold. They are capable to differentiate *in vitro* and *in vivo* to several mesenchymal tissues, including bone, cartilage, tendon, muscle, adipose tissue and possibly bone marrow stroma (Pittenger *et al*., 1999; Tao and Ma, 2003). These cells are also a rich source of growth factors, and they have a wide range of immunomodulatory effects. MSCs secrete paracrine soluble factors that modulate immune responses as well as alter the responses of endothelium or epithelium to injury through the release of growth factors (Parekkadan *et al*., 2007; Rasmusson *et al*., 2007;

Lee *et al*., 2009; Nemeth *et al*., 2009). MSCs can also upregulate the expression of major histocompatibility complex class II (MHC II) antigens when exposed to low levels of inflammatory cytokines, and function as antigen-presenting cells stimulating the adaptive immune system (Chan *et al*., 2006; Stagg *et al*., 2006). They have also been claimed to prevent neutrophil apoptosis and degranulation in culture without inhibiting their phagocytic or chemotactic capabilities (Raffaghello *et al*., 2008). Furthermore, systemically administrated MSCs have been proved to have protective effect against a bacterial infection in a mouse model of bacterial sepsis (Nemeth *et al*., 2009).

Due to their anti-proliferative, immunomodulatory and anti-inflammatory properties (Le Blanc, 2006; Lee *et al*., 2009), MSCs are expected to develop into therapeutic agents in various clinical settings. Recent studies have suggested that MSCs may have potential in treatment of several clinical disorders, including myocardial infarction (Li *et al*., 2005; Miyahara *et al*., 2006; Iso *et al*., 2007), diabetes (Lee *et al*., 2006), sepsis (Nemeth *et al*., 2009), hepatic failure (Parekkadan *et al*., 2007), acute renal failure (Tögel *et al*., 2005), and acute lung injury (ALI), and adult respiratory distress syndrome (ARDS) (Lee *et al*., 2009) even though contradictory results have been published as well.

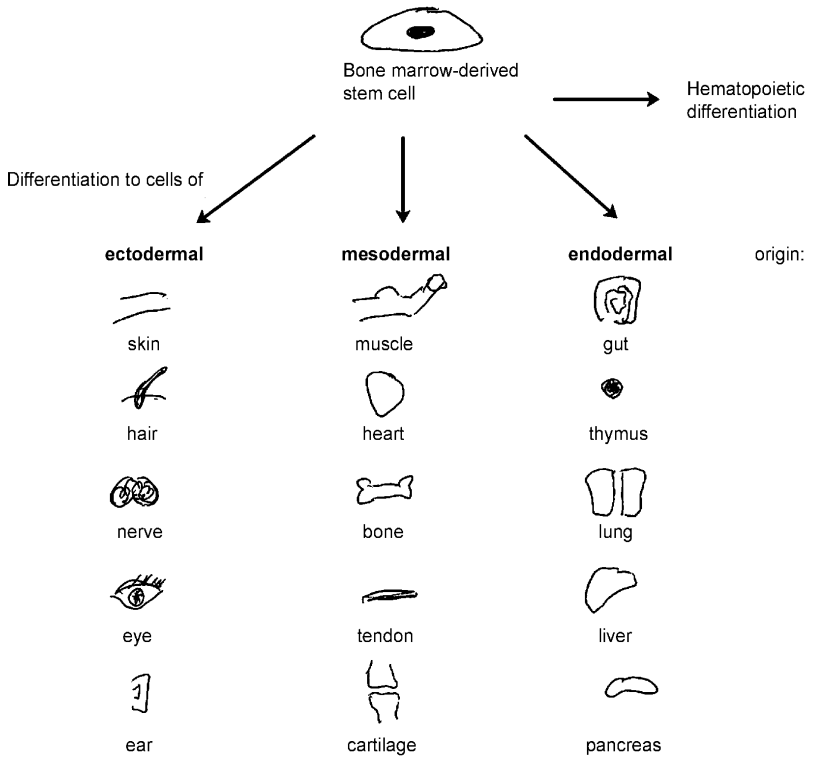

Figure 2. HSCs are mainly responsible for blood cell production, but MSCs also release proangiogenic factors essential for blood cell proliferation. Both of these stem cell types are able to differentiate into several non-hematopoietic cell lineages. Modified from Tao and Ma, 2003, Figure 2.)

2.2.2 Stem cell trafficking

Hematopoietic stem and progenitor cell mobilization from the bone marrow enables migration to peripheral blood and homing to peripheral tissues. The process is tightly controlled by various chemoattractant cytokines, such as stromal derived factor-1 alpha (SDF-1α) (Magnon and Frenette, 2008), growth factors, such as granulocyte colony-stimulating factor (Kong *et al*., 2004) (Magnon and Frenette, 2008), and hormones like (mostly by parathyroid hormone) (Brunner *et al*., 2007). HSCs and their progeny exit the bone marrow through the bone marrow-blood barrier and are then distributed into the circulation, from where they can home back to the bone marrow. HSCs can also home to peripheral extramedullary tissues, stay there for several days and then return to the bloodstream through the lymphatic system (Massberg *et al*., 2007; reviewed in Schulz *et al*., 2009)

It is not known why HSCs migrate between the bone marrow, blood, extramedullary tissues, and lymph compartment. It is proposed that circulating hematopoietic stem and progenitor cells help to locally replenish tissue-resident subsets of specialized myeloid cells like monocytes and macrophages. Migratory HSCs also seem to possess the ability to inspect peripheral tissues and respond rapidly to situations, such as tissue injury and infections, which require prompt influx of large amount of innate immune cells (reviewed in Schulz *et al*., 2009).

Compared to HSCs, less is known about the trafficking of MSCs. Host MSCs seem to mobilize in response to inflammation or injury, but the mechanisms of this process are still unresolved.

2.3 NEOVASCULARIZATION

For a long time all neovascularization was thought to happen via angiogenesis, the process in which new capillaries are formed from pre-existing vessels. Angiogenesis occurs in several physiological and pathological conditions, for example embryonic development, wound healing, diabetic retinopathy, and tumors. A suitable microenvironment is a requirement for angiogenesis, and in postnatal life an appropriate inflammatory response is needed (Naldani and Carraro, 2005). The most common pro-inflammatory and angiogenic cytokines and growth factors are presented in included to the table III in chapter 2.5.3.1.

Vasculogenesis is defined as the initial differentiation of mesoderm into angioblast precursor cells that form the primary capillary network in the developing embryo. In contrast to earlier concepts, accumulating evidence suggests that postnatal vasculogenesis may also take place (Ribatti *et al*., 2001; reviewed in Bauer *et al*., 2005). In the adults, bone marrow-derived, circulating endothelial progenitors contribute to postnatal

neovascularization and enhance vascular repair following ischemic injury (Bailey and Flemming, 2003). In later studies, it has even been claimed that vasculogenesis and hematopoiesis are parts of the same process, in which formation of a blood vessel is accompanied by the simultaneous *in situ* production of blood cells within the vessel (hemo-vasculogenesis) (Sequiera Lopez *et al*., 2003).

2.4 HEMOGLOBIN EXPRESSION

In the vertebrate embryo, hematopoietic and vascular endothelial cells are the first cells to differentiate in response to induction of the mesoderm (Baron, 2001). Primitive hematopoiesis is restricted to the formation of nucleated erythrocytes (Wong *et al*., 1986) and macrophages (Cline and Moore, 1972). During primitive hematopoiesis, nucleated erythrocytes express embryonic hemoglobin (Wong *et al*, 1986). Definitive hematopoiesis is the process whereby all types of blood cells are formed followed by their differentiation, including the enucleation of erythrocytes (Sequiera Lopez *et al*., 2003). During the latter stages of erythroid cell development, large amounts of globin chains and heme molecules are synthesized (reviewed in Sadlon *et al*., 1999).

In mammals, hemoglobin serves as the main oxygen transporter in the blood. The hemoglobin molecule is an assembly of four globular protein subunits. These subunits are covalently bound to each other, and usually consist of two α subunits and two other subunits, which vary according to the developmental state and in some case, according to disease states. During the embryonic period the two other subunits are ε type, which are replaced by γ subunits during the fetal period. In adult humans, the most common type is Hb A, in which the two other subunits are β type. Less than 3% of adult hemoglobin consists of Hb A-2, in which two alpha chains are combined with two delta chains. This hemoglobin may be present in high amounts in hemoglobinopathies such as sickle cell anemia and beta thalassemia. The conventional globin family includes also other members, namely myoglobin, neuroglobin and cytoglobin. Myoglobin stores oxygen temporally and enhances oxygen diffusion to the mitochondria in the cardiac and striated muscle (Nishi *et al*., 2008). The physiological roles of neuroglobin are still uncertain, but it has been assumed to possess an essential, conserved function beneficial to neurons (Burmester and Hankeln, 2009). Cytoglobin is present in almost all tissues and organs, and serves as an antioxidative protein (Nishi *et al*., 2008).

Regulation of hemoglobin synthesis is perhaps the best-known example of feedback regulated protein synthesis. Hemoglobin genes are situated in the genome in clusters that are regulated by upstream locus control regions. The expression of the various

globin genes, especially the non-α-cluster genes corresponding to the embryonic, fetal, and adult developmental stages, has been used as a measure of the maturation stage of erythropoiesis in primates and humans (Peschle *et al*., 1984). In a recent study, Lu *et al*. (2008) managed to differentiate human embryonic stem cells into functional oxygen-carrying erythrocytes, and a significant amount of adult β-globin synthesis was noticed after 28 days in a cell culture. β-globin expression correlated directly to the expression of the mature erythrocyte marker glycophorin A. Overall, hemoglobin expression is regulated by a numerous different factors, such as hormones, enzymes, iron, hypoxia, stress, and growth factors (reviewed in Tsiftsoglou *et al*., 2009). One essential enzyme is an erythroid-specific isoform of 5-aminolevulinate synthase (ALAS2), which is specifically required for the expression of β-globin (Sadlon *et al*., 1999).

Adult vertebrate hemoglobin expression has traditionally been thought to concentrate entirely to the bone marrow area. However, recent evidence shows that extramedullary hematopoiesis occurs also during adult life in situations, when blood availability is affected. Observations of extramedullary hematopoiesis have been made in various tissues and organs, i.e. skull (Joseph *et al*., 2000), brain (Fucharoen *et al*., 1985), liver, spleen, kidneys, adrenal glands, breast, paravertebral and presacral areas (Tunaci *et al*., 1999), skin, testicles (Ruberto *et al*., 1995), heart (Hill and Swanson., 2000), lung (Yusen and Kollef, 1995), gastrointestinal tract (Sunderland *et al*., 1994), pancreas (Crider *et al*., 1998), prostate (Humphrey and Vollmer, 1991), endometrium (Dassen *et al*., 2008), and mesangial cells (Nishi *et al*., 2008). This phenomenon has been explained by the fact that hemo-vasculogenesis is widespread throughout the whole embryo during development, and therefore hematopoiesis can be reactivated as a compensatory mechanism in organs or regions, where it previously occurred during embryonic and fetal life (Sequiera Lopez *et al*., 2003). Despite the numerous observations of hemoglobin expression in nonhematopoietic organs, the exact function of extramedullary hemoglobin expression is still mostly unknown.

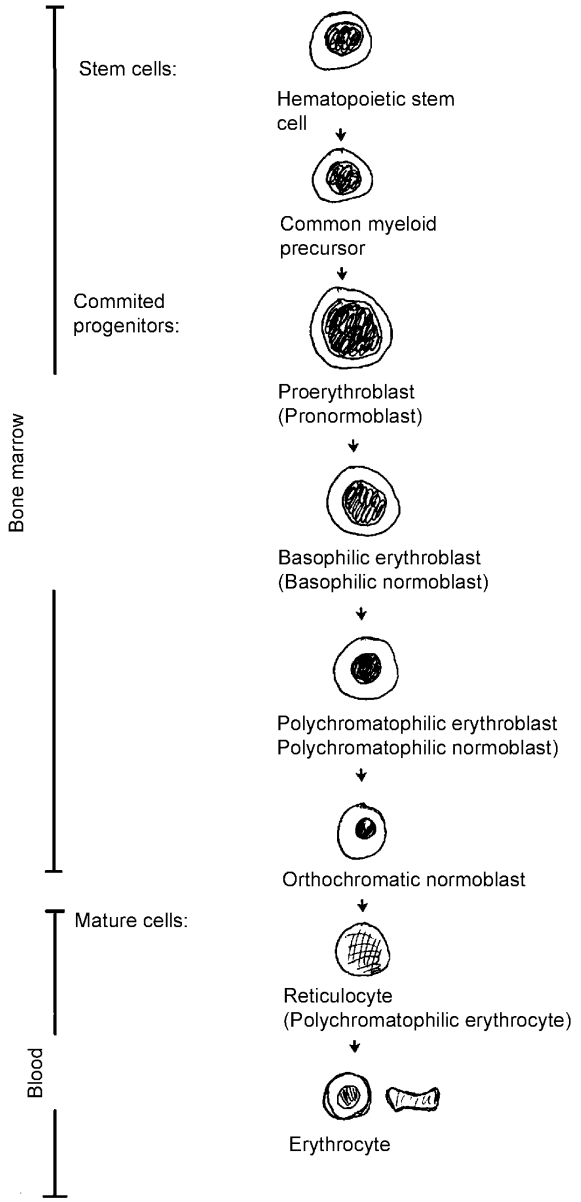

Figure 3. Erythropoiesis is a complex process involving many steps.

2.5 HEALING OF CONNECTIVE TISSUE

Tissue repair is normally a rapid process that has been devised through evolution to allow animals to rapidly recover tissue integrity after an injury using scarring to join the wound edges or to fill tissue voids (Caplan, 2003). However, the potential of connective tissues to heal varies greatly. For example, articular cartilage has a low healing

potential, whereas bone can regenerate itself without scar or functional degradation. Most connective tissues heal via three distinct but overlapping phases; hemostasis and inflammation, proliferation, and remodeling. Thus, the repair process itself is a series of complex biological events, which are coordinated via various intra- and intercellular pathways. All these phases are important and a disturbance in any of them will result in defective tissue repair.

In addition, the immune system is also activated as a response to the injury. Mammals have a very highly developed adaptive immunity but a relatively poor capacity to regenerate, whereas urodeles regenerate structures more easily but have a less robust immune system (reviewed in Godwin and Brockes, 2006). Emerging evidence suggests that the immune system might play a more positive role in tissue regeneration in immune privileged sites than the inflammation process (Mastellos *et al*., 2001; Mastellos and Lambris 2002; Kimura *et al*., 2003; reviewed in Godwin and Brockes, 2006). The outcomes of wound repair and regeneration are profoundly different, but may be mechanistically linked by subtle differences in signaling pathways (Whitby and Ferguson, 1991 A,B; reviewed in Metcalfe and Ferguson, 2007).

Here, the stages of wound healing are individually examined and the healing of bone is more briefly discussed as its early repair stages share similarities with soft connective tissue healing.

2.5.1 The biology of wound healing

The repair of wounds is essential for maintaining life. The wound healing process resembles embryogenesis, but after the fetal period wound repair is controlled via inflammation and healing leads to a scar formation (Martin, 1997; reviewed in Ferguson and O`Kane, 2004). The entire wound healing process is an intricate series of events that begins at the moment of injury and can continue for months to years. Most of the information on biochemistry and cell biology of wound healing and development of the granulation tissue has been obtained from animal studies using model wounds or inductive scaffolds (Davidson, 2001; Dorsett-Martin, 2004; Lindblad, 2008).

2.5.1.1 The inflammatory phase

Inflammatory response after injury has an essential role in orchestrating a series of cellular events aiming to wound healing. Scars are the end point of the normal continuum of mammalian tissue repair. On the contrary, fetal wound repair until a gestational age equivalent to the first trimester of human development occurs without scarring and fibrosis (reviewed in Ferguson and O`Kane, 2004; Buchanan *et al*., 2009). Fetal scarless healing is characterized by minimal inflammation and complete restoration of normal

skin structure. Therefore, early fetal wound repair is essentially a regenerative process (reviewed in Ferguson and O`Kane, 2004).

In later periods of life, inflammation is needed to initiate proper healing response. It occurs immediately after tissue damage and lasts approximately the following 48 hours. The components of the coagulation cascade, inflammatory cells and components of the immune system are needed to prevent ongoing blood and fluid losses, to remove dead and devitalized tissues and to prevent infection. The inflammatory response is composed of two major components, a vasomotor-vasopermeability response with increased capillary permeability and active leukocyte infiltration (Steele and Wilhelm, 1970; reviewed in Stadelmann *et al*., 1998; Kim M-H *et al*., 2009). A key chemical mediator responsible of these changes is believed to be histamine.

The initiation of the repair process is platelet activation and fibrin clot formation, which leads to hemostasis. Fibrin and fibronectin form a primitive or provisional matrix, which functions as a scaffold for infiltrating cells. Platelets not only participate to hemostasis, but are also storehouses for a number of growth factors and vasoactive substances, and are important in initiating the complement cascade with the formation of the complement factors C3a and C5a (reviewed in Steed, 1997; Amara *et al*., 2008). The secreted substances act as cues for other cells. The next cells to arrive at the wound site are the polymorphonuclear cells, i.e. neutrophils, which respond to the activation of the complement, the degranulation of platelets and the products of bacterial degradation. Like platelets, polymorphonuclear cells are an important source of proinflammatory cytokines, providing signals that activate adjacent fibroblasts and keratinocytes (Hubner *et al*., 1996; reviewed in Aukhil, 2000). Furthermore, these cells are important in protecting the wound from infection by killing bacteria and assisting in the removal of devitalized tissue fragments and debris (reviewed in Stadelmann *et al*., 1998; Brinkmann *et al*., 2004; reviewed in Martin and Leibovich, 2005). Activated neutrophils release free oxygen radicals and lysosomal enzymes, including matrix metallo- and other proteinases, collagenases and elastases, which help fight infection and clean the wound (Kajiki *et al*., 1988; reviewed in Steed, 1997).

Circulating monocytes appear shortly after the polymorphonuclear cells at the wound site, where they are activated and subsequently transformed into macrophages. Wound macrophages are voracious phagocytes that clear the wound of all matrix and cell debris, including fibrin and spent neutrophils. They also play a central role in fibroblast chemotaxis, proliferation, and the subsequent collagen synthesis and degradation (Leibovich and Ross, 1976; reviewed in Stadelmann *et al*., 1998). Macrophages continue growth factor and cytokine secretion into the wound environment, thus maintaining the wound repair signals initiated by degranulating platelets and polymorphonuclear

cells (Leibovich and Ross, 1975; Rappolee *et al*., 1988; reviewed in Aukhil, 2000; Lucas *et al*., 2010). Although essential in the commencement of the wound repair, the disappearance of the inflammation is a prerequisite for the normal progression of the healing process.

2.5.1.2 The proliferative phase

The second stage of wound repair, the proliferative phase, also called the granulation tissue formation phase, is characterized by rapid new tissue formation and migration of different cell types. The provisional ECM is gradually replaced with granulation tissue. Normally this stage starts two to three days after injury and lasts approximately two to three weeks. Granulation tissue consists mainly of new capillaries, macrophages, fibroblasts, and loose connective tissue within loosely associated fibrils of collagen, proteoglycans and other ECM proteins (reviewed in Aukhil, 2000). The cellular events in the healing process are further regulated by growth factors and cytokines, derived mostly from macrophages.

Fibroblasts produce numerous proteins of the ECM, such as collagens, elastin, and proteoglycans. Collagen synthesis is a seminal feature of fibroplasia and its synthetic activity reaches its maximum rate at one week after wounding. From numerous different collagens, type III and type I are the major factors causing the mechanical strength of the wound. The synthesis of type III collagen dominates during the first few days of wound healing, followed then by a massive synthesis of type I collagen (Kavitha and Thampan, 2008). Thick collagen fibers of ECM are mainly formed from type I collagen. Additionally, type V and VI collagens are also significant for achieving a proper tensile strength (Hildebrand and Frank, 1998; Breuls *et al*., 2009). Collagen levels rise continually approximately for three weeks until a point of homeostasis is achieved. At this point the rate of collagen degradation equals the collagen synthesis (reviewed in Stadelmann *et al*., 1998).

After hemostasis, the fibrin clot is degraded by plasmin and different matrix metalloproteinases (MMPs), which are a family of enzymes specifically cleaving a subset of matrix proteins (Agren *et al*., 2001; Hieta *et al*., 2003; Pirilä *et al*., 2007; Toriseva *et al*., 2007; reviewed in Toriseva and Kähäri, 2009). Degradation process begins from under the clot and allows the growth of granulation tissue. Degradation of the more superficial layers is necessary for the epithelialization that is needed to control the growth of the repair tissue and wound closure. In addition, neovascularization is also dependent on proteolytic activity.

The goal of the proliferative phase is to diminish the area of tissue loss by contraction and fibroplasia and to establish a viable epithelial barrier by the activities of the keratinocytes. Seven to ten days after wounding, fibroblasts in the wound transform

into myofibroblasts and express α-smooth muscle actin (reviewed in Aukhil, 2000; Opalenik and Davidson, 2005; reviewed in Gurtner *et al*., 2008). Such transformation allows these myofibroblasts to generate strong contractile forces responsible for wound contraction (Hinz *et al*., 2001). Expression of smooth muscle α-actin has also been shown to correspond with the initiation of cell apoptosis (programmed cell death) and may reflect a terminal differentiation event (Garbin *et al*., 1996). When the wound matures, mechanical stress decreases, and myofibroblasts are eliminated via apoptosis (Hinz, 2007).

Neovascularization accompanies this fibroblastic phase and is essential for the tissue to grow. Another important feature of skin wound healing is wound re-epithelialization. This process is regulated mainly by the contact inhibition. In skin wounds, contact inhibition is lost, and epithelial cells immediately adjacent to the injury site begin to divide and migrate (reviewed in Stadelmann *et al*., 1998) until the wound is covered and the wound edges are met.

2.5.1.3 The remodeling phase

By three weeks after injury, the balance between collagen synthesis and degradation is achieved and wound remodeling and maturation begins (reviewed in Stadelmann *et al*., 1998; reviewed in Gurtner *et al*., 2008). The main objective of this remodeling phase of tissue repair is to achieve maximum tensile strength by reorganization, degradation and resynthesis of the ECM.

The wound tensile strength increases progressively. The randomly distributed collagen fibers are cross-linked, aggregate into fibrillar bundles, and organize according to mechanical stress (Levenson *et al*., 1965; reviewed in Singer and Clark, 1999; reviewed in Toriseva and Kähäri, 2009). The rate of collagen synthesis within the wound is higher than in normal tissues for up to six-twelve months (Barnes *et al*., 1975), and also the composition of the ECM changes, for example the type III collagen matrix is replaced by a type I collagen matrix (Bailey *et al*.,1973). This final process may last even several years, before the new granulation tissue rich in cells and vascular capillaries has matured into a relatively acellular and avascular scar that lacks appendages, including hair follicles, sebaceous glands, and sweat glands (reviewed in Gurtner *et al*., 2008).

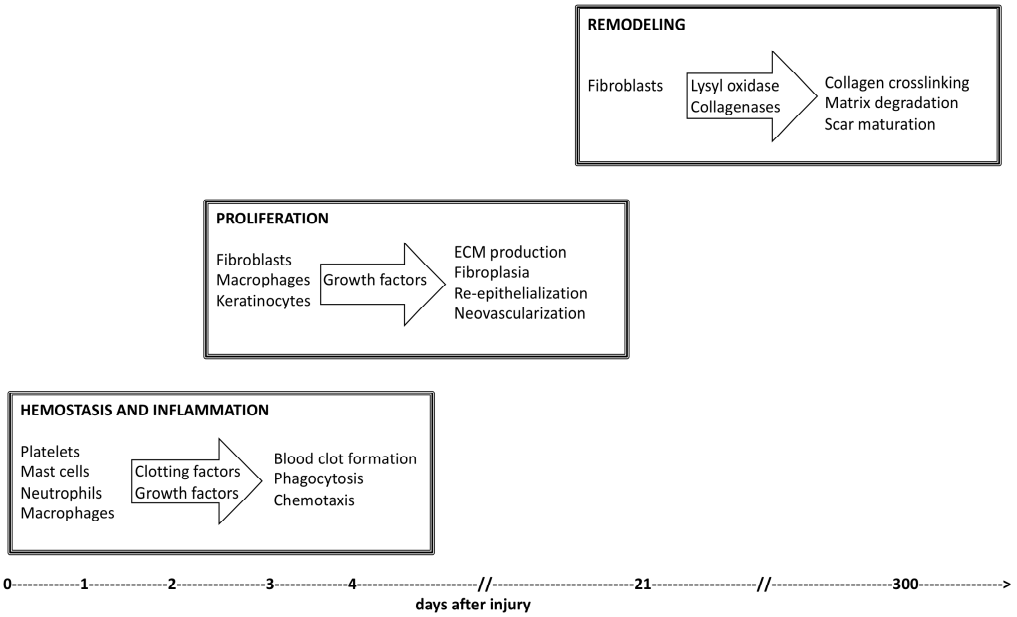

Figure 4. The phases of normal cutaneous wound healing. Each phase utilizes specific cell types, which release chemical mediators to carry out specific biological functions. (Modified from Murphy *et al*., 2006)

2.5.2 Bone healing

Bone healing is a complex, but well-orchestrated regenerative process that is initiated in response to injury. The mechanisms that regulate fetal skeletogenesis are common to the regulation of adult skeletal regeneration, and as a result, bones possess a remarkable ability to heal completely (reviewed in Tsiridis *et al*., 2007). Thus, bone formation is a life-long event, which begins during fetal development and persists as a continuous remodeling process throughout the life (Ekholm, 2001).

2.5.2.1 Fracture healing

The earliest stage of bone fracture healing has clear connections with skin wound healing; the hematoma formed immediately after fracture between the bone fragment ends is rapidly invaded by richly vascular granulation tissue. This loose ECM contains small diameter fibrils made of type III collagen. Characteristic for long bone healing is that the undifferentiated soft connective tissue is gradually replaced by hyaline cartilage containing large amount of type II collagen. The cartilage is replaced by type I collagen-rich woven bone in a process that closely resembles endochondral ossification. During the remodeling phase, the new bone eventually matures into compact lamellar bone and returns to its original shape (Ekholm, 2001). In addition to bone forming cells, osteoclasts

are also needed in order to achieve the fine architecture of bone tissue (Han and Zhang, 2006).

Optimal bone regeneration requires adequate blood flow. Ossification of the cartilaginous matrix occurs in parallel with revascularization (Allori *et al*., 2008 Part II). During the final stages of endochondral ossification and the remodeling phase, specific MMPs degrade cartilage and bone, allowing infiltration of blood vessels (Gerstenfeld *et al*., 2003). Neovascularization is regulated by different angiogenic factors, such as vascular endothelial growth factors (VEGFs) and angiopoietins (reviewed in Tsiridis *et al*., 2007).

Table II. The most common collagen types in wound and bone healing and their distribution (References Stadelmann *et al*.,1998; Kadler *et al*., 2007; Kavitha and Thampan 2008).

Type	Structure	Distribution
I	fibrillar	Bone, tendon, skin, dentin, ligament, fascia, arteries, uterus, granulation tissue
II	fibrillar	Hyaline cartilage, eye tissues
III	fibrillar	Granulation tissue, skin, arteries, uterus, bowel wall
IV	nonfibrillar	Basement membrane
V	fibrillar	Cell surfaces, hair, placenta; associated with type I collagen
VI	nonfibrillar	Most interstitial tissue; associated with type I collagen
IX	FACIT	Cartilage; associated with type II and XI fibrils
X	nonfibrillar	Hypertrophic and mineralizing cartilage
XI	fibrillar	Cartilage

2.5.2.2 Healing of bone defects

In humans, there are several conditions, such as bone tumor removal, severe fractures with bone defects and posttraumatic impaired bone healing (delayed unions, nonunions and malunions), which include bone loss. New bone formation in mechanically stable defects, as well as in rigidly fixed fractures, differs from natural fracture healing in that bone formation occurs primarily through intramembraneous ossification (Karp *et al*., 1992) without intermediate cartilage template. Animal models are commonly used to study the healing of bone defects (reviewed in Horner *et al*., 2009).

Especially in weight bearing bones, the method of fixation is important. Sufficient rigidity is needed to ensure bone healing, but too rigid fixation might also prevent it (Panagiotis, 2005). Numerous other factors, like the location and configuration of the fracture, large distances between the healing bone surfaces, inadequate daily biomechanical forces, associated soft tissue damage, and the age, affect the healing ability of bone defects (Panagiotis, 2005; Strube *et al*., 2008). The size of the defect is important as well. If the defect size exceeds certain limits (often called the “critical size”), the defect never heals.

Different vertebrates have different critical sizes, but in general the healing rate seems to be faster in small animals (Schmitz *et al*., 1990; reviewed in Horner *et al*., 2009).

2.5.2.3 Current clinical treatment of bone loss situations

Treatment of large size bone defects takes place usually by bone transplantation. Already in 1950´s it was noticed that bone contained native osteoinductive factors that regulate bone metabolism (Urist, 1953). Since then the use of autogenous bone in bone-grafting has been the gold standard in orthopedics. Autologous bone grafts are osteogenic, osteoconductive, osteoinductive and completely biocompatible. However, the use of the autologous bone is associated with postoperative pain and the potential donor site morbidity. The supply of the autologous bone might also be limited, and the quality of the autologous bone in some cases might be unsatisfactory. Because of these disadvantages, several bone-graft substitutes have been widely used. Allografts and demineralized bone matrices are osteoinductive choices, but they have many negative aspects as well. Fresh allografts possess bacterial, viral and prion contaminant and immune response risks, but the preparation and sterilization of allografts and demineralized bone matrices weaken their osteoinductive properties (reviewed in Keating and McQueen 2001; reviewed in Finkemeier, 2002).

However, due to reasons listed above proper biomaterial scaffolds are expected to replace bone grafts in the future therapies of bone defects. Numerous bone-graft substitutes of artificial materials have been developed. The ideal substitute material should provide mechanical support, and possess same characteristics as autologous bone, namely osteogenicity, osteoinductivity and osteoconductivity. The ideal material does not exist yet, so the hunt for the perfect bone substitute, the perfect biomaterial, is still going on.

2.5.3 Regulation of wound healing and bone repair

Wound and bone healing are complex processes involving intricate interactions among a variety of different cell types, structural proteins, growth factors, cytokines, proteinases and gaseous mediators. Both tissues use their own specific signaling pathways, but the general principles of the repair processes are relatively similar. Inflammation response is important in initiation and guiding of both processes. The main difference between bone and wound healing is the end result, because fracture repair leads to the total bone regeneration, whereas the end result of wound healing is a fibrous scar with impaired tensile strength.

Cutaneous wound healing is an extremely dynamic process where the protective barrier function of skin is quickly reconstituted in a timely and spatially coordinated manner. One crucial mediator of cellular interactions during the healing is the ECM, where many important signals are mediated via cell-ECM interactions. During cell migration, the

proteins of ECM are cleaved in a strictly controlled proteolysis leading to increased cell motility and division. Several proteases, like MMPs, PA-Plasmin system, ADAMs and ADAMTs proteases, are involved in this process, which often produces biologically active cleavage products (reviewed in Toriseva and Kähäri, 2009). Proteases also activate various growth factors and other proteases, in addition to regulating growth factor signaling by shedding growth factor receptors on cell surface (reviewed in Toriseva and Kähäri, 2009).

Nitric oxide (NO) is an important gaseous signaling molecule in wound repair (reviewed in Schwentker *et al*., 2002). All cell types participating in the healing process are capable of producing NO via three isoforms of nitric oxide synthases (NOS). Two NOS isoforms, neuronal and endothelial are expressed constitutively, whereas the third NOS isoform, inducible NOS (iNOS), is produced in large quantities in response to inflammatory cytokines and/or bacterial products (Shi *et al*., 2001, Weller, 2003). After tissue injury, iNOS expression is upregulated (Bogdan *et al*., 2000), and the produced NO affects several aspects of wound healing, including modulation of cytokine production, angiogenesis, inflammation, cell proliferation, matrix deposition, and remodeling (Shi *et al*., 2001; reviewed in Schwentker *et al*., 2002; reviewed in Luo and Chen, 2005). Consequently NO influences all steps in the wound repair process.

Like wound healing, bone repair is a cascade of complex biological events involving intracellular and extracellular molecular signaling for bone induction and conduction. During the repair process, the pathway of embryonic development is repeated with coordinated participation from several cell types (Ferrara and Davis-Smyth, 1997). At the injury site, the cortex, the periosteum, the bone marrow, and the external soft tissues contribute to the healing process (Tsiridis *et al*., 2007). The extent to which each component is involved depends on the conditions present at the injured tissue, such as the level of growth factors, hormones, nutrients, pH, oxygen tension, the electrical environment, an the mechanical stability of the fracture (Riedel and Valentin-Opran, 1999). The activation of the immune system and the initiation of the inflammatory response are crucial in the beginning of the repair process (Tsiridis *et al*., 2007).

Historically, the ECM was viewed as an inert environment that provided only stromal support. To date, the matrix is seen as an active and dynamic biochemical system that conveys important regulatory cues to nearby cells, effecting gene expression and changes at the cytostructural level (Allori *et al*., 2008, Part I). Various hormones, cytokines, and growth factors serve as signaling molecules, which affect to cellular migration, adhesion, proliferation, differentiation, transcription and translation. These signaling molecules may mediate their message to cells either locally or systematically, that is, in autocrine, paracrine, juxtacrine or endocrine fashion.

Besides growth promoting molecules, numerous inhibitory molecules that regulate different signaling pathways are needed as well. These inhibitory molecules help to

maintain the adequate balance of growth promoting factors. Inhibition may happen via various mechanisms, mainly via negative feedback loops and crosstalk of different pathways, for example at the extracellular level (noggins, DAN family, Chordin/ Twisted Gastrulation, Follistatin, BMP-3, Ahsg), at the receptor level (BAMBI), or at the intracellular level (Inhibitory Smads, Smurf-1, Smurf-2, Ski, Tob, Smad-8B, CIZ). (Tsiridis *et al*., 2007)

In addition to signaling molecules, the ECM contains molecules, which participate actively to cell adhesion and mineral nucleation. The key components of ECM include fibrillar and nonfibrillar proteins, minerals and enzymes, such as the MMPs. By binding to receptors (e.g., integrins and transmembrane proteoglycans) on bone cells, ECM molecules cause cytoskeletal deformations that activate second-messenger systems and lead to altered gene expression, adhesion, migration, proliferation, differentiation, growth and cell death (Allori *et al.*, 2008, Part II). ECM molecules may influence the expression of genes encoding other ECM molecules, so these molecules are continuously under a dynamic balance via this feedback process.

2.5.3.1 Growth factors and cytokines in wound and bone healing

In addition to the importance of cell-cell and cell-matrix interactions, all stages of the repair process are controlled by a wide variety of different soluble factors such as growth factors and cytokines. Growth factors are naturally occurring proteins regulating a variety of cellular processes. They represent the intercellular signaling that orchestrates the complex sequence of cell migration, division, differentiation, and protein expression. The term cytokine is sometimes used interchangeably with the term growth factor. However, many cytokines also exhibit growth factor activity, but some cytokines have an inhibitory effect to cell growth or proliferation.

Growth factors and cytokines are produced by a variety of cell types and can exert autocrine, paracrine, juxtacrine, and endocrine effects. They bind to the surface receptors of the target cell, which in turn triggers the intracellular domain that generally activates a protein kinase. The kinase cascade activates transcription of a gene into mRNA, which is then translated into proteins to be used within cells or exported. Many signaling molecules may be bound and immobilized in the matrix or at the cell surface, and therefore, are no longer soluble. Consequently, the activity of signaling molecules *in vivo* reflects a delicate balance between free and bound forms.

To date, numerous growth factors and cytokines are held as key regulators of the wound healing process (Puolakkainen *et al*., 1995; reviewed in Werner and Grose, 2003; Barrientos *et al*., 2008; reviewed in Toriseva and Kähäri, 2009). After trauma the expression of growth factors and cytokines is frequently upregulated, and different cell types express multiple growth factors and cytokines and their receptors in healing skin wounds. When the skin

is injured, the epidermal barrier is disrupted and the keratinocytes release prestored IL-1 alerting surrounding cells to the barrier damage. Degranulating platelets secrete growth factors as epidermal growth factor (EGF), platelet derived PDGF and TGF-β. PDGF and proinflammatory cytokines, like IL-1, attract bacteria removing neutrophils, whereas TGF-β helps monocytes to convert into macrophages. The macrophages, in turn, release a variety of proinflammatory cytokines like interleukins and growth factors like fibroblast growth factor (FGF), EGF, TGF-β and PDGF promoting for example granulation tissue formation and re-epithelialization of the wound. VEGF released from platelets together with FGF promote endothelial cell proliferation and angiogenesis. Synthesis, deposition and organization of new ECM are controlled i.e. by FGF, TGF-β and PDGF. The accurate balancing of the pro- and anti-inflammatory signals is essential for successful wound healing, so therefore the concentration and duration of exposure to each growth factor and cytokine is strictly controlled in relation to other factors (reviewed in Gurtner *et al*., 2008; Barrientos *et al*., 2008).

In bone healing, the most crucial growth factors and cytokines involved are much the same that participate in cutaneous wound healing, namely the proinflammatory cytokines, the members of the TGF-β superfamily, and angiogenic factors (Tsiridis *et al*., 2007). The TGF-β superfamily contains numerous growth and differentiation factors that include bone morphogenetic proteins (BMPs), TGF-βs, activins, inhibins, and the Mullerian inhibiting substance. Although multiple factors are important in bone healing, probably the most critical group in regulating osteoblast growth, differentiation, and apoptosis is the BMPs. Each type of BMPs has a unique role in fracture repair process, and especially BMP-2 and BMP-7 have been noticed to possess enhanced osteoinductive activity (Israel *et al*., 1996; Geesink *et al*., 1999; Riedel and Valentin-Opran, 1999). At the moment, both of these factors are commercially available in a recombinant form for clinical treatment of impaired bone repair (Tsiridis *et al*., 2007).

Chemokines are small chemotactic pro-inflammatory cytokines released by the cells at the site of the injury. They stimulate the migration of multiple cell types at the wound site, particularly inflammatory cells (Barrientos *et al*., 2008). Chemokines are suggested to contribute to the regulation of re-epithelialization, tissue remodeling and angiogenesis (reviewed in Raja *et al*., 2007). The CXC, CC, and C families of ligands act by binding to G protein-coupled surface receptors, CXC-receptors and the CC-receptor. Especially interesting is SDF-1α, (CXCL12), which is a member of CXC family and works via the CXCR4 receptor. It plays a role in the inflammatory response, promotes angiogenesis, and furthermore, recruits proangiogenic subpopulations of hematopoietic cells (bone marrow progenitors) from circulation to peripheral tissues (Grunewald *et al*., 2006; Schantz *et al*., 2007).

Table III. The most common growth factors in wound and bone healing. (reviews of Werner and Grose, 2003; Tsiridis *et al*., 2007; Barrientos *et al*., 2008; Gurtner *et al*., 2008; Lau *et al*., 2009).

Growth factor	Cells	Function	Special notes
Angiogenic factors (Angiopoietins and Vascular endothelial growth factors)			
Angiopoietin-1, -2	Endothelial cells	Formation of larger vessels Development of collateral branches	Regulators of vascular morphogenetic molecules
VEGF-A	Platelets Neutrophils Macrophages Endothelial cells Smooth muscle cells Fibroblasts Keratinocytes	Granulation tissue formation Chemotaxis Angiogenesis Lymphangiogenesis	Hypoxia is a major stimulus for VEGF-A release Binds to the tyrosine kinase receptors Flt-1 and KDR
VEGF-C	Macrophages	Inflammation Hematopoietic cell recruitment Angiogenesis Lymphangiogenesis	Receptors VEGFR-2 and-3 Increases vascular permeability via VEGFR-2
Epidermal growth factor –family			
EGF	Platelets Macrophages Fibroblasts	Re-epithelialization Increases tensile strength	Acts mostly via EGFR Ligand also to several other receptors
Fibroblast growth factor			
FGF-2 (basic FGF)	Keratinocytes Mast Cells Fibroblasts Endothelial cells Smooth muscle cells Chondrocytes	Granulation tissue formation Re-epithelialization Matrix formation and remodeling	FGF-7 and FGF-10 other important family members
Granulocyte macrophage colony-stimulating factor			
GM-CSF	Macrophages T-cells Mast Cells Endothelial cells Fibroblasts	Inflammation Re-epithelialization Increase endothelial cell migration and proliferation	Upregulates IL-6
Hepatocyte growth factor			
HGF	Mesenchymal cells	Stimulation of keratinocyte migration Targets and acts epithelial and endothelial cells	Role in embryonic organ development and adult organ regeneration
Insulin-like growth factors			
IGF-I	Bone matrix Endothelial cells Osteoblasts Chondrocytes	Bone matrix formation by fully differentiated osteoblasts	Growth hormone regulates the serum level of IGF-I
IGF-II	Same as IGF-I	Later stage of endochondral bone formation	

Growth factor	Cells	Function	Special notes
Platelet derived growth factor -family			
PDGF	Platelets Macrophages Endothelial cells Fibroblasts Keratinocytes	Inflammation Granulation tissue formation Re-epithelialization Matrix formation and remodeling Blood vessel maturation	A family of several homo and heterodimers: -AA, -AB, -BB, -CC, DD Important in each stage of healing Counteracts with many other factors
Proinflammatory cytokines			
Interleukin-1	Neutrophils Monocytes Macrophages Keratinocytes	Inflammation Re-epithelialization	Increases FGF-7 secretion IL-1β elevated in chronic wounds
Interleukin-6	Neutrophils Macrophages	Inflammation Re-epithelialization	Important in initiating the healing response
TNF-α	Neutrophils Macrophages	Inflammation Re-epithelialization	Levels elevated in chronic wounds
Transforming growth factor β -superfamily			
TGF-β1	Platelets Keratinocytes Macrophages Lymphocytes Fibroblasts Osteoblasts Chondrocytes	Inflammation Granulation tissue formation Wound contraction Re-epithelialization Matrix formation and remodeling	Predominates in cutaneous wound healing Potent stimulator collagen synthesis → scar formation
TGF-β2	Same as TGF-β1	Same as TGF-β1 Chemotaxis for bone forming cells	Scar formation More important in bone repair than TGF-β1
TGF-β3	Same as TGF-β1	Inflammation Keratinocyte migration Neovascularization Keratinocyte DNA synthesis inhibition	Stop signal for terminal wound differentiation Inhibits scarring Promotes better collagen organization
BMP-2	Osteoprogenitors Mesenchymal cells Osteoblasts Chondrocytes	Recruitment of mesenchymal cells Chondrogenesis Initiation of healing cascade	Potent inducer of MSC differentiation
BMP-4	Same as BMP-2	Early stage callus formation	
BMP-6	Same as BMP-2	Ossification regulation Chondrocyte maturation	
BMP-7	Same as BMP-2	Ossification regulation	

2.5.4 Stem cells in wound healing

Plasticity and ability to differentiate into various tissue types is valuable during wound healing. Both hematopoietic and mesenchymal stem cells have been shown to play an important role in many tissue injuries. HSCs are capable to differentiate into neovasculature (Asahara *et al*., 1999), and they seem to be the key mediators in endothelial repair. Injection of HSCs into mice accelerates revascularization of ischemic limbs (Schatteman *et al*., 2000) and wound healing (Sivan-Loukianova, 2003). In addition to their vascular repair ability, HSCs have an ability to differentiate into non-hematopoietic cells (see chapter 2.2.1). Thus, their role in wound healing might be much wider than has been assumed.

HSCs and MSCs seem to have functional interaction in the bone marrow microenvironment (Valtieri and Sorrentino, 2008) and it is likely that both cell types are needed in wound healing. MSCs home to the wound site and differentiate to the connective tissue cells that produce the ECM of the granulation tissue (Yamaguchi *et al*., 2005; Liu *et al*., 2006; McFarlin *et al*., 2006; Cha and Falanga, 2007; Kwon *et al*., 2008). Furthermore, MSCs release factors that not only enhance non-hematopoietic tissue repair but also yield proangiogenic factors essential for HSCs/EPCs survival, proliferation and differentiation (Kinnaird *et al*., 2004). This indicates that MSCs are important in blood vessel formation as well.

The injured area sends both attractive and repulsive cues to bone marrow-derived stem cells to target them exactly to the correct location (Chan-Ling *et al*., 2006). Stem cells migrate to sites of injury because they are attracted by locally produced growth factors and/or cytokines, including the chemokine stromal-derived factor-1 (SDF-1) (Aiuti *et al*., 1997; Tachibana *et al*., 1998). The fate of stem cells is ultimately dictated by the local environment.

For tissue engineering, there are several optional sources of stem cells. Embryonic stem cells are derived from the inner cell mass of the embryonic blastocysts, and represent an attractive alternative for cell-replacement therapy due to their pluripotency (reviewed in Metcalfe and Ferguson, 2007). Their use is, however, limited because of many controversial ethical and technical issues. A more acceptable choice might be cord blood cells, which are more primitive than adult bone marrow cells, and have increased capacity for multilineage differentiation (Ballen, 2007).

Multipotent adult stem cells do not possess the full range of plasticity of embryonic stem cells, but they offer practical advantages including ease of isolation and propagation (Lee *et al*., 2009). The treatment with autologous adult stem cells also helps to avoid rejection problems, and the harvesting of stem cells is relatively easy from the bone marrow area or from peripheral blood (reviewed in Metcalfe and Ferguson, 2007). Nowadays more

attention has been focused on adult somatic stem cell sources, especially to the skin and fat tissue (Shi *et al*., 2006; Jeong, 2010), which both represent a large reservoir of adult stem cells. The advantages of these tissues are that they are easily accessible and immune privileged. Adult stem cells reside in specific niches and the niche exposes the stem cells to different differentiation cues (reviewed in Metcalfe and Ferguson, 2007). These cells are essential for tissue repair, wound healing and regeneration, because they are responsible for all cell replacement within a tissue. It has also been speculated that the use of cells with stem cell properties might reduce scar formation (Shi *et al*., 2006). A new source of stem cells, so called induced pluripotent stem cells, can be generated directly from somatic differentiated cells by silencing several key genes of differentiation by various techniques. Examples of such techniques are expected to revolutionize the stem cell technology (Nakagawa *et al*., 2008; Voigt and Serikawa, 2009; Aoki *et al*., 2010; Tat *et al*., 2010).

2.5.5 Impaired wound healing and chronic wounds

Normally the end result of uncomplicated wound healing in postnatal life is a scar with little fibrosis, minimal if any wound contraction, and a return to near normal tissue architecture and organ function. The wound is considered chronic, if the healing process does not occur in orderly or timely sequence or does not result in structural integrity (Robson, 1997). Impaired wound healing is characterized by granulation tissue formation that is not resolved in a proper time and often leads to the excessive fibrosis causing hypertrophic scars or keloids.

Skin ulcers are probably the most common types of chronic wounds. They are characterized by missing or insufficient granulation tissue and are caused by an underlying disease, environmental factors (such as elevated skin pressure), prolonged inflammation and infections. The most common ethiological reason for chronic ulcers is the lack of adequate blood circulation, usually due to venous insufficiency or arterial diseases or both. Other important etiological factors are diabetes, vasculitis, trauma and neoplasia (Sarkar and Ballantyne, 2000, Mekkes *et al*., 2003, Simon *et al*., 2004). Whatever the cause behind the ulcer, chronic wounds often demand a long-lasting treatment, worsen the individual's quality of life and are a remarkable burden on the community.

Despite the etiology of a chronic wound, local molecular and cellular pathophysiologies are much alike (Mast and Schultz, 1996). The secretion or function of many molecular and cellular factors is disturbed. In non-healing wounds, inflammation is usually counterproductively prolonged. Thus, the presence of macrophages, B cells and plasma cells are elevated in chronic wounds and diabetic ulcers (Kössi, 2000). The levels of glucose, total protein and albumin are decreased in chronic leg ulcers compared to healing wounds (Trengove *et al*., 1996) suggesting a diminished availability of energy.

In addition, the amount of many crucial growth factors, e.g. PDGF, FGF-2, EGF, and TGF-β, has been shown to be reduced compared with the amounts in acute wounds (Cooper *et al*., 1994). Chronic wounds have often lost the delicate balance between adequate growth factor and cytokine production versus prolonged activation of pro-inflammatory cytokines and diminished anti-inflammatory cytokine function (reviewed in Barrientos *et al*., 2008). As a matter of fact, fluid collected from acute wounds stimulates DNA synthesis up to three times that seen in non-treated fibroblasts cell cultures, whereas fluid collected from chronic wounds failed to stimulate any synthesis at all (Bennett and Schultz, 1993). It has also been claimed that both keratinocytes and endothelial cells isolated from chronic wounds are unable to upregulate chemokines and cytokines necessary for the resolution of chronic inflammation (Galkowska *et al*., 2006).

Chronic ulcers contain elevated MMP levels and activity (Impola *et al*., 2005) as well as interleukin-1α (IL-1α and tumor necrosis factor alpha (TNF-α) compared to normally healing surgical wounds (Saarialho-Kere *et al*., 1993; Barone *et al*., 1998; Wallace and Stacey, 1998; reviewed in Toriseva and Kähäri, 2009) and the levels of growth factor degrading proteases are up to 100-fold greater than in acute wound fluids (Tarnuzzer and Schultz, 1996). The degradative effect of chronic wound fluid is associated with increased levels of elastase and diminished levels of proteinase inhibitors (Yager at al., 1997), so even if relevant growth factors are present in a chronic wound, the presence of wound proteinases may play a significant role in neutralizing their effect.

In normal skin, types I and III collagen exist in a ratio approximately 4:1. In hypertrophic and immature scars, the percentage of type III collagen may be as high as 33%, altering the ratio of type I to type III collagen to 2:1 (reviewed in Stadelmann *et al*., 1998). As the wound heals, granulation tissue converts from a cell-rich, highly vascularized medium to a much less vascular and cellular matrix of collagen. To achieve this stage, the cells present in granulation tissue must be removed from the wound by apoptosis. A failure of this process leads to a high degree of cellularity and abundant scar formation.

Many impaired wound healing states have been associated with depleted NO production. For example, disturbance in the availability of NO explain many of the poor wound healing characteristics found in diabetes (Curran *et al*., 2006, Boykin JV Jr, 2010). Although NO is a prerequisite for successful wound healing, a surfeit of this gaseous signaling molecule may be as detrimental as its underproduction, affecting among other things collagen synthesis (reviewed in Schwentker *et al*., 2002).

Wound infection often leads to impaired wound healing. Infection inhibits the multiple processes involved in wound healing (Tachi *et al*., 2004). Bacteria and bacterial products attenuate fibroblast proliferation, collagen synthesis, growth factors activities, and increase degradation of collagen (Tarnuzzer and Schultz, 1996). The process is mediated by inflammatory cells. Adequate tissue oxygen tension is important for adequate bacterial

killing by polymorphonuclear cells via oxidative intracellular mechanisms (reviewed in Stadelmann *et al*., 1998). Hemoglobin plays an important role in a tissue oxygen delivery, and an insufficient hemoglobin level has been shown to be an independent risk factor causing delayed healing (Takahashi *et al*., 2009).

Chronic ulcers are a common problem especially among elder people. Recent evidence suggests also, that age-related decline in repair and regeneration potential might be a significant factor behind non-healing wounds in the skin (Wall *et al*., 2008). Chronicity, maintained by chronic inflammation or bacterial infection, may lead to localized cellular/tissue aging within the wound site. The failure to resolve inflammation might be a consequence of fibroblast dysfunction, caused by the lack of the precise cues required to guide infiltrating leukocytes to the injury site (Wall *et al*., 2008). This theory is based to the new concept of a stromal address code, reviewed by Parsonage *et al*., (2005).

Fibroblast dysfunction is seen when an increased proportion of cells undergo irreversible growth arrest or replicative senescence (Dimri *et al*., 1995). Senescent fibroblasts, which are still metabolically active, are highly stable and express numerous ECM-related enzymes and structural molecules (Wall *et al*., 2008), while downregulating type I collagen (Varani *et al*., 2004). Fibroblasts isolated from chronic wounds have decreased proliferation potential in cultures and increased replicative senescence compared with normal non-wound skin fibroblasts (Stanley and Osler, 2001). However, not all studies have shown similar findings, and the role of aging and altered life span of fibroblasts within the wound is still not fully understood (Wall *et al*., 2008) but might be related to the limited number of mitotic divisions of specialized cells (Goldstein *et al*., 1974).

2.5.5.1 Current clinical treatments of chronic ulcers

At the present time, the basic treatment of chronic ulcers is to take care of the underlying reasons causing ulceration, for example by balancing blood glucose levels of diabetic patients, optimizing treatment of cardiovascular diseases, for selected patients surgical interventions, and correcting anemia. Decreasing and preventing tissue edema is crucial (O´Meara *et al*., 2009). Topical care includes a careful conservative treatment with various methods according to the clinical situation of the wound, e.g. mechanical cleaning of the wound bed, antiseptic baths, enzymatic gels and different wound covering materials. Some commonly used methods and new clinical tests for wound bed cleaning are listed in the table IV below. Antibiotics are needed in cases of bacterial infections (O´Meara *et al*., 2010). The most complicated ulcers are treated with skin grafts or even with microvascular reconstructions. Simultaneous vascular reconstructions are often essential to prevent fast relapses. Some milder cases can be treated with skin strip grafts performed by dermatologists or general practitioners. This technique is simpler and the demand of resources is much lower than in techniques of plastic surgeons.

Table IV. Methods used for wound bed cleaning.

Treatment methods	References
Enzymatic gels	Ramundo and Gray, 2008
Honey	Jull *et al*., 2008
Hyperbaric oxygen	Hunter *et al*., 2010
Larvae	Dumville *et al*., 2009
Resin salve	Sipponen *et al*., 2008
Silver	Bergin and Wraight, 2006
Topical Activated Protein C	Whitmont *et al*., 2008
Wound dressings	Palfreyman *et al*., 2006

In general, a serious injury to the skin requires an immediate coverage to facilitate repair and restore skin function. This is valid in severe burns and traumas, but active interference is as well important in chronic ulcers. The gold standard is autologous skin graft. However, autologous skin grafting is not always possible, therefore numerous commercial artificial skin constructs have been developed. For example, Alloderm® is made from cadaver skin and processed acellular to become immunologically inert whereas Integra®, Dermagraft®, Apligraf® and Epigel® are artificial ECM constructs made from natural or synthetical materials (reviewed in Metcalfe and Ferguson, 2007). These substitutes are mainly used for a temporary coverage of wounds. None of these materials are able to fully recreate the functions and esthetics of the skin. Common problems with existing skin substitutes include reduced vascularization, scarring, absence of differentiated structures, poor biocompatibility, poor mechanical and handling properties, and in some cases, delay involving cell culture or persistence of cells in heterologous grafts (reviewed in Metcalfe and Ferguson, 2007). These materials are also often very expensive.

Because there are no artificial skin substitutes that completely replicate normal uninjured skin (reviewed in Metcalfe and Ferguson, 2007), the interest to enhance wound healing by exogenous growth factors is growing. The outcome of studies concerning growth factor therapy has been inconsistent. A delicate balance of different growth factors and cytokines is a key question, because an overexpression of one subject might disturb the complex entity of the healing process. Furthermore, topical delivery of growth factors to chronic wounds is difficult because of the rapid degradation of agents caused by the proteolytic environment of chronic wounds (reviewed in Barrientos *et al*., 2008). Currently, gene therapy and developing biomaterial technology have provided new possibilities, and there are multiple novel delivery systems, including adenovirus and slow-releasing polymers, which are being investigated as growth factor delivery systems. The most promising growth factors for clinical testing are bFGF (Robson *et al*., 1992), GM-CSF (Bianchi *et al*., 2002; Cianfarani *et al*., 2006), EGF (Fernández-Montequin *et al*., 2009), and VEGF (Saaristo *et al*., 2006). At the moment, the only growth factor in

real clinical use, approved by the FDA, is topically applied PDGF-BB, which is used in the treatment of chronic ulcers with good results (Margolis *et al*., 2000; Margolis *et al*., 2004).

A new area under intensive investigation is stem cell application to engineered constructs. With different scaffolds, stem cells can be selectively delivered to certain areas. Locally implanted MSCs seem to be effective in bone, cartilage and tendon repair (Burns *et al*., 2010; Charbord *et al*., 2010). The treatment can be targeted also for example straight to the cardiac muscle (Lesman *et al*., 2010), or neural tissue (Gelain *et al*., 2006). In wound healing, new multifunctional biological scaffolds are often combined with wound dressing and films. These scaffolds are able to release shielded bioactive substances, such as MSCs, or to anchor endogenous circulating repairing cells (Kobayashi and Spector, 2009; Dainiak *et al*., 2010; Drago *et al*., 2010).

2.6 BIOMATERIALS

A biomaterial is defined as a material, either man-made or natural, which is used and adapted for a medical application. It does not have a chemical effect in the organism, nor does it need to be metabolized to be active like for example drugs (Ratner *et al*., 2004).

2.6.1 General aspects of biomaterials

The major classes of biomaterials include metals, ceramics, polymers and composites, but an autograft, allograft, or xenograft used as a transplant material can be considered as a biomaterial as well. Metals and phosphate ceramics have already been used for a half of a century and polymers for over 30 years to replace diseased or damaged tissues, so they are supposed to be non-toxic and biocompatible. At first biomaterials were selected to be as bio-inert as possible, meaning that they are not degraded in tissues and not recognized as a foreign material. Some bioactive materials are, however, capable to form a bond between an implant and a living tissue (Hench, 2006). Most new biomaterials are also biodegradable and metabolized into non toxic compounds, which are either utilized by the host or secreted into urine. To date, many biomaterials combine bioactive and bioresorbable properties in order to activate *in vivo* mechanisms of tissue regeneration, stimulating the body to heal itself and leading to replacement of the scaffold by the regenerating tissue (Boccaccini and Blaker, 2005).

Current strategies for producing improved biomaterials are alteration of implant chemistry and changing the physical properties of the implant (Khang *et al*., 2008). Novel sophisticated scaffolds are combined for example with different growth factors or stem cells, aiming to enhance the tissue repair (reviewed in Metcalfe and Ferguson, 2007). The clinical applications of biomaterials are numerous, from silicon breast implants and

contact lenses to heart valves. The field of applications is continuously expanding and consists of dentistry (Smith *et al*., 2008), head and neck surgery (Bücheler and Haisch, 2003), and orthopedics (Navarro *et al*., 2008), cartilage repair (Stoddart *et al*., 2009), skin tissue regeneration (Priya *et al*., 2008), and tissue engineering of cornea (Shah, 2008), myocardium (Jawad, 2008), and genitourinary tract (Wood *et al*., 2008). There are also biomaterials for central nervous system (Zhong *et al*., 2008), for targeted and prolonged drug delivery (Kim S *et al*., 2009), and even for nanoparticle oxygen carries, which act as a hemoglobin substituent (Piras *et al*., 2008).

2.6.2 Cellulose as a wound healing material

Cellulose is a natural polymer, which is degraded by microbial enzymes. It is a linear homopolymer of glucose ($C_6H_{12}O_6$), consisting of β-D-glucopyranose units linked by β(1-4) bonds to form long, straight chains strengthened by cross-linked hydrogen bonds (Mayes, 1988). Cellulose is the most common organic compound on Earth, being the structural component of the primary cell wall of green plants, many forms of algae and the oomycetes. For industrial purposes cellulose can be manufactured from wood pulp or other cellulose materials. The production of the cellulose is cheap, and it is easily fabricated to different structures and derivatives.

Cellulose and its derivatives are well tolerated by several tissues and cells (Miyamoto *et al*., 1989; Barbié *et al*., 1990; Kino *et al*., 1998). Because of its good biocompatibility and non-toxicity, cellulose and its derivatives have also been used in biomedicine. The examples of the different biomedical applications are, among the others, coating materials for drugs, blood coagulants, additives of pharmaceutical products, supports for immobilized enzymes, artificial kidney membranes and stationary phases for optical resolution (Miyamoto *et al*., 1989). Cellulose induces a moderately strong foreign body reaction in tissues. When implanted subcutaneously, vital and well-vascularized granulation tissue grows rapidly into the cellulose sponge (Viljanto and Kulonen, 1962; Raekallio and Viljanto, 1975). This process resembles wound healing assayed by histological or chemical criteria (Edwards *et al*., 1957; Holund *et al*., 1979; Pajulo *et al*., 1996; Inkinen *et al*., 2003). Cellulose acts as a chemoattractant for cells that are involved in the repair process, but it does not interfere with the normal wound healing process.

Because of this good granulation tissue formation ability, cellulose sponges have been used in experimental surgery since 1959 (Viljanto and Kulonen, 1962), and the subcutaneous implantation of the cellulose sponge is a widely accepted method to study wound healing (Edwards *et al*., 1957; Holund *et al*., 1979; Pajulo *et al*., 1996; Inkinen *et al*., 2003). Cellulose sponges have also been used to stimulate granulation tissue in the wound base after deep burns and traumatic injuries (Viljanto, 1972; Viljanto and Jääskeläinen, 1973; Viljanto and Raekallio, 1976 A;B). Several cellulose products for wound healing purposes

(e.g. Cellspon®, Cellstick®, Sponcal®, Visella® and Absorpal®) are commercially available. These products are made from sponge form viscose cellulose, which has a homogenous porous structure, characterized by thin pore walls with one or more interpore openings. The sponge is elastic and can be compressed and expanded repeatedly without damage to the internal structure, thus providing a free entrance for the cells to inner parts of the sponge (Viljanto, 1995). Because of these properties, viscose cellulose sponge is easy to shape to a suitable form prior implantation.

The greatest disadvantage in cellulose use as a biomaterial is its low biodegradability. Animal cells do not have enzymes that can cleave the β1-4- bond between two glucose moieties. Thus cellulose degradation in tissues takes place by a slow nonenzymatic hydrolysis of the β1-4 bond. Because of this slow degradation, cellulose can be considered as an almost stable matrix. The time needed for the total disappearance of the cellulose sponge from subcutaneous tissue is longer than 60 weeks (Märtson *et al*., 1999). The biocompatibility of the cellulose sponge with the bone is also good. The osteogenic potential of the cellulose has been investigated, and the cellulose sponge has been shown to allow osteoconduction, but not to have osteoinductive properties (Märtson *et al*., 1997; Märtson *et al*., 1998). In practice, this means that when implanted into bone, cellulose does not inhibit the growth of new bone at sites where bone should be formed.

2.6.3 Hydroxyapatite coating of biomaterials

Mature compact bone consists of about 70% inorganic salts and 30 % organic matrix by weight. The mineral components of bone are mainly calcium and phosphate in the form of HA crystals. Because of their physicochemical similarity to the teeth and bones, HA ceramics have been used in oral, maxillofacial, and orthopedic applications (Hench, 1991). Hydroxyapatite has good bioactivity and osteoconductivity (Park and Lakes, 1992, Hench, 1998, Domingo *et al*., 2003), but the material itself has poor mechanical properties. HA coating for stiff materials, like stainless steel, titanium or titanium and magnesium alloy implants (Habibovic *et al*., 2002, Kim *et al*., 2005, Zhang *et al*., 2009), have been used in order to enhance their biocompatibility and adherence to bone.

Bioactive glasses are a group of synthetic silica-based bioactive materials with bone bonding properties. They were developed in 1969 by Larry Hench, as the first and the original 45S5 Bioglass®. The bioactive glass 45S5 is a silica-based melt-derived glass characterized by a SiO_2 content of less than 60 %, a high Na_2O and CaO content, and a high $CaO:P_2O_5$ ratio (Hench, 2006). These silica, calcium, and phosphate groups form a reactive surface allowing bonding with bone. Especially silica is believed to play a critical role in bioactivity (Välimäki and Aro, 2006). Indeed, bioglass 45S5 and other similar bioactive glass formulations have been shown to stimulate bone regeneration (Jell and Stevens, 2006), and thus have been used in a variety of clinical applications, for

example in dentistry, head and neck surgery, and orthopedics. The mechanical properties of bioglasses are, however, poor for clinical purposes, because they are relatively stiff, brittle and difficult to form (Thomas *et al*., 2005).

Bioactive glasses can also be used to produce a HA coating for different materials. This so called biomimetic method was first presented by Kokubo *et al*., 1991. In this method, a substrate is set in contact with particles of bioactive CaO-SiO_2-based glass soaked in a simulated body fluid (SBF) with inorganic ion concentrations nearby equal to those of human blood plasma. This leads to apatite nuclei formation *in situ*, resulting in a coating resembling a natural bone surface.

3. AIMS OF THE STUDY

In this study, the biocompatibility of silica-rich HA coating of cellulose sponges was tested both in femoral bone defects and in the subcutaneous space in rats. All studies have been executed in experimental rat models.

The detailed aims were:

- To test the compatibility of the HA-coated cellulose sponge for a bone substitute material (I)
- To investigate the granulation tissue forming ability of the HA-coated cellulose sponge in rat subcutaneous tissue (II)
- To study the reasons behind the induced granulation tissue forming ability of HA-coated cellulose sponges, and the possible presence and attachment mechanisms of bone marrow-derived stem cells (III)
- To examine hemoglobin expression in experimental granulation tissue (IV)

4. MATERIALS AND METHODS

4.1 HYDROXYAPATITE COATING OF CELLULOSE

The viscose cellulose sponge was manufactured by Cellomeda Oy, Turku, Finland. A specific bioactive glass, S53P4 (Abmin Technologies Ltd/Vivoxid, Turku, Finland) was used as the source of ions in the hydroxyapatite coating process, which is explained in more detail in study I.

4.2 EXPERIMENTAL ANIMALS

A total of 94 male Sprague-Dawley rats aged 8-13 weeks and weighing 310 $\pm$ 75 grams (SD) at the time of implantation were used in this study. During the experiments the rats were housed individually in cages with free access to food pellets and 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 (Good Laboratory Practice) Compliance. The study design was approved by the Animals Ethical Committee of the State Provincial Office of Southern Finland.

4.3 IMPLANTATION PROCEDURE

The experimental animals were anesthetized with a subcutaneous injection 10 ml/kg of a mixture of midazolam 5 mg/ml (DormicumR, Roche, Switzerland) and fentanyl-fluanisole 0.3-10 mg/ml (HypnormR, Jansen Pharmaceuticals, Belgium) in sterile water 1:1:2. The backs of the animals were shaved, disinfected with a solution of chlorhexidine gluconate 5 mg/ml (KlorhexolR, Leiras, Finland) and draped with sterile towels.

Study I: An incision, 1.5 cm of length, was made on the midline of the antero-lateral aspect of the femur. By dissecting femoral muscles and periosteus, a unicortical defect (diameter 2.4 mm), perpendicular to the longitudinal axis of the diaphysis just below the *trochanter major femoris* was created, using serial drills with increasing diameters. Using an applicator, another unicortical defect was created 8 mm distal to the same direction. The holes were connected by drilling leaving a unicortical defect of ca. 10 x 2.3 mm. Before insertion into defect, each implant was moistened with 0.9% NaCl solution to retard blood clotting at the dry implant surface and to allow easier migration of repair cells into the implants. Moisturized sponges also swell by ca. 10-15% and adhere better than dry sponges into the defect cavity, and do not break during implantation. The edges of fascia and dissected muscles were sutured with an X-type 6-0 stitch (Vicryl, Ethicon).

Studies II, III and IV: Two separate 2 cm long midline incisions, cranial and caudal, were made on the back of the animals. The sterilized sponge implants ca. 10 mm x 5 mm were moistened with 0.9% NaCl solution, after which the implants were inserted bilaterally into pockets of subcutaneous space without fixation. Skin wounds were closed with interrupted 4-0 sutures (Vicryl-rapid, Ethicon).

In each experiment, mineralized and non-mineralized implants were inserted alternatively into the left and right sides to reduce biological variation.

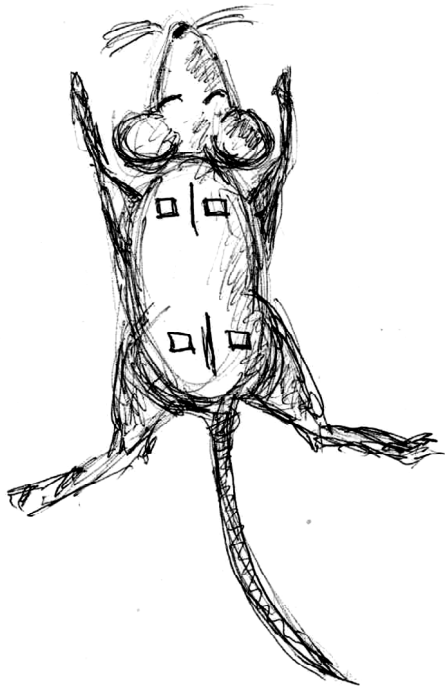

Figure 5. A schematic representation of the subcutaneous implantation model used in studies II-IV. Two midline incisions were made on the back of the rats, and sponge implants were inserted bilaterally into subcutaneous pockets.

4.4 METHODS

The methods and analytical techniques used in this study are listed in Table V and described in detail in the original publications (I-IV) or in the references. The cDNA clones used as probes in the Northern analyses are listed in Table VI and the antibodies used in immunohistological stainings, Western analyses, immunofluoresence stainings and flow cytometry analyses are listed in Table VII.

Table V. Methods used in this study.

Methods	Described in	Used in
Imaging techniques		
Scanning electron microscopy	I	I
Fourier transform infrared spectroscopy	I	I
Histology		
Hematoxylin-eosin	I	I, II
Weigert-van Gieson	I	I, II
Histomorphometry	I	I
Radiographic examination	I	I
DAPI DNA staining	II	II
Giemsa staining	III	III
RNA analyses		
RNA isolation	Chomczynski and Sacchi 1987, IV	I, II, III, IV
Northern blot	I	I, II, IV
RT-PCR	IV	IV
Illumina microarray	IV	III, IV
Protein analyses		
Binding of TGF-β1 to cellulose sponges	I	I
Picrosirius Red staining	II	II
Avidin-biotin immunolabeling	I, II	I, II, III, IV
Immunofluorescence	III	III
Cell sorting		
Flow cytometry	III	III

Table VI. The cDNA clones used as hybridization probes used in this study.

mRNA	cDNA probe	Used in	Reference
mouse proα1(I) collagen	pMCol1α1-1	I, II	Metsäranta *et al*., 1991
mouse proα1(II) collagen	pMCol2α1-1	I	Metsäranta *et al*., 1991
rat proα1(III) collagen	pRGR5	I, II	Glumoff *et al*.,1994
mouse decorin	pMDcn-1	I, II	Säämänen *et al*., 2001
α globin		IV	Satoh *et al*., 1987
β globin		IV	Satoh *et al*., 1987

Table VII. Antibodies used in different protein analyses and cell sorting

Antibody	Host animal	Used in	Supplier/reference
CaR	mouse	III	Santa Cruz, USA
CD31	rabbit	II	Santa Cruz, USA
CD34	rat	III	Santa Cruz, USA
CD117 (c-kit)	rabbit	III	Santa Cruz, USA
Collagen type I	rabbit	I, II	Research Diagnostic, USA
Collagen type III	rabbit	I, II	Research Diagnostic, USA
Decorin (LF-113)	rabbit	I, II	Fisher *et al*.,1989
ED-1	mouse	II	Acris Antibodies GmbH, GER
Glycophorin A	mouse	IV	Santa Cruz, USA
Hemoglobin α	rabbit	IV	Santa Cruz, USA
Hemoglobin β, δ, γ	rabbit	IV	Santa Cruz, USA
PCNA	mouse	II	Zymed Laboratories, USA
PDGF-A	rabbit	II	Research Diagnostic, USA
Stro-1	mouse	III	Zymed Laboratories, USA
TGF-β1	rabbit	I, II	Santa Cruz, USA
TGF-β1	rabbit	I	RD, UK
TNF-α	goat	II	Santa Cruz, USA
VEGF	rabbit	II	Research Diagnostic, USA

4.5 STATISTICAL ANALYSIS

In study I, statistical significance was calculated by the two-way analysis of variance (ANOVA). In study II, all data were expressed as the mean ±SD. The raw data was analyzed with the statistical program SPSS 12.0 (SPSS Inc., USA). Paired *t*-tests were used to test difference between coated and uncoated groups. The differences were considered statistically significant at $p < 0,05$. In study III, the two-tailed *t*-test was used.

5. RESULTS

5.1 CHARACTERIZATION OF THE HYDROXYAPATITE SURFACE

The HA-coated sponges were approximately 30% heavier than uncoated ones of similar size. The SEM images taken from the surfaces of cellulose sponges before and after mineralization revealed successful homogenous coating with small spherical HA precipitates. Despite the coating, the interconnecting pores inside the sponges remained open. The quality of coating was identified as carbonated HA by Fourier transform infrared spectroscopy (FTIR) spectroscopy (I; Fig 2).

5.2 EXPERIMENTAL ANIMALS AND IMPLANTS

The rats recovered quickly from the operation, walked freely, and continued to gain weight throughout the observation period. No wound or implant infections were detected, and all animals were alive and healthy at the end of the experiment.

5.3 BIOCOMPATIBILITY OF HYDROXYAPATITE COATED CELLULOSE SPONGES WITH BONE (I)

5.3.1 General histological findings, histomorphometric results and radiographic examination

The aim of the study was to test the compatibility of HA-coated cellulose sponges in an experimental rat femoral cortical defect model up to one year.

Light microscopy examination of hematoxylin-eosin (not shown) and van Gieson stained sections revealed that the HA-coated sponges induced a much stronger inflammatory reaction with rapid connective tissue proliferation than uncoated sponges. This inflammatory reaction persisted throughout the observation period, and even after 52 weeks HA-coated sponges were richer in giant cells and macrophages than the controls (I; Fig 3).

Bone formation was clearly delayed in the HA-coated sponges compared to the uncoated sponges. At four and six weeks, new bone was formed all over the marrow side of the uncoated sponges, but the HA-coated sponges were mostly filled with a fibrous granulation tissue rich in foreign body giant cells. Hardly any progress of bone formation was seen at twelve weeks after the operation in the HA-coated sponges. At the end of the experiment, at 52 weeks, most parts of the implants were still filled with connective

tissue and giant cells, only the sponge edges being ossified. New bone growing beneath the HA-coated implants had, however, pushed the implants upwards and started to fill the bottom of the defects. This phenomenon was not seen in the uncoated sponges, in which the new bone grew straight into the sponge structure. (I; Fig 4)

Histological findings were supported by morphometric analysis, where less bone tissue was observed in HA-coated sponges at 2-12 weeks compared to the uncoated sponges. Still at 52 weeks, the total amount of new bone in the defect area with both types of sponges was almost similar, because new bone had started to fill the cavity beneath the HA-coated sponges (I; Fig 5). This fact was also seen in radiographs of 52-week-old implants (I; Fig 6).

5.3.2 Expression of connective tissue genes in bone implants

The expression of four skeletal connective tissue specific proteins was investigated by Northern analysis of the implants. In the HA-coated implants, type III collagen (p=0,016) and decorin (p=0,036) expression at 2 and 4 weeks remained lower than in the uncoated implants. At 6 weeks, the last time point in RNA analysis of this study, the differences were non-significant. The differences in type I collagen expression between the HA-coated and uncoated sponges were also non-significant in each time-point (I; Fig 7). No cartilage-specific type II collagen mRNA signals were observed in the implants at any time.

5.3.3 Binding of TGFβ1 to cellulose sponges *in vitro* and *in vivo* and immunodetection of TGFβ1

One reason for the observed *in vivo* fibrous reaction of the HA-coated sponges could be a strong binding of the fibrogenic growth factor, TGF-β1, to the sponges. To test this hypothesis, HA-coated and uncoated cellulose sponges were incubated with TGF-β1 in SBF solution. Indeed, even after washing the sponges incubated withTGF-β1 for 1 h in SBF at 37°C, the HA-coated sponges showed more TGF-β1 immunoreactivity indicating a higher binding affinity of TGF-β1 to the HA-coated sponges compared to the uncoated sponges. A stronger immunoreactivity of TGF-β1 was also seen *in vivo* in the coated samples. (I; Fig 8).

5.4 BIOCOMPATIBILITY OF HYDROXYAPATITE COATED CELLULOSE SPONGES IN SUBCUTANEOUS TISSUE (II)

5.4.1 Visual evaluation and general histological findings of subcutaneous implants

In study I, HA-coated cellulose sponges did not permit osteoid ingrowth, instead of bone tissue the sponges were filled with a fibrous tissue rich in inflammatory cells. This indicated that HA-coated cellulose was not suitable for bone substitute material, but in

other clinical applications a rapid granulation tissue growth might be needed. Thus, in study II we investigated further the granulation tissue forming capacity of the HA-coated cellulose sponges in the rat subcutaneous tissue.

Observations were made at a total of nine time points; 1, 3, 5, 7, 10, 14, 30, 90 and 365 days post-surgery. During the whole experiment, both implant types were easily visualized at the sites of implantation. Especially from postoperative day 3 to day 7, the HA-coated sponges were darker in color as a sign of high cellularity and rich vascularization, whereas the uncoated sponges were pale (II; Fig 1a). Up to 14 days, the HA-coated sponges were also heavier compared to control sponges (a statistically significant weight increase at days 3 ($p=0,018$) and 7 ($p=0,021$; II; Fig 1b)).

In microscopic examination, already at day 1 a vigorous inflammatory response with lymphocytes, granulocytes, monocytes, macrophages and extravasated erythrocytes was seen in the HA-coated sponges. The inflammatory cells were gathered around the mineralized sponge fibers and filled the inner space of the sponge. The growth of granulation tissue started in the area of the capsule which had powerful angiogenesis. The pores of the HA-coated implants were entirely filled with the granulation tissue already at day 14 postoperatively, whereas the pores in the inner space of the controls were sparsely populated. After this time-point, these differences gradually vanished, and at later observations points the structure of the granulation tissue looked similar in both types of sponges. (II; Fig 2) No differences in the number of apoptotic cells were seen between the two sponge types in staining of nuclei with DAPI DNA stain (II; not shown).

5.4.2 Expression of connective tissue proteins in subcutaneous implants

The total amount of RNA was on average twice as high in the coated samples until day 14 postoperatively. The relative expression of collagens I and III and decorin were similar in the coated and control sponges despite the greater amount of total RNA in the coated samples. At later time points the total RNA content was approximately the same in both types of sponges. The expressions of type I and III collagens had greatly diminished by postoperative day 14, whereas decorin expression continued throughout experiment in both implant groups.

On the other hand, the measurement of total collagen from picrosirius red-stained sections revealed statistically significant difference (1 day, $p = 0.028$; 30 days, $p = 0.037$) in the HA -coated sponges compared with the control sponges. This difference lasted until 30 days, after which both types of sponges seemed to be filled with similar amounts of connective tissue and differences in collagen staining were no more observed (II; Fig 3a, b). Immunohistological stainings of collagens type I and III, and decorin, showed also more rapid tissue reaction in the coated samples, but similar to other results, differences remain only during the first two weeks (II; Fig 3c).

5.4.3 Immunohistochemical detection of macrophages, blood vessels, growth factors and PCNA

To investigate the level of inflammation in both types of sponges, the sponge sections were stained with ED1 antibody to reveal macrophages and giant cells. Under light microscopy examination it was obvious that more macrophages had gathered earlier to mineralized sponges compared to controls (II; Fig 4b), but no statistical significance was reached (II; Fig 4a). Sections were also stained with a pro-inflammatory growth factor, namely TNF-α, and in both implants especially the macrophage and giant cell areas were strongly positive (II; Fig 4c).

The amount of blood vessels in the implants was detected immunohistologically with anti-CD31. A forceful neovascularization was noticed particularly in capsule areas of HA-coated sponges (II; Fig 5a). Statistically significant differences in the amounts of CD31-positive blood vessels were observed at post-operative days 5 ($p = 0.007$) and 10 ($p = 0.043$) (II; Fig 5c). Additionally, similar positive areas were obtained from VEGF stainings (II; Fig 5b).

Immunohistological staining with TGF-β1, PDGF-A and the cell proliferation marker PCNA (II; Fig 6), followed a similar pattern; a heavy staining, the most intense in the capsule areas, was observed in the coated sponges during the first two weeks. After several weeks the capsules became inactive, and the maximal staining was observed in the center of the sponges.

5.5 STEM CELL HOMING INTO HYDROXYAPATITE-COATED CELLULOSE SPONGES (III)

5.5.1 Visual inspection of hematoxylin-eosin and Giemsa staining

In study II, enhanced granulation tissue formation and a vigorous neovasculature in subcutaneously implanted HA-coated sponges was noticed. We hypothesized that this reaction might be caused by increased bone marrow-derived stem cells homing to the HA-coated sponges mediated by calcium-sensing G-protein-coupled receptors (CaR). To verify this assumption, we studied more specifically to two time points after subcutaneous implantation, namely 3 and 7 days. These time points were chosen based on the results of study II.

Histological sections of both sponge types were stained with hematoxylin-eosin (HE) and Giemsa dyes (III, Fig. 1). Like in the earlier study, granulation tissue grew notably faster into the HA-coated sponges. The foreign body reaction started from the sponge surfaces from where the cells invaded further towards the sponge centers. Those cells gathered to the sponge centers resemble remarkably histologically stained bone marrow cells.

5.5.2 Flow cytometry and immunohistochemical detection of stem cell markers

The appearance of bone marrow-derived stem cells in the both types of implants was screened with flow cytometric analysis. C-kit, a common premature cell marker, was chosen as a stem cell marker. Flow cytometry revealed a significantly higher ($p < 0.05$) c-kit expression in the HA-coated sponges than in the uncoated ones (III; Fig 2).

After this finding, sponge sections were examined more closely with immunohistological stainings of c-kit and two other common stem cells markers, namely CD34 for hematopoietic stem cells, and stro-1 for mesenchymal stem cells. All markers stained numerous cells in the HA-coated sponges, whereas in the uncoated sponges only a few positive cells were observed. C-kit and CD34 positive cells were accumulated to the sponge center, whereas stro-1 positive cells were, on the other hand, found closer to the sponge surface areas, which were rich in new granulation tissue. All studied stem cells seemed to seek to close contact with cellulose fragments, especially in HA-coated sponges (III; Fig 3).

5.5.3 Immunofluorescence detection of CaR-positive cells

To investigate the possible mechanism of bone marrow-derived stem cell homing into the HA- coated implants, the sponge sections were stained with CaR-antibody and visualized by immunofluorescence. Although CaR is expressed in various cell types, including macrophages and monocytes, these receptors are especially important in retaining HSCs in close physical nearness of the endosteal surface in bone marrow. Indeed, a greater amount of CaR-positive cells was observed in the HA-coated implants in the new granulation tissue, but also in the central area of the sponges, where most of them were gathered near the HA-coated cellulose fragments. In the uncoated implants, only few CaR-positive cells were observed, mainly near the sponge surfaces (III; Fig 4).

5.6 HEMOGLOBIN EXPRESSION IN GRANULATION TISSUE (IV)

In study IV, hemoglobin expression was discovered for the first time in the developing granulation tissue.

5.6.1 Hemoglobin gene expression in experimental granulation tissue

β-globin expression was observed in 10-day old HA coated and uncoated cellulose implants by RT-PCR (IV; Fig. 1a). Sequencing of the β–globin PCR product verified that the product was a specific amplification of the β–globin gene transcript. The hemoglobin gene expression was verified by Northern blot analysis, which revealed a biphasic expression pattern of α- and β-globin mRNAs in both sponge types. The first

peak appeared during the most active phase of the inflammatory stage and the second one when other than inflammatory cells invaded the sponges. Although similar but postponed expression pattern was observed in the uncoated samples, the maximal globin expression levels did not reach those in the coated ones (IV; Fig. 1 b,c).

5.6.2 Expression of inducible nitric oxide synthase

The expression of iNOS was studied with conventional RT-PCR from 3 and 10 day-old samples (IV, Fig. 2). In the HA coated sponges, iNOS expression was intense at day 3, correlating with the first hemoglobin expression peak observed during the most active inflammatory stage. However, there was no sign of iNOS expression at day 10 in the HA coated sponges. In contrast, in the uncoated sponges iNOS expression was detected only in the later time point studied.

5.6.3 Microarray data of globin and hemoglobin-related genes

Microarray analysis was made from 1- and 3-day old sponges to characterize early gene expression in experimental granulation tissue formation. The data obtained supported the earlier findings of globin expression. In addition to α- and β-globin mRNAs, also embryonic and fetal ξ-, ε-, γ- and θ- globins were found. Furthermore, several important hemoglobin-related genes were expressed, such as erythropoietin (Epo), hypoxia-inducible factor 1 alpha (Hif1α), GATA-1 and -2, erythroid-associated factor (Eraf) and ALAS-2. The latter one is an erythroblast specific enzyme, and its expression was confirmed by RT-PCR from 14 days old sponge samples (IV; Fig. 3).

5.6.4 Immunohistological findings of hemoglobin and glycophorin A-positive cells

istological sections taken from HA-coated and control implants were stained with polyclonal antibodies against rat hemoglobin α-chain, rat hemoglobin β-, δ- and γ-chains (IV, Fig. 4), and glycophorin A (IV, Fig. 6). More abundant positive cells were noticed in the HA-coated implants, especially in the sponge center, whereas the uncoated implants contained clearly less positive cells.

The hemoglobin immunoreactive cells in the 7 day-old samples were rounded in shape, and approximately 8-10 μm in size, resembling morphologically primitive erythroid precursor cells. The fewer hemoglobin immunoreactive cells found from the uncoated sponges were larger and more irregularly shaped, befitting for example giant cells. The staining with glycophorin A, which is an erythroid-specific transmembrane protein, showed positive cells throughout the implant area specifically in the HA- sponges, indicating the presence of erythroid lineage cells.

6. DISCUSSION

6.1 EXPERIMENTAL ANIMALS

Animal experiments have been discussed among the public for many years. Due to public pressure the interest to replace animal experiments with alternative methodologies has grown. To date, various optional techniques are available, such as cell and organ cultures, and unliving systems or computer simulations depending on the purpose of the experiments. The "Three Rs" principle has been implemented to biomedical research, meaning replacement, reduction and refinement. This principle is thought to be a key foundation concept in optimizing the welfare of animals used in experiments (Obora and Kurosawa, 2009).

Unfortunately all the developed alternative models are unable to mimic the complex interactions of living tissues (Grossblatt and Clark, 1996) and *in vitro* tests are often incapable of predicting the behavior of a biomaterial *in vivo* (Märtson, 1999). For this reason animal experiments are still very important in biomedical research (Rusche, 2003; Hendriksen, 2006). The "Three Rs" principle has, however, increased critical discussion concerning animal experiments and the total number of animal experiments is slowly decreasing (Rusche, 2003). The role of animal experimentation has shifted into the direction of confirmation of results obtained by animal-free methods.

Apatite coating of a culture plate or other material has been used in numerous cell culture experiments and found to maintain the growth and differentiation of osteoblastic cells. In addition, apatite coatings are useful in the adhesion of metal implants to the host bone. Therefore it was presumed that apatite coating could make the cellulose sponges more suitable for bone tissue engineering, and the fate of the HA-coated cellulose was studied by using an experimental rat bone defect model. Rats were chosen instead of mice because of their bigger size, which facilitates bone implantation, and because of the earlier experience of rats in bone healing studies in our laboratory (Penttinen, 1972; Ekholm *et al*.,1995; Ekholm *et al*., 2000). Rats also have a mobile subcutaneous tissue (Kawamata *et al*., 2003), which makes subcutaneous implantation easier. However, in relation to mice, rats have one severe disadvantage – less commercial antibodies are available for rats.

At the time of implantation the age of the animals varied between 9-13 weeks. For the bone implantation work, rats weighing at least 300 grams were used to facilitate the operation and prevent fractures at the implant sites. For long-term follow-up it was meaningful to use relatively young animals to diminish the risk of natural removal. On the other hand, when young animals are used, there might be a chance for too optimistic

results. Young animals have better healing capabilities, and have more numerous and perhaps more viable stem cell population, compared to older rats. In fact, MSCs of old mice have been shown to inhibit rather than promote wound healing when applied to wounds in diabetic mice (Schatteman and Ma, 2006).

6.2 EXPERIMENTAL MODEL AND METHODOLOGICAL ASPECTS

The implantation of biomaterials to experimental animals is a widely used technique to test the biocompatibility and the behavior of materials in living organisms. The implantation procedure initiates a posttraumatic tissue response, and the recognition of an implant as foreign material causes a foreign body reaction (Morehead and Holt, 1994). The intensity of the reaction varies widely according to the implanted material, from frank toxicity and rejection to biocompatibility and bioactivity. Subcutaneous implantation of porous biocompatible material induces the formation of granulation tissue, which repeats the normal phases wound healing, except the re-epithelialization. If host-specific general responses are studied, samples can be taken from the implantation skin wound, however. The subcutaneous implantation of a biocompatible porous material, such as cellulose, has been accepted as an experimental model for wound healing studies (Edwards *et al*., 1957; Holund *et al*., 1979; Pajulo *et al*., 1996; Inkinen *et al*., 2003; Puolakkainen *et al*., 2005).

Non-healing segmental long bone defect model (Bonnarens and Einhorn, 1984, Einhorn *et al*., 1984) is well established for testing biomaterial destiny in bone. Experimental animal model offers an appropriate microenvironment reflecting the clinical situation. When a porous biomaterial is implanted in bone, the pores are first filled with blood clots, then osteoprogitor cells, and finally, after about 4 weeks, with bony trabeculae (Märtson, 1999). The ingrown bone is then remodeled as a response to mechanical stress. In this model, many factors influence bone formation, such as the animal species, the location and the size of the defect, the method of creation, fixation method, and the duration of the study (Horner *et al*., 2009). For testing the feasibility of scaffold materials to repair bone defects, small animals, like rats, could be more advantageous, because of the bigger number of test samples with similar expenses. Controversially, larger animals may be more appropriate for testing the mechanical stability (Horner *et al*., 2009).

Compared to dense bodies or granules, porous scaffolds allow tissue ingrowth. Porous materials have a high surface area to volume ratio (Lakes, 1995), which allows easier access of migrating cells and offers resources for sufficient blood circulation. Porosity, distribution of pore size and shape, as well as the degree of interconnection between the pores are important for tissue ingrowth into material (Lakes, 1995). The fate of the implanted material depends on its chemical properties. In cases when the degradation is incomplete, the remaining implant is covered with a connective tissue capsule.

In this study, several proteins were analyzed both at the RNA and protein level. Changes in mRNA levels make it possible to gather interesting information about gene expression alterations. The disadvantage of RNA analyses is that they are not always able to predict the amount of gene product that is made. The actual implementation of gene transcription and translation of the transcript and the amount of post-translational modifications of the proteins strongly affects the results at the protein level. Likewise, protein analyses alone are not sufficient methods to investigate incidents in living tissues, mainly due to technical difficulties. For example, maintaining the functionally important secondary and tertiary structures of proteins during analysis, and the lack of amplification possibilities compared to nucleic acids, may cause problems especially in the quantitative protein analysis. Therefore, RNA and protein analysis complement each other, and it is justified to use various methods from both categories (reviewed in Metcalfe and Ferguson, 2007).

Antibody selection for rats was relatively problematic from the beginning. It was especially hard to find suitable commercial markers for stem cell studies. In addition to this, markers between different species vary, and there is no proper data so far on which stem cell markers to use for specific stem cell lines. In general, stem cells are difficult to define, and most of the cell type classifications have been made by exclusion using several positive and negative markers per each cell type. Antibodies in this study were selected on the basis of the most commonly used stem cell markers in the literature.

6.3 STATISTICAL ANALYSIS

The number of animals per analyses (2-5 animals/ time point/ group) was obviously small. Generally, statistical analyses of at least three values in the group are possible. Normal biological variation is, however, more obvious in relatively small animal groups, which make statistically significant results more difficult to achieve. In this work, the emphasis is on observing events caused by the biomaterial in living organisms, and for this reason relatively small animal groups is justified not only for economical but for ethical reasons as well. All the conclusions in this study were based on the consistent and uniform results of several methods. Therefore, experiments in this thesis can be considered as valid description of certain biological events.

6.4 THE EFFECT OF HYDROXYAPATITE COATING OF CELLULOSE TO BONE FORMATION

The HA coating of biomaterials has been used widely to increase the osteoinductive properties of materials (Sartoris *et al*., 1986; Bucholz *et al*., 1989; Soballe *et al*., 1991; Vaughn *et al*., 1991). Based on this, the biomimetic treatment of cellulose implants

with bioactive glass was expected to improve the bone forming capacity of cellulose. Instead of improvements, chronic inflammation and poor ossification was observed in the HA-coated sponges, whereas the untreated cellulose adhered better to the old bone and permitted new bone ingrowth. In the HA-coated sponges, most of the new bone was formed beneath the implants leaving them filled with a fibrous tissue rich in inflammatory cells.

Theoretically, a precipitation of a thick apatite layer that clogged the implant pores could cause reduced ossification, but the SEM examination showed a thin apatite layer and open pores of the HA-coated cellulose. Histological inspection revealed that the femoral HA-coated implants were completely filled with repair cells within two weeks, as they favored rapid granulation tissue ingrowth throughout the whole implant area. Thus, some other explanation than the blocking of porous interconnections is more probable for the poor of bone.

The silica-rich HA coating induced a strong inflammatory reaction with rapid connective tissue proliferation, which might be a better explanation for the weak bone forming outcome. Persistent activation of inflammatory cells causes prolonged secretion of proinflammatory cytokines, like interleukins and TNF-α, and these agents may inhibit differentiation of bone progenitor cells and bone matrix biosynthesis, and stimulate lytic activities as well resulting in altered bone formation and remodeling. Especially the expression of TNF-α downregulates the bone specific transcription factor Cbfa1, which depresses the function of differentiated osteoblasts (Gilbert *et al*., 2002; Nanes, 2003).

Like TNF-α, also TGF-β1 has been proven to repress transcription of Cbfa1, and for this reason to inhibit osteoblast differentiation (Alliston *et al*., 2001). TGF-βs are potent promoters of cellular proliferation and collagen production (Allori *et al*., 2008, Part I). Their effects are mediated via cellular mitogen-activated protein kinase signaling pathways, which crosstalk with Smad signaling (Woodward, 2009). Smad proteins are intracellular molecules responsible of canonical signaling cascade of TGF-β superfamily growth factors (Song *et al*., 2009). TGF-β1 has been shown to be a powerful stimulator of fibroblast activity, and excessive supply of TGF-β1 may lead to fibrosis (Branton and Kopp, 1999).

As shown by *in vitro*–studies, the HA-coated sponges bound TGF-β1 more effectively compared to untreated sponges. This might explain the early fibroblastic proliferation and formation of a fibrous tissue in the bone implants instead of osteoid ingrowth. The tightly attached TGF-β1 is not released from the apatite layer either. Thus, there is less available TGF-β1 in the HA-coated sponges to block the inflammatory cytokine production of macrophages (Xiao *et al*., 2002), which might favor the development of chronic inflammation. Although not beneficial in bone repair, the rapid granulation tissue ingrowth capacity of HA coating might be useful in other applications.

6.5 THE EFFECT OF HYDROXYAPATITE COATING OF CELLULOSE ON THE GRANULATION TISSUE FORMATION

Biocompatibility has often been said to be one of the most important properties of biomaterials. However, in some situations a strong foreign body reaction might be useful. In acute wound healing, the prerequisites for the normal progress of the healing process are adequate inflammatory response, connective tissue formation and neovascularization. In chronic wounds, some key elements of compromised healing are a protracted, non-resolving inflammatory phase, the presence of contaminating bacterial populations and high levels of oxidative stress (Wall *et al*., 2008).

Growth factors, cytokines and chemokines are major regulators during the wound healing process, coordinating the functions of multiple cell types. These agents control the healing process in a complex spatio-temporal order, in connection to wound environment signals modulating their activity. This delicate balance is disturbed in chronic wounds, which are arrested in a state of chronic inflammation. As a consequence, the generation of a proteolytic environment by inflammatory cells infiltrating the wound site as well as a prolonged up-regulation of pro-inflammatory cytokines and chemokines inhibit the normal progression of wound healing (Barrientos *et al*., 2008).

Cellulose sponge has been used for wound healing purposes in experimental and clinical settings for several decades (Viljanto, 1972, Viljanto and Jääskeläinen, 1973). Despite reported good treatment results (Pajarre, unpublished data), the clinical use of cellulose has been modest. As shown in study II, the HA coating of cellulose improved the granulation tissue inducing capacity of the cellulose. The inflammatory response in the HA-coated sponges was faster and more vigorous compared to the uncoated implants. This response seems to enhance the cleaning of bacteria and other harmful material from the wound bed, and resident inflammatory cells act as important activators and mediators of growth factors, cytokines and chemokines.

Hydroxyapatite coating enhanced significantly granulation tissue formation estimated by macroscopic observations and histological criteria. Further analyses revealed more abundant accumulation of inflammatory and stem cells, and production of connective tissue components in the HA-coated implants. Total RNA production was increased in the coated sponges compared to the controls, but the specific expressions of several connective tissue protein mRNAs were at similar levels to those of the uncoated sponges. This finding suggests that even though the growth of granulation tissue was quicker in the HA-coated sponges, the biochemical composition of the ECM produced by the forming granulation tissue was similar in both sponge types. This is important if apatite coated sponges are to be used in clinical wound care. During the long-term follow-up of both implant types, a mild permanent foreign body reaction remained in both sponges.

The HA-coated sponges also activated vigorous neovascularization. Many growth factors essential for wound healing were detected, again in greater amounts in the HA-coated sponges. Due to all these results, the HA layer seems to activate the body's own healing mechanisms. Because of the continuous foreign body reaction and slow ossification seen in the HA implants, these sponges are not suitable in bone tissue engineering. On the other hand, they might be efficient in short-term treatments of poorly healing dermal wounds, e.g. chronic ulcers or serious burn injuries. This material may have a potential to correct the impaired stromal address code common in chronic wound cells (Wall *et al*., 2008), and reactivate the sequences of the healing process by recruiting the inflammatory cells necessary to drive an acute-phase inflammatory response that could possibly overcome the non-resolving inflammation of chronic wounds.

The effect of the silica-rich HA coating in subcutaneous tissue is most likely caused by several factors. Macrophages are functionally responsible for the clearance of foreign particles and when they try to resorb the HA coating, large amounts of calcium, phosphate, and silica are released into the implant area. All of these agents are key players in many biological events. Calcium has an established role in the normal homeostasis of mammalian skin, and it serves as a modulator in keratinocyte proliferation and differentiation (Lansdown, 2002). Experimental models suggest that management of calcium is essential in wound healing, and although the exact roles of calcium remain unclear (Lansdown, 2002), extracellular calcium has been shown to promote stem cell mobilization and homing (Wu *et al*., 2009). Furthermore, local release of phosphate offers more building material for various biochemical syntheses, such as biosynthesis of DNA, RNA, and proteins, activation of amino acids, signaling, and for sugar metabolism.

Silicon (Si) participates in the biosynthesis of collagen, strengthens and makes the walls of blood vessels more flexible, and accelerates wound healing process (Puzanowska-Tarasiewicz *et al*., 2009). Silica (SiO2) has been shown to activate macrophages and to induce pro-inflammatory cytokine release (Liu *et al*., 2007; Hamilton *et al*., 2008). Thus, the irritating effect of silica might be the essential component in the HA coating that greatly enhances granulation tissue formation. Eventually, the forming fibrous capsule around the HA-coated implant seems to isolate the silica-rich HA surface from the direct cell contact of host animal, and the strong tissue response gradually settles down.

6.6 BONE MARROW-DERIVED STEM CELL ACTIVATION CAPACITY OF HYDROXYAPATITE COATED CELLULOSE

The HSC niche is known to be located close to the endosteal surface in the bone marrow, where active bone modeling and remodeling takes place causing an increased extracellular calcium ion concentration. The interaction between the HSCs and

endosteum is mediated via CaR (Adams *et al*., 2006). Tissue injury releases growth factors, cytokines and chemokines, which mobilize HSCs and EPCs from the bone marrow into peripheral blood. These circulating cells home to the sites of nascent neovascularization and differentiate into mature vascular endothelial cells (Kawamoto *et al*., 2003).

Both HSCs and MSCs are needed in wound healing process (Lau *et al*., 2009), but their exact roles in tissue repair are still unknown. As HSC /EPCs are nowadays believed to be in a central role in postnatal blood vessel formation (Velasquez, 2007), MSCs regulate tissue repair via multiple mechanisms including promotion of the survival and proliferation of endogenous cells, and inhibition of inflammatory and immune responses (Phinney and Prockop, 2007). In addition to enhancing the regeneration of non-hematopoietic tissues, MSCs are also essential for of HSCs/EPCs survival (Kinnaird *et al*., 2004), supporting successful angiogenesis and vasculogenesis.

In study II, the HA coating of cellulose was shown to induce a more effective formation of highly vascularized granulation tissue when implanted subcutaneously in rat. The HA coating was formed by treating the cellulose with a bioactive glass in SBF, which creates a silica-rich calcium phosphate layer mimicking the natural composition of bone. In the HA-coated sponges, plentiful giant cells/macrophages were gathered around the mineralized cellulose fragments, and while these cells try to phagocyte or hydrolyze the silica-rich foreign material, $Ca^{2}+$ is released creating an environment that resembles the stem cell niche in bone marrow, attracting hematopoietic progenitors. This theory was supported by the observation of a greater amount of CaR-positive primitive cells in the centers of the HA coated implants.

The increased expression of stromal-derived factor-1 (SDF-1), a vital chemokine in stem and progenitor cell recruitment for tissue repair after injury (Hattori *et al*., 2003; Kollet *et al*., 2003), and its receptor CXCR4 (our unpublished data) most probably facilitate the homing of circulating stem cells to the HA-coated subcutaneous implants.

In some wounds, like chronic ulcers or non-healing burns, it is thought that the mesenchymal cells filling the dermis become phenotypically altered or senescent (Wall *et al*., 2008). The plasticity of bone marrow-derived stem cells means that they have an inherent capacity to produce new granulation tissue if the conditions for growth were correct (reviewed in Metcalfe and Ferguson, 2007). Because of their plasticity, stem cells are widely used in wound healing studies, and they have proved to be powerful enhancers of the healing process (Schatteman *et al*., 2000; Sivan-Loukianova, 2003; McFarlin *et al*., 2006).

6.7 HEMOGLOBIN EXPRESSION AND ITS ROLE IN WOUND HEALING

Local hypoxia may severely jeopardize the survival of the traumatized tissue, and as a main oxygen carrier, hemoglobin is important in wound healing process (Takahashi *et al*., 2009). Although extramedullary hemoglobin expression has been well documented, the local hemoglobin expression during wound granulation tissue formation was demonstrated for the first time in this thesis work. Specifically the finding of β-globin mRNA confirms the presence of active hemoglobin synthesis in the granulation tissue. In another repair situation, Setton-Avruj and co-workers (2007) reported α-globin expression and bone marrow cell migration in injured sciatic nerves.

Wound healing and embryogenesis share many similarities (Martin, 1997). After the fetal period, inflammation is needed to initiate the healing process, but the final tissue repair follows the same principles as during embryonic tissue development. There are a couple of optional explanations for the ability of adult tissue to generate blood under circumstances of abnormal physiology or disease after a period of latency: The reactivation of a gene program in differentiated cells that have retained the capacity to reacquire an embryonic phenotype, or more likely the differentiation of resident precursor cells with hemo-vasculogenic potential when the physiological conditions demand it to increase tissue oxygenation (Sequiera Lopez *et al*., 2003).

In this study, Northern blots showed a biphasic expression pattern of α- and β-globin mRNAs in both HA coated and uncoated implants. This finding suggests that during granulation tissue formation, globin expression is induced in different cell types. The first peak occurred during the most active phase of the inflammatory stage. Activated macrophages have been shown to express globin (Liu *et al*., 1999) most likely in connection to the regulation of the inflammation process (Nishi *et al*., 2009). As demonstrated in study II, macrophages invaded both types of subcutaneously implanted sponges, but more rapidly the HA-coated implants. For this reason it is logical to assume that the first globin expression peak referees to the activated macrophages. There were no statistically significant differences between the relative levels of globin mRNAs in HA-coated and uncoated samples due to the biological variation between the animals and the low animal number. However, when looking at the globin expression levels in each individual animal, there was a clear difference at day 3 with average 3.5 (± 1.9) times higher levels in HA-coated samples correlating with the earlier onset of the inflammatory phase. The observation that globin expression was also linked to the intensy of the inflammatory reaction mirrors the approximately same mRNA levels at day 5, 7, and 10. During this time period, the inflammatory reaction started to decline in the coated samples, whereas it was activated in the uncoated ones.

Oxygen transport is probably the most important task of hemoglobin, but it is obvious that hemoglobin has various other not yet fully understood functions as well. Activated macrophages have been shown to express globin (Liu *et al*., 1999), which has been connected to the regulation of the inflammation process. Hemoglobin has also been shown to serve as a carrier of NO and regulate the activity of NO (Veeramachaneni *et al*., 1999; Gladwin *et al*., 2003). NO is produced by iNOS, which is upregulated during wound healing (reviewed in Schwentker *et al*., 2002). Here, the expression of iNOS, which reflects the production of NO, paralleled the inflammatory situation in both implant types. It is likely that the enhanced hemoglobin production in HA-coated sponges compared to uncoated ones during the early inflammatory phase eliminates the excess NO and prevents its damaging effects on matrix deposition, neovascularization and apoptosis.

After the most vigorous inflammation reaction had started to decline, neovascularization took place. The timing of the second globin expression peak fits the observations of new vessel formation. It is likely that the latter globin expression arises from immature cells of the erythroid series. The detection of positive erythroid-specific markers, such as ALAS2 and glycophorin A, supports this theory. The invasion of cells able to differentiate into immature erythropoietic cells was more rapid in HA-coated implants, which explained the 2 (± 0.2) and 3 (± 1) fold globin expression level in these sponges 2 and 4 weeks after implantation. Again, a slower cell invasion but over a longer time period is obvious in uncoated implants reflected by the similar relative α- and β-globin mRNA levels at 6 weeks.

This study shows a novel finding of local hemoglobin synthesis during the wound healing process. The HA coating of cellulose sponges strengthens hemoglobin production in the forming granulation tissue, which might be one more factor why these sponges could improve impaired wound healing.

6.8 FUTURE PERSPECTIVES

The hypothesis to improve the bone forming capacity of cellulose sponges by HA coating proved to be incorrect. The subcutaneous testing of these same sponges was started before the results of the bone implantation study were obtained. In general, a good biomaterial should be non-toxic, non-antigenic, sterile, and easy to handle, have appropriate physical and mechanical properties, undergo controlled degradation, and also evoke minimal inflammatory reaction (reviewed in Metcalfe and Ferguson, 2007). This is crucial for a bone substituent material, but for other applications various properties e.g. induction of granulation tissue growth might be useful.

In addition to the pilot usage in 1970´s by Viljanto, pure cellulose sponge has also been used in treatment of chronic leg ulcers (Pajarre, unpublished data) and in severe burn injuries (Lagus, unpublished data) in the 1990´s with good results. The cellulose sponge attracts inflammatory cells, and absorbs debris and bacteria from the wound site. In this case, a short-term, powerful inflammatory response is actually necessary. After cleaning the wound bed, the cellulose induces vital granulation tissue formation, and smoothens and prepares the wound bed for successful skin transplantation. Based on the present findings, the silica-rich HA coating of cellulose sponges improves the tissue repair enhancing properties of the cellulose. The granulation tissue growth was considerably accelerated and magnified, but the quality of the induced granulation tissue was equal to that in the untreated cellulose implants. For this reason, it is logical to expect that the HA-coated cellulose sponges are more efficient in the treatment of poorly healing wounds, and should be tested in a proper clinical trial.

Different biomaterials are combined with various growth factors and stem cells intending to improve tissue repair. Another tissue engineering concept is cell guidance aimed at total *in vivo* tissue engineering without the need for cell seeding (Schantz *et al*., 2007). The fascinating property of the HA-coated sponges is its ability to amplify the healing mechanisms of the body. It acts as a cell-guiding material, attracting stem cell reserves. The novel innovations of stem cell therapies have raised great enthusiasm, but also a lot of criticism. The ethics of stem cells therapies has been questioned, but mostly the concern is focused on the use of embryonic stem cells. From the ethical point of view, HA-coated sponges are acceptable as there is no need to harvest and culture autologous cells for implantation.

Both the cellulose and the biomimetic HA coatings have been used in research and clinical applications in experimental animals and in humans. Separately these materials have been proven to be safe for living organisms. On the basis of their chemical constituents, there is no reason to expect any harmful effects when they are used together. The planned treatment model for future clinical experiment includes application of sponges on the wound and their frequent changes at three days intervals. The treatment continues until the wound base is smooth and ready for skin transplantation, and according to expectations the maximum exposure time would be less than two weeks. The contact to the sponge would be only local and temporary, so the risk of possible disadvantages remains very minimal. In addition to safety issues, the HA-coated cellulose sponges are also easy to handle and form, and they can be sterilized in an autoclave. In addition, the manufacturing process of the HA sponges is relatively simple and cheap to do in large quantities.

The interesting finding of hemoglobin expression during wound healing brought into day-light new data concerning blood formation and development of neovascularization

in the wound healing process. Compared to housekeeping gene expression, there were no differences in the relative expression of hemoglobin alpha and beta genes in either sponge type. However, in the HA-coated sponges, the expressions started earlier and, as much more hemoglobin synthesizing cells were present, much more hemoglobin RNA and consecutively hemoglobin protein was produced. The clinical relevance of this individual subject is the development of more, abundant well vascularized granulation tissue in the critical early phases (1-10 days of treatment) of wound healing. If this can be confirmed in clinical trials it will shorten the hospitalization time of chronic wound patients.

7. SUMMARY AND CONCLUSIONS

Current clinical treatment methods of impaired bone and wound healing are insufficient, and novel therapeutical applications are needed. In this study, the biocompatibility of silica-rich HA coating of cellulose sponges was tested both in femoral bone defects and in the subcutaneous space in rats.

Based on the present studies, following conclusions can be made:

1. The HA coating reduced osteoid tissue ingrowth to cellulose sponges. Instead of bone formation, the HA coating favored rapid fibrous tissue proliferation. Also abundant amounts of activated macrophages and giant cells were seen as a sign of chronic inflammation. *In vitro* studies revealed tight binding of TGF-β1 to the apatite layer. Excessive TGF-β1 supply, as well as TNF-α expression, might inhibit osteoblast differentiation by repressing Cbfa1 transcription. According to these results, silica-rich HA coating of cellulose does not improve bone healing, and for this reason HA-coated cellulose was not considered to have any value as a bone replacement material.

2. When cellulose sponges were implanted subcutaneously, the biomimetic HA coating clearly enhanced granulation tissue formation compared to the uncoated cellulose sponges. The HA-coated sponges activated inflammatory response and the secretion of growth factors important to wound healing, such as TGF-β1, TNF-α, VEGF, and PDGF-A. Despite the magnitude of the early events in the HA sponges, the granulation tissue was similar in both implants at the end of the long-term follow-up.

3. The HA-coated sponges attracted more HSCs and MSCs compared to the uncoated sponges. The more abundant homing of HSCs to HA implants is suggested to be caused by the calcium of hydroxyapatite coating. In the bone marrow, HSCs localize to their stem cell niche via calcium sensing receptors. HA-coated sponges contained indeed more CaR-positive cells, which most likely participate to the more abundant vascularization of the granulation tissue.

4. Hemoglobin expression, both at mRNA and protein levels, was detected for the first time during granulation tissue formation. The expression pattern was biphasic, the first peak appeared during the inflammatory response in the initiation of the healing process, and the second peak appeared simultaneously when hematopoietic progenitor cells invaded the implants. iNOS expression was noticed earlier in the HA-coated implants reflecting the progress of the inflammatory process. Furthermore, the expression of erythroid markers was also found indicating that local hematopoiesis occurs in experimental granulation tissue.

HA-coated cellulose is able to facilitate homing of both hematopoietic and mesenchymal progenitors more efficiently than the untreated cellulose. Because of this cell guiding property, in combination with the capacity to promote proliferation of richly vascularized connective tissue, HA-coated cellulose might have potential for clinical application in the treatment of poorly healing wounds.

ACKNOWLEDGEMENTS

This study was carried out at the Department of Medical Biochemistry and Genetics, University of Turku, and in part, at the Department of Anesthesiology, Intensive Care, Emergency Medicine and Pain Medicine, Turku University Hospital. I wish to express my gratitude to both Professors Jukka Finne and Klaus Elenius, the previous and the present head of the Department of Medical Biochemistry, for the possibility to work at the department. I am also grateful to Professor Klaus Olkkola, the head of the Anesthesiology Department, Docent Riku Aantaa, the Administrative Chief of the Anesthesiology Department, and Docent Tuula Manner, for their support especially in the final stages of this study.

I own my warmest thanks to my supervisors, Professor Risto Penttinen and Ph.D. Erika Ekholm. They have taught me the ABC´s of scientific work from step by step, and under their inspiring guidance I have had an excellent possibility to grow from a rookie to a researcher. I greatly appreciate their devotion to my work and help in many ways.

Professors Pauli Puolakkainen and Seppo Vainio are kindly acknowledged for reviewing the thesis and for valuable comments. I thank my supervisory committee Docent Sami Areva, Professor Veli-Matti Kähäri, and Professor Hannu Järveläinen, whose patience and plentifulmultiple advices are especially appreciated. I also wish to thank Docent Anna-Marja Säämänen for her interest in my work.

I wish to express my gratitude to all my co-authors, Professors Jukka Salonen, Antti Yli-Urpo, Pekka Saukko, Drs. Ari-Pekka Forsback, Matis Märtson, Asko Kuusilehto, Johanna Holmbom, Virpi Ääritalo, Christa Finnberg, Jutta Jokinen, Timothy Wilson, Anne Jokilammi, Perttu Terho, Christoffer Stark and Ville Peltonen, for successful collaboration.

I wish to thank Päivi Auho, Marja Uola, and Maria Ström, for the excellent technical assistance, and Rakel Mattsson for secretarial assistance. The rest of the staff at the Department of Medical Biochemistry is also appreciated for their help during these years.

I express my gratitude to my colleagues at the Anesthesiology Department for their encouragement during the writing period of this thesis. Especially the members of the “Nokka-klubi”, Riikka Heikkinen, Petri Aaltonen, and Olli Vänttinen, are acknowledged for keeping up the spirit throughout the residency years. In addition, I wish to encourage my colleague and friend Hanna Illman, whose turn will be sooner than she knows.

I thank my family, in-laws and friends for their support. My deepest gratitude belongs to my dear Timo, who gave me the greatest gift ever, our son Frans. Lastly, even I am aware

that canines are not able to read, I appriciate that our dog Sylvi every day reminds me of the basic facts of life – scientific writing is important, but not as important as eating, resting and playing.

This work was financially supported by the Finnish Medical Foundation, the Orion Pharmos Research Foundation, TEKES (the National Agency of Finland), and the Turku University Foundation.

Turku, June 2010

Miretta Tommila

REFERENCE LIST

Adams GB, Chabner KT, Alley IR, Olson DP, Szczepiorkowski ZM, Poznasnsky MC, Kos CH, Pollak MR, Brown EM, Scadden DT: Stem cell engraftment at the endosteal niche is specified by the calcium-sensing receptor. Nature 2006; 439:599-603.

Agren MS, Mirastschijski U, Karlsmark T, Saarialho-Kere U: Topical synthetic inhibitor of matrix metalloproteinases delays epidermal regeneration of human wounds. Exp Dermatol 2001; 10(5): 337-348.

Aiuti A, Webb IJ, Bleul C, Springer T, Gutierrez-Ramos JC: The chemokine SDF-1 is a chemoattractant for human CD34+ hematopoietic progenitor cells and provides a new mechanism to explain the mobilization of CD34+ progenitors to peripheral blood. J Exp Med 1997; 185:111-120.

Alliston T, Choy L, Ducy P, Karsenty G, Derynck R: TGF-β-induced repression of Cbfa1 by Smad3 decreases Cbfa1 and osteocalcin expression and inhibits osteoblast differentiation. The EMBO Journal 2001; 20:2254-2272.

Allori AC, Sailon AM, Warren SM: Biological basis of bone formation, remodeling, and repair – Part I: Biochemical signaling molecules. Tissue Engineering: Part B 2008; 14:259-273.

Allori AC, Sailon AM, Warren SM: Biological basis of bone formation, remodeling, and repair – Part II: Extracellular matrix. Tissue Engineering: Part B 2008; 14:275-283.

Amara U, Da niel Rittirsch, Flierl M, Bruckner U, Klos A, Gebhard F, Lambris JD, Huber-Lang M: Interaction between the coagulation and complement system. Adv Exp Med Biol. 2008; 632: 71-79.

Aoki T, Ohnishi H, Oda Y, Tadokoro M, Sasao M, Kato H, Hattori K, Ohgushi H: Generation of induced pluripotent stem cells from human adipose-derived stem cells without c-MYC. Tissue Eng Part A 2010; 16(7): 2197-2206.

Arnett TR: Extracellular pH regulates bone cell function. J Nutr 2008; 138:415S-418S.

Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T: Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997; 275:964-967.

Asahara T, Masuda H, Takahashi T, Kalka C, Pastore C, Silver M, Kearne M, Magner M, Isner JM: Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. Circ Res 1999; 85:221-228.

Aukhil I: Biology of wound healing. Periodontology 2000; 22: 44-50.

Bailey AJ, Bazin S, Delaunay A. Changes in the nature of the collagen during development and resorption of granulation tissue. Biochem Biophys Acta 1973; 323:383-390.

Bailey AS and Fleming WH: Converging roads: evidence for an adult hemangioblast. Experimental Haematology 2003; 31:987-993.

Ballen K: Targeting the stem cell niche: squeezing blood from bones. Bone Marrow Transplantantion 2007; 2007:1-6.

Balsam LB, Wagers AJ, Christensen JL, Kofidis T, Weissman IL, Robbins LC: Haematopoietic stem cells adopt mature haematopoietic fates in ischemic myocardium. Nature 2004; 428:668-673.

Barbié C, Chaveaux D, Barthe X, Baquey C, Poustis J: Biological behaviour of cellulosic materials after bone implantation: Preliminary results. Clinical Materials 1990; 5:251-258.

Barnes MJ, Morton LF, Bennet RC, Bailey AJ. Studies in collagen synthesis in the mature dermal scar in the guinea pig. Biochem Soc 1975; 3:917-920.

Baron M: Induction of embryonic hematopoietic and endothelial stem/progenitor cells by hedgehog-mediated signals. Differentiation 2001; 68:175-185.

Barone EJ, Yager DR, Pozez AL, Olutoye OO, Crossland MC, Diegelmann RF, Cohen IK: Interleukin-1α and collagenase activity are elevated in chronic wounds. Plast Reconstr Surg 1998; 102:1023-1029.

Barrientos S, Stojadinovic O, Golinko MS, Brem H, Tomic-Canic M: Growth factors and cytokines in wound healing. Wound Rep Reg 2008; 16:585-601.

Bauer SM, Bauer RJ, Velasquez OC: Angiogenesis, vasculogenesis, and induction of healing in chronic wounds. Vasc Endovasc Surg 2005; 39:293-306.

Bennett NT, Schultz GS. Growth factors and wound healing: part II. Role in normal and chronic wound healing. Am J Surg 1993; 166:74-81.

Bergin SM, Wraight P: Silver based wound dressings and topical agents for treating diabetic foot ulcers. Cochrane Database Syst Rev. 2006 Jan 25; (1): CD005082.

Bianchi L, Ginebri A, Hagman JH, Francesconi F, Carboni I, Chimenti S: Local treatment of chronic cutaneous leg ulcers with recombinant human

granulocyte-macrophage colony-stimulating factor. J Eur Acad Dermatol Venereol 2002; 16:595-598.

Boccaccini AR, Blaker JJ: Bioactive composite materials for tissue engineering scaffolds. Expert Rev Med Devices 2005; 2:303-17.

Bogdan C, Rollinghoff M, Diefenbach A: The role of nitric oxide in innate immunity. Immunol Rev 2000; 173:17-26.

Bonilla S, Silva A, Valdes L, Geijo E, Garcia-Verdugo JM, Martinez S: Functional neural stem cells derived from adult bone marrow. Neuroscience 2005; 133:85-95.

Bornstein P, Sage EH: Matricellular proteins: extracellular modulators of cell function. Curr Opin Cell Biol 2002; 14:608-616.

Borue X, Lee S, Grove J, Herzog EL, Harris R, Diflo T, Glusac E, Hyman K, Theise ND, Krause DS: Bone marrow-derived cells contribute to epithelial engraftment during wound healing. Am J Path 2004; 165:1767-1772.

Boykin JV Jr. Wound nitric oxide bioactivity: a promising diagnostic indicator for diabetic foot ulcer management. J Wound Ostomy Continence Nurs 2010; 37:25-32.

Branton MH, Kopp JB: TGF-beta and fibrosis. Microbes Infect 1999; 1:1349-1365.

Breuls RGM, Klumpers DD, Everts V, Smit TH: Collagen type V modulates fibroblast behavior dependent on substrate stiffness. Biochem Biophys Res Com 2009; 380:425–429.

Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, Weinrauch Y, Zychlinsky A: Neutrophil extracellular traps kill bacteria. Science 2004; 303: 1532-1535.

Brunner S, Theiss HD, Murr A, Negele T, Franz WM: Primary hyperparathyroidism is associated with increased circulating bone marrow-derived progenitor cells. Am J Physiol Endocrinol Metabol. 2007; 293(6): E1670-1675.

Buchanan EP, Longaker MT, Lorenz HP: Fetal skin wound healing. Adv Clin Chem 2009; 48:137-161.

Bucholz RW, Carlton A, Holmes R: Interporous hydroxyapatite as a bone graft substitute in tibial plateau fractures. Clin Orthop Relat Res 1989; 240: 53-62.

Burmester T and Hankeln T: Commentary: What is the function of neuroglobin? J Exp Biol 2009; 212:1423-1428.

Burns JS, Rasmussen PL, Larsen KH, Schroder HD, Kassem M: Parameters in 3D "Osteospheroids" of telomerized human mesenchymal (stromal) stem cells grown on osteoconductive scaffolds that predict in vivo bone forming potential. Tissue Eng Part A, 2010; 16(7): 2331-2342.

Bücheler M, Haisch A: Tissue engineering in otorhinolaryngology. DNA Cell Biol. 2003; 22(9);549-564.

Caplan AI: Embryonic development and the principles of tissue engineering. Novartis Found Symp. 2003; 249:17-25.

Cha J, Falanga V: Stem cells in cutaneous wound healing. Clin. Dermatol 2007; 25:73-78.

Chan JL, Tang KC, Patel AP, Bonilla LM, Pierobon N, Ponzio NM, Rameshwar P: Antigen-presenting property of mesenchymal stem cells occurs during a narrow window at low levels of interferon-γ. Blood 2006; 107:4817-4824.

Chan-Ling T, Baxter L, Afzal A, Sengupta N, Caballero S, Rosinova E, Grant MB: Hematopoietic stem cells provide repair functions after laser-induced Bruch´s membrane rupture model of choroidal neovascularization. Am J Path 2006; 168:1031-1044.

Charbord P, Livne E, Gross G, Häupl T, Neves NM, Marie P, Bianco P, Jorgensen C: Human bone marrow mesenchymal stem cells: A systematic reappraisal via the Genostem Experience. Stem Cell Rev 2010, Mar 3 (Epub ahead of print).

Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987; 162:156-159.

Cianfarani F, Tommasi R, Failla CM, Viviano MT, Annessi G, Papi M, Zambruno G, Odorisio T: Granulocyte/macrophage colony-stimulating factor treatment of human chronic ulcers promotes angiogenesis associated with de novo vascular endothelial growth factor transcription in ulcer bed. Br J Dermatol 2006; 154:34-41.

Cline MJ and Moore MA: Embryonic origin of the mouse macrophage. Blood 1972; 39:842-849.

Cooper DM, Yu EZ, Hennessey P, Ko F, Robson MC. Determination of endogenous cytokines in chronic wounds. Ann Surg 1994; 219:688-692.

Crider S, Kroszer-Hamati A, Krishnan K: Isolated pancreatic extramedullary hematopoiesis. Acta Haematol 1998; 99:38-40.

Crigler L, Kazhanie A, Yoon TJ, Zakhari J, Anders J, Taylor B, Virador VM: Isolation of a mesenchymal cell population from murine dermis that contains progenitors of multiple cell lineages. FASEB J 2007; 21(9): 2050-2063.

Curran JN, Winter DC, Bouchier-Hayes, D. Biological fate and clinical implications of arginine metabolism in tissue healing. Wound Repair Regen. 2006; 14:376-386.

Dainiak MB, Allan IU, Savina IN, Cornelio L, James ES, James SL, Mikhalowsky SV, Jungvid H, Galaev IY: Gelatin-fibrinogen cryogel dermal matrices for wound repair: preparation, optimisation and in vitro study. Biomaterials 2010; 31:67-76.

da Silva Meirelles L, Chagastelles PC, Nardi NB: Mesenchymal stem cells reside in virtually all post-natal organs and tissues. J Cell Sci 2006; 119: 2204-2213.

Dassen H, Kamps R, Punyadeera C, Dijicks F, de Goeij A, Ederveen A, Dunselman G, Groothuis P: Haemoglobin expression in human endometrium. Human Reproduction 2008; 23:635-641.

Davidson JM: Experimental Animal Wound Models. Wounds 2001; 13:9-23.

Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens M, Rubelj I, Pereira-Smith O, Peacocke M, Campisi J: A biomarker that identifies senescent human cells in culture and in aging skin *in vivo*. Proc Natl Acad Sci USA 1995; 92:9363-9367.

Domingo C, Arcis RW, Osorio E, Osorio R, Fanovich MA, Rodriguez-Clemente R, Toledano M: Hydrolytic stability of experimental hydroxyapatite-filled dental composite materials. Dent Mater 2003; 19:478- 486.

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop Dj, Horwitz E: Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006; 8:315-317.

Dorsett-Martin WA: Rat models of skin wound healing: a review. Wound Repair Regen. 2004; 12: 591-599.

Drago H, Marín GH, Sturla F, Roque G, Mártine K, Diaz Aquino V, Lamonega R, Gardiner C, Ichim T, Riordan N, Raimondi JC, Bossi S, Samadikuchaksaraei A, van Leeuwen M, Tau JM, Núñes L, Larsen G, Spretz R, Mansille E: The next generation of burns treatment:intelligent films and matrix, controlled enzymatic debridement, and adult stem cells. Transplant Proc 2010; 42:345-349.

Dumville JC, Worthy G, Soares MO, Bland JM, Cullum N, Dowson C, Iglesias C, McCaughan D, Mitchell JL, Nelson EA, Torgerson DJ; VenUS II team: VenUS II: a randomised controlled trial of larval therapy in the management of leg ulcers. Health Technol Assess. 2009; 13(55): 1-182, iii.-iv.

Edwards LC, Pernokas LN, Dunphy JE: The use of plastic sponge to sample regenerating tissue in healing wounds. Surg Gynecol Obstet 1957; 105:303-309.

Ekholm EC, Hietaniemi K, Määttä A, Vuorio E, Paavolainen P, Penttinen RPK: Extended expression of cartilage components in experimental pseudoarthrosis. Connect Tissue Res 1995; 31: 211-218.

Ekholm EC, Ravanti L, Kähäri VM, Paavolainen P, Penttinen RPK: Expression of extracellular matrix genes, transforming growth factor (TGF)-β1 and *ras* in tibial fracture healing of lathyric rats. Bone 2000; 27: 551-557.

Ekholm E: Bone repair. An experimental study on factors affecting the healing and the callus size. Annales Universitatis Turkuensis 2001; D 450. Doctoral thesis.

Ferguson MWJ, O`Kane S: Scar-free healing: from embryonic mechanisms to adult therapeutic intervention. Phil Trans R Soc B 2004; 359:839-850.

Ferrara N, Davis-Smyth T: The biology of vascular endothelial growth factor. Endocrin Rev 1997; 18:4-25.

Fernández-Montequin JI, Betancourt BY, Leyva-Gonzales G, Mola EL, Galán-Naranjo K, Ramírez-Navas M, Bermúdez-Rojas S, Rosales F, García-Iglesias E, Berlanga-Acosta J, Silva-Rodriguez R, Garcia-Siverio M, Martinez LH: Intralesional administration of epidermal growth factor-based formulation (Heberprot-P) in chronic diabetic foot ulcer: treatment up to complete wound closure. Int Wound J 2009; 6(1): 67-72.

Finkemeier CG. Bone-grafting and bone-graft substitutes. J Bone Joint Surg Am 2002; 84:454-464.

Fisher LW, Termine JD, Young MF: Deduced protein sequence of bone small proteoglycan I (biglycan) shows homology with proteoglycan II (decorin) and several nonconnective tissue proteins in a variety of species. J Biol Chem 1989; 264:4571-4576.

Friedenstein AJ, Petrakova KV, Kurolesova AI, Frolova GP: Heterotopic bone marrow. Analysis of precursor cells for osteogenic and hematopoietic tissues. Transplantation 1968; 6:230-247.

Fucharoen S, Suthipongchai S, Poungvarin N, Ladpli S, Sonakul D, Wasi P: Intracranial extramedullary hematopoiesis inducing epilepsy in a patient with β-thalassemia-hemoglobin E. Arch Intern Med 1985; 145:739-742.

Galkowska H, Wojewodzka U, Olszewski WL: Chemokines, cytokines, and growth factors in keratinosytes and endothelial cells in the margin of chronic diabetic foot ulcers. Wound Repair Regen 2006; 14:558-565.

Garbin S, Pittet B, Montandon D, Gabbiani G, Desmouliere A: Covering by a flap induces

apoptosis of granulation tissue myofibroblasts and vascular cells. Wound Repair Regen 1996; 4:244.

Geesink RGT, Hoefnagels NHM, Bulstra SK: Osteogenic activity of OP-1 bone morphogenetic protein (BMP-7) in a human fibular defect. J Bone Joint Surg (Br) 1999; 81:710-718.

Gehron Robey P: The biochemistry of bone. Endocrinol Metab Clin North Am 1989; 18:859-902.

Gelain F, Bottai D, Vescovi A, Zhang S: Designer Self-Assembling Peptide Nanofiber Scaffolds for Adult Mouse Neural Stem Cell 3-Dimensional Cultures. PLoS ONE 2006; 1:e119. doi:10.1371/journal.pone.0000119.

Gerstenfeld LC, Cullinane DM, Barnes GL, Graves DT, Einhorn TA: Fracture healing as a post-natal developmental process: molecular, spatial, and temporal aspects of its regulation. J Cell Biochem 2003; 88:873-884.

Gherghiceanu M, Popescu LM: Cardiomyocyte precursors and telocytes in epicardial stem cell niche: electron microscope images. J Cell Mol Med 2010; 14(4): 871-877.

Gilbert L, He X, Farmer P, Rubin J, Drissi H, van Wijnen AJ, Lian JB, Stein GS, Nanes MS: Expression of the osteoblast differentiation factor RUNX2 (Cbfa1/AML3/ Pebp2alpha A) is inhibited by tumor necrosis factor-alpha. J Biol Chem 2002; 277:2695-2701.

Gladwin MT, Crawford JH, Patel RP: The biochemistry of nitric oxide, nitrite, and haemoglobin: Role in blood flow regulation. Free Radic Biol Med 2004; 36:707-717.

Glumoff V, Mäkelä JK, Vuorio E: Cloning of cDNA for rat pro alpha 1(III) collagen mRNA. Different expression patterns of type I and type III collagen and fibronectin genes in experimental granulation tissue. Biochim Biophys Acta 1994; 1217:41-48

Godwin JW, Brockes JP: Regeneration, tissue injury and the immune response. J Anat 2006; 209:423-432.

Goldstein S, Singal DP: Scenescence of cultured human fibroblasts: mitotic versus metabolic time. Ex. Cell Res 1974; 88:359-364.

Grossblatt N, Clark D (Eds): Guidance for the care and use of laboratory animals. National Academy of Science, Washington D.C. 1996.

Grunewald M, Avraham I, Dor Y, Bachar-Lustig E, Itin A, Jung S, Chimenti S, Lnadsman L, Abramovitch R, Keshet E: VEGF-induced adult neovascularazation: recruitment, retention, and role of accessory cells. Cell 2006; 124:175-189.

Gurtner GC, Werner S, Barrandon Y, Longaker MT: Wound repair and regeneration. Nature 2008; 15; 453:314-321.

Habobovic P, Barrere F, Blitterswijk CA, de Kroot K, Layrolle P: Biomimetic hydroxyapatite coating on metal implants. J Am Ceram Soc 2002; 85:517-522.

Hamilton RF Jr, Thakur SA, Holian A: Silica binding and toxicity in alveolar macrophages. Free Radic Biol Med 2008; 44:1246-1258.

Han D, Zhang Q: An essential requirement for osteoclasts in refined bone-like tissue reconstruction *in vitro*. Med Hypotheses 2006; 67:75-78.

Hattori K, Heissig B, Rafii S: The regulation of hematopoietic stem cell and progenitor mobilization by chemokine SDF-1. Leuk Lymphoma 2003;44:575-582.

Haussler MR, Haussler CA, Bartik L, Whitfield GK, Hsieh JC, Slater S, Jurutka PW: Vitamin D receptor: molecular signaling and actions of nutritional ligands in disease prevention. Nutr Rev 2008; 66 (Suppl2):S98-112.

Hench L: Bioceramics: from concept to clinic. J Amer Ceram Soc. 1991; 74:1487-1510.

Hench LL: Bioceramics. J Am Ceram Soc 1998; 81:1705-1728.

Hench L. The Story of BioglassR. J Mater Sci: Mater Med 2006; 17:967-978.

Hendriksen CF: English abstract from article ”Developments in the area of animal experiments in biomedical research”. Ned Tijdschr Geneeskd 2006; 150:2857-2862.

Hieta N, Impola U, López-Otin C, Saarialho-Kere U, Kähäri VM: Matrix metalloproteinase-19 expression in dermal wounds and by fibroblasts in culture. J Invest Dermatol 2003; 121(5): 997-1004.

Hildebrand KA, Frank CB: Scar formation and ligament healing. Can J Surg 1998; 41:425-429.

Hill DA and Swanson PE: Myocardial extramedullary hematopoiesis: a clinico-pathologic study. Mod Pathol 2000; 13:779-787.

Hinz B, Mastrangelo D, Iselin CE, Chaponnier C, Gabbiani G: Mechanical tension controls granulation tissue contractile activity and myofibroblast differentiation. Am J Path 2001; 159:1009-1020.

Hinz B: Formation and function of the myofibroblast during tissue repair. J Invest Dermatol 2007; 127:526-537.

Holund B, Junker P, Garbarsch P, Cristoffersen P, Lorenzen I: Formation of granulation tissue in subcutaneously implanted sponges in rats. Acta Pathol Microbiol Scand 1979; 87:3671-3741.

Horner EA, Kirkham J, Wood D, Curran S, Smith M, Thomson B, Yang XB: Long bone defect models

for tissue engineering applications: Criteria for choice. Tissue Engineering Part B: 2009. DOI: 10.1089/ten.TEB.2009.0224.

Hubner G, Branchle M, Smola H, Madlener M, Fassler R, Werner S: Differential regulation of pro-inflammatory cytokines during wound healing in normal and glucocorticoid treated mice. Cytokine 1996; 8: 548-556.

Humphrey PA and Vollmer RT: Extramedullary hematopoiesis in the prostate. Am J Surg Pathol 1991; 15:486-490.

Hunter S, Langemo DK, Andersson J, Hanson D, Thompson P: Hyperbaric oxygen therapy for chronic wounds. Adv Skin Wound Care 2010; 23(3): 116-119. Review.

Hynes RO: Integrins: bidirectional, allosteric signaling machines. Cell 2002; 110:673-687.

Impola U, Jeskanen L, Ravanti L, Syrjänen S, Baldursson B, Kähäri VM, Saarialho-Kere U: Expression of matrix metalloproteinase (MMP)-7 and MMP-13 and loss of MMP-19 and p16 are associated with malignant progression in chronic wounds. Br J Dermatol 2005; 152(4): 720-726.

Inkinen K, Wolff H, Lindroos P, Ahonen J: Connective tissue growth factor and its correlation to other growth factors in experimental granulation tissue. Connect Tissue Res 2003; 44:19-29.

Iso Y, Spees JL, Serrano C, Bakondi B, Pochampally R, Song YH, Sobel BE, Delafontaine P, Prockop DJ: Multipotent human stromal cells improve cardiac function after myocardial infarction in mice without long-term engraftment. Biochem Biophys Res Commun 2007; 354:700-706.

Israel DI, Nove J, Kerns KM, Kaufman RJ, Rosen V, Cox KA, Wozney JM: Heterodimeric bone morphogenetic proteins show enhanced activity in vitro and in vivo. Growth Factors 1996; 13:291-300.

Jaffredo T, Nottingham W, Liddiard K, Bollerot K, Pouget C, de Bruijn M: From hemangioblast to hematopoietic stem cell: An endothelial connection? Experimental Hematology 2005; 33:1029-1040.

Jawad H, Lyon AR, Harding SE, Ali NN. Myocardial tissue engineering. British Medical Bulletin 2008; 87:31-47.

Jell G, Stevens MM: Gene activation by bioactive glasses. J Mater Sci: Mater Med 2006; 17:997-1002.

Jeong JH: Adipose stem cells and skin repair. Curr Stem Cell Res Ther 2010; 5(2): 137-140.

Joseph M, Rajshekhar V, Chandy MJ: Haematopoietic tissue presenting as a sphenoid sinus mass: case report. Neuroradiology 2000; 42:153-154.

Jull AB, Rodgers A, Walker N: Honey as a topical treatment for wounds.Cochrane Database Syst Rev. 2008 Oct 8; (4): CD005083.

Järveläinen H, Puolakkainen P, Pakkanen S, Brown EL, Höök M, Iozzo RV, Sage EH, Wight TN: A role for decorin in cutaneous wound healing and angiogenesis. Wound Repair Regen 2006;14: 443-452.

Järveläinen H, Sainio A, Koulu M, Wight TN, Penttinen R: Extracellular matrix molecules: Potential targets in pharmacotherapy. Pharmacol Rev 2009; 6:198-223.

Kadler KE, Baldock C, Bella J, Boot-Hanford RP: Collagens at a glance. J Cell Sci 2007; 120:1955-1958.

Kajiki A, Higuchi K, Nakamura M, Liu LH, Pula PJ, Dannenberg AM Jr: Sources of extracellular lysosomal enzymes released in organ-culture by developing and healing inflammatory lesions. J Leukoc Biol. 1988; 43(2): 104-116.

Karp NS, McCarthy JG, Schreiber JS, Sisson HA, Thorne CH: Membranous bone lengthening: a serial histological study. Ann Plast Surg 1992; 29:2-7.

Kaushansky K: Lineage-Specific hematopoietic growth factors. New Eng J Med 2006; 354:2034-2045.

Kavitha O and RV Thampan: Factors influencing collagen biosynthesis. J Cell Biochem 2008: 104;1150-1160.

Kawamata S, Ozawa J, Hashimoto M, Kurose T, Shinohara H: Structure of the rat subcutaneous connective tissue in relation to its sliding mechanism. Arch Histol Cytol 2003; 66:273-279.

Kawamoto A, Tkebuchava T, Yamaguchi J, Nishimura H, Yoon YS, Milliken C, Uchida S, Masuo O, Iwaguro H, Ma H, Hanley A, Silver M, Kearney M, Losordo DW, Isner JM, Asahara T: Intramyocardial transplantation of autologous endothelial progenitor cells for therapeutic neovascularization of myocardial ischemia. Circulation 2003; 107:461-468.

Keating JF, McQueen MM. Substitutes for autologous bone graft in orthopaedic trauma. J Bone Joint Surg Br 2001; 82-B:3-8.

Khang D, Carpenter J, Chun YW, Pareta R, Webster TJ: Nanotechnology for regenerative medicine. Biomed Microdevices 2008; DOI 10.1007/s10544-008-9264-6.

Kiel MJ, Morrison SJ: Uncertainty in the niches that maintain haematopoietic stem cells. Nat Rev Immunol 2008; 8:290-301.

Kim H-W, Knowles JC, Kim H-E: Hydroxyapatite porous scaffold engineered with biological

polymer hybrid coating for antibiotic Vancomycin release. J Mat Sci: Mat Med 2005; 16:189-195.

Kim M-H, Curry F-R.E, Simon SI: Dynamics of neutrophil extravasation and vascular permeability are uncoupled during aseptic cutaneous wounding. Am J Physiol Cell Physiol 2009; 296: 848-856.

Kim S, Kim J-H, Jeon O, Kwon IC, Park K. Engineered polymers for advanced drug delivery. Eur J Pharm Biopharm 2009; 71:420-430.

Kimura Y, Madhavan M, Call MK, Santiago W, Tsonis PA, Lambris JD, Del Rio-Tsonis K: Expression of complement 3 and complement 5 in newt limb and lens regeneration. J Immunol 2003; 170: 2331-2339.

Kinnaird T, Stabile E, Burnett MS, Shou M, Lee CW, Barr S, Fuchs S, Epstein SE: Local delivery of marrow-derived stromal cells augments collateral perfusion through paracrine mechanisms. Circulation 2004; 109:1543-1549.

Kino Y, Sawa M, Kasai S, Mito M: Multiporous cellulose microcarrier for the development of hybrid artificial liver using isolated hepatocytes. J Surg Res 1998; 79:71-76.

Kobayashi M, Spector M: In vitro response of the bone marrow-derived mesenchymal stem cells seeded in a type-I-collagen -glycosaminoglycan scaffold for skin wound repair under the mechanical loading condition. Mol Cell Biomech 2009; 6:217-227.

Kolf CM, Cho E, Tuan RS. Mesenchymal stromal cells. Biology of adult mesenchymal stem cells: regulation of niche, self-renewal and differentiation. Arthritis Res Ther. 2007; 9: 204.

Kokubo T, Hata K, Nakamura T, Yamamuro T: Apatite formation on ceramics, metals and polymers induced by CaO SiO2 based glass in simulated body fluid. Bioceramics 1991; 4:113-120.

Kollet O, Shivtiel S, Chen YQ, Suriawinata J, Thung SN, Dabeva MD, Kahn J, Spiegel A, Dar A, Samira S, Goichberg P, Kalinkovich A, Arenzana-Seisdedos F, Nagler A, Hardan I, Revel M, Safritz DA, Lapidot T: HGF, SDF-1 and MMP-9 are involved in stress-induced CD34+ stem cell recruitment to the liver. J Clin Invest 2003; 112:160-169.

Kong D, Melo LG, Gnecchi M, Zhang L, Mostoslavsky G, Liew CC, Pratt RE, Dzau VJ: Cytokine-induced mobilization of circulating endothelial progenitor cells enhances repair of injured arteries. Circulation 2004; 110(14): 2039-2046.

Kwon DS, Gao X, Liu YB, Dulchavsky DS, Danyluk AL, Bansal M, Chopp M, McIntosh K, Arbab AS, Dulchavsky SA, Gautam SC: Treatment with bone marrow-derived stromal cells accelerates wound healing in diabetic rats. Int Wound J 2008; 5:453-463.

Kyriakides TR, Bornstein P: Matricellular proteins as modulators of wound healing and the foreign body response.Thromb Haemost 2003; 90:986-992.

Kössi J: Hexose sugars and sucrose in wound healing. Annales Universitatis Turkuensis 2000; D 409. Doctoral thesis.

Lakes R: Composite biomaterials. In: The Biomedical Engineering. Ed. Bronzino JD, CRC Press and IEEE Press, 1995. pp. 598-610.

Lansdown AB: Calcium: a potential central regulator in wound healing in the skin. Wound Rep Reg 2002; 10:271-285.

Lau K, Paus R, Trede S, Day P, Bayat A: Exploring the role of stem cells in cutaneous wound healing. Exp Dermatol 2009; 18:s921-933.

Le Blanc K: Mesenchymal stromal cells: tissue repair and immune modulation. Cytotherapy 2006; 8:559-561.

Lee JW, Gupta N, Serikov V, Matthay MA: Potential application of mesenchymal stem cells in acute lung injury. Expert Opin. Biol. Ther. 2009; 9(10): 1259-1270.

Lee RH, Seo MJ, Reger RL, Spees JL, Pulin AA, Olson SD, Prockop DJ: Multipotent stromal cells from human marrow home to and promote repair of pancreatic islets and renal glomeruli in diabetic NOD/scid mice. Proc Natl Acad Sci USA 2006; 103:17438-17443.

Leibovich SJ, Ross R: The role of the macrophage in wound repair: A study with hydrocortisone and antimacrophage serum. Am J Pathol 1975; 78: 71-100.

Leibovich SJ, Ross R: A macrophage-dependent factor that stimulates the proliferation of fibroblasts *in vitro*. Am J Pathol 1976; 84: 501-514.

Lesman A, Gepstein L, Levenberg S: Vascularization shaping the heart. Ann NY Acad Sci 2010; 1188:46-51.

Levenson SM, Geever EF, Crowley LV, Oates JF, Bernard CW, Rosen H. The healing of rat skin wounds. Ann Surg 1965; 161:293-308.

Li TS, Hayashi M, Ito H, Furutani A, Murata T, Matsuzaki M, Hamano K: Regeneration of infarcted myocardium by intramyocardial implantation of ex vivo transforming growth factor-β-preprogrammed bone marrow stem cells. Circulation 2005; 111:2438-2445.

Lindblad WJ: Considerations for selecting the correct animal model for dermal wound-healing studies. J Biomater Sci Polym Ed. 2008; 19:1087-1096.

LiuH,ZhangH,FormanHJ:Silicainducesmacrophage cytokines through phospatidylcholine-specific phospholipase C with hydrogen peroxidase. Am J Respir Cell Mol Biol 2007; 36:594-599.

Liu L, Zeng M, Stamler JS: Hemoglobin induction in mouse macrophages. Proc Natl Acad Sci USA 1999; 96(12). 6643-6647.

Liu Y, Dulchavsky DS, Gao X, Kwon D, Chopp M, Dulchavsky S, Gautam SC: Wound repair by bone marrow stromal cells through growth factor production. J. Surg. Res. 2006; 136:336-341.

Liu Z, Martin LJ: The adult neural stem and progenitor cell niche is altered in amyotrophic lateral sclerosis mouse brain. J Comp Neurol 2006; 497(3): 468-488.

Lord BI: The architecture of bone marrow cell populations. Int J Cell Cloning 1990; 8:317-331.

Lorenz E, Uphoff D, Reid TR, Shelton E. Modification of irradiation injury in mice and guinea pigs by bone marrow injections. J Natl Cancer Inst 1951; 12:197-201.

Lu S-J, Feng Q, Park JS, Vida L, Lee B-S, Strausbauch M, Wettstein PJ, Honig GR, Lanza R: Biologic properties and enucleation of red blood cells from human embryonic stem cells. Blood 2008; 112:4475-4484.

Lucas T, Waisman A, Ranjan R, Roes J, Krieg T, Müller W, Roers A, Eming SA: Differential roles of macrophages in diverse phases of skin repair. J Immunol 2010; 184: 3964-3977.

Luo JD, Chen AF. Nitric oxide: a newly discovered function on wound healing. Acta Pharmacol Sin 2005; 26:259-264.

Magnon C, Frenette PS: Hematopoietic stem cell trafficking. StemBook 2008; ed. The Stem Cell Research Community, StemBook, doi/10.3824/stembook.1.8.1

Margolis DJ, Cromblehome T, Herlyn M: Clinical protocol: phase I trial to evaluate the safety of H5.020CMV.PDGF-B for the treatment of a diabetic insensate foot ulcer. Wound Rep Reg 2000; 8:480-493.

Margolis DJ, Crombleholme T, Herlyn M, Cross P, Weinberg L, Filip J, Propert K. Clinical protocol. Phase I trial to evaluate the safety of H5.020CMV. PDGF-b and limb compression bandage for the treatment of venous leg ulcer: trial A. Hum Gene Ther 2004; 15:1003-1019.

Marks SC Jr, Hermey DC (1996): The structure and development of bone. In principles of bone biology. Eds Bilezikion JP, Raisz LG, Rodan GA, Academic Press Inc. pp 3-14.

Martin P: Wound healing – aiming for perfect skin regeneration. Science 1997; 276:75-81.

Martin P, Leibovich SJ: Inflammatory cells during wound repair: the good, the bad and the ugly. Trends in Cell Biology 2005; 15(11): 599-607.

Mast BA, Schultz GS: Interactions of cytokines, growth factors, and proteases in acute and chronic wounds. Wound Rep Reg 1996; 4:411-420.

Mastellos D, Papadimitriou JC, Franchini S, Tsonis PA, Lambris JD: A novel role of complement: mice deficient in the fifth component of complement (C5) exhibit impaired liver regeneration. J Immunol 2001; 166: 2479-2486.

Mastellos D, Lambris JD: Complement: more than a "guard" against invading pathogens? Trends Immunol 2002; 23: 485-491.

McFarlin K, Gao X, Liu YB, Dulchavsky DS, Kwon D, Arbab AS, Bansal M, Li Y, Chopp M, Dulchavsky SA, Gautam SC: Bone marrow-derived mesenchymal stem cells accelerate wound healing in the rat. Wound Repair Regen. 2006; 14:471-478.

Massberg S, Schaerli P, Knezevic-Maramica I, Kollnberger M, Tubo N, Moseman EA, Huff IV, Junt T, Wagers AJ, Mazo IB, von Andrian UH: Immunosurveillance by hematopoietic progenitor cells traffickg through blood, lymph, and peripheral tissues. Cell 2007; 131(5): 994-1008.

Matsumoto T, Kuroda R, Mifune Y, Kawamoto A, Shoji T, Miwa M, Asahara T, Kurosaka M: Circulating endothelial / skeletal progenitor cells for bone regeneration and healing. Bone 2008; 43:434-439.

Mayes PA: Carbohydrates of physiologic significance. In: Harper´s Biochemistry, 21st edition, pp. 125-129 Ed. Murray *et al*., Appelton & Lange, 1988.

Mekkes JR, Loots MAM, Van Der Wal AC, Bos JD: Causes, investigation and treatment of leg ulceration. Brit J Dermatol 2003; 148:388-401.

Mendes SC, Robin C, Dzierzak E. Mesenchymal progenitor cells localize within hematopoietic sites throughout ontogeny. Development 2005;132:1127-1136.

Metcalfe AD, Ferguson MWJ: Tissue engineering of replacement skin: the crossroads of biomaterials, wound healing, embryonic development, stem cells and regeneration. J R Soc.Interface 2007; 4:413-437.

Metsäranta M, Toman D, De Crombrugghe B, Vuorio E: Spesific hybridization probes for mouse type I, II, III and IX collagen mRNAs. Biochim Biophys Acta 1991; 1089:241-243.

Miyahara Y, Nagaya N, Kataoka M, Yanagawa B, Tanaka K, Hao H, Ishino K, Ishida H, Shimizu T, Kangawa K, Sano S, Okano T, Kitamura S, Mori H: Monolayered mesenchymal stem cells repair

scarred myocardium after myocardial infarction. Nat Med 2006; 12:459-465.

Miyamoto T, Takahashi S, Ito H, Inagaki H: Tissue compatibility of cellulose and its derivatives. J Biomed Mat Res 1989; 23:125-133.

Morehead JM, Holt RG: Soft-tissue response to synthetic biomaterials. Otolaryng Clin of NA 1994; 27:195-201.

Murphy WL, Grorud K, Vanderby R Jr: Healing of bone and connective tissue. Encyclopedia of Biomat Biomed Eng 2006. DOI:10.1081/E-EBBE-120041710.

Märtson M, Viljanto J, Laippala P, Saukko P: Bone formation in implant is a milieu dependent phenomenon. An experimental study with cellulose sponge in the rat. Surgery in Childhood International 1997; 5:251-255.

Märtson M, Viljanto J, Hurme T, Saukko P: Biocompatibility of cellulose sponge with bone. Eur Surg Res 1998; 30:426-432.

Märtson M: Cellulose sponge as tissue engineering matrix – The effect of cellulose content, implant size and location on connective tissue and bone formation in the rat. Annales Universitatis Turkuensis 1999; D349. Doctoral thesis.

Märtson M, Viljanto J, Hurme T, Laippala P, Saukko P: Is cellulose sponge degradable or stable as implantation material? An in vivo subcutaneous study in the rat. Biomaterials 1999; 20:1989-1995.

Nakagawa M, Koyanagi M, Tanabe K, Takahashi K, Ichisaka T, Aoi T, Okita K, Mochiduki Y, Takizawa N, Yamanaka S: Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. Nat Biotechnol 2008; 26 101-106.

Naldani A and Carraro F: Role of inflammatory mediators in angiogenesis. Curr Drug Targets Inflamm Allergy 2005; 4:3-8.

Nanes MS: Tumor necrosis factor-α: molecular and cellular mechanisms in skeletal pathology. Genes 2003; 231:1-15.

Navarro M, Michiardi A, Castaño O, Planell AJ: Biomaterials in orthopaedics. J R Soc Interface 2008; 5:1137-1158.

Nelimarkka L, Salminen H, Kuopio T, Nikkari S, Ekfors T, Laine J, Pelliniemi L, Järveläinen H: Decorin is produced by capillary endothelial cells in inflammation-associated angiogenesis. Am J Pathol 2001;158:345-353.

Nemeth K, Leelahavanichkul A, Yuen PS, Mayer B, Parmalee A, Doi K, Robey PG, Leelahavanichkul K, Koller BH, Brown JM, Hu X, Jelinek I, Star RA, Mezey E: Bone marrow stromal cells attenuate sepsis via prostaglandin E2-dependent reprogramming of host macrophages to increase their interleukin-10 production. Nat Med 2009; 15:42-49.

Nilsson SK, Johnston HM, Whitty GA, Williams B, Webb RJ, Denhardt DT *et al*.: Osteopontin, a key component of the hematopoietic stem cell niche and regulator of primitive hematopoietic progenitor cells. Blood 2005; 106:1232-1239.

Nishi H, Inagi R, Kato H, Tanemoto M, Kojima I, Son D, Fujita T, Nangaku M: Hemoglobin is expressed by mesangial cells and reduces oxidant stress. J Am Soc Nephrol 2008; 19:1500-1508.

Nowell PC, Cole LJ, Habermeyer JG, Roan PL: Growth and continued function of rat marrow cells in irradiated mice. Cancer Res 1956; 16:258-261.

Obora S, Kurosawa T: Implementation of the Three Rs in biomedical research – has the turn of the century turned the tide? Altern Lab Anim 2009; 37:197-207.

Ohishi M and Schipani E: Bone marrow mesenchymal stem cells. J Cell Biochem 2010; 109:277-82.

O´Meara S, Cullum NA, Nelson EA: Compression for venous leg ulcers. Cochrane Database Syst Rev 2009 Jan 21; (1): CD000265.

O´Meara S, Al-Kurdi D, Ologun Y, Ovington LG: Antibiotics and antiseptics for venous leg ulcers. Cochrane Database Syst Rev 2010 Jan 20; (1): CD003557.

Opalenik SR, Davidson JM: Fibroblast differentiation of bone marrow-derived cells during wound repair. FASEB 2005; 19: 1561-1563.

Orgel JPRO, Eid A, Antipova O, Bella J, Scott JE: Decorin core protein (decoron) shape complements collagen fibril surface structure and mediates its binding. PLoS One 2009; 4:e7028.

Orlic D: Adult bone marrow stem cells regenerate myocardium in ischemic heart disease. Ann NY Acad Sci 2003; 996:152-157.

Pajulo O, Viljanto J, Lönnberg B, Hurme T, Lönnqvist K, Saukko P: Viscose cellulose sponge as an implantable matrix: changes in the structure increase the production of granulation tissue. J Biomed Mater Res 1996; 32:439-446.

Palfreyman SJ, Nelson EA, Lochiel R, Michaels JA: Dressings for healing venous leg ulcers. Cochrane Database Syst Rev 2006 Jun 19; (3): CD001103.

Palis J and Yoder MC: Yolk-sac hematopoiesis: the first blood cells of mouse and man. Exp Hematol 2001; 29:927-936.

Pallafacchina G, Francois S, Regnault B, Czarny B, Dive V, Cumano A, Montarras D, Buckingham M: An adult tissue-specific stem cell in its niche: a gene profiling analysis of in vivo quiescent and activated muscle satellite cells. Stem Cell Res. 2010; 4(2): 77-91.

Panagiotis M: Classification of non-union. Injury 2005; 36:S30-S37.

Parekkaddan B, van Poll D, Suganuma K, Carter EA, Berthiaume F, Tilles AW, Yarmush ML: Mesenchymal stem cell-derived molecules reverse fulminant hepatic failure. PLoS ONE 2007; 2:e941.

Park C, Ma YD, Choi K: Evidence for the Hemangioblast. Exp Hematol 2005; 33:965-970.

Park HC, Yasuda K, Kuo MC, Ni J, Ratliff BB, Chandler PN, Goligorsky MS: Renal capsule as a stem cell niche. Am J Physiol Renal Physiol 2010 Mar 3 (Epub ahead of print).

Park JB, Lakes RS: Biomaterials: An Introduction. New York: Plenum Press; 1992. p. 185-222.

Parsonage G, Filer AD, Haworth O, Nash GB, Rainger GE, Salmon M, Buckley CD: A stromal address code defined by fibroblasts. Trends Immunol 2005; 26:150-156.

Peschle C, Migliaccio AR, Migliaccio G, Petrini M, Calandrini M, Russo G, Mastroberardino G, Presta M, Gianni AM, Comi P, Giglioni B, Ottolenghi S: Embryonic-Fetal Hb switch in humans: studies on erythroid bursts generated by embryonic progenitors from yolk sac and liver. Proc Natl Acad Sci U S A. 1984;81:2416-2420.

Penttinen R: Biochemical studies on fracture healing in the rat with special reference to the oxygen supply. Acta Chirurgia Scandinavica 1972; 432. Doctoral thesis.

Petersen BE, Bowen WC, Patrene KD, Mars WD, Sullivan AK, Murase N, Boggs SS, Greenberger JS, Goff JP: Bone marrow as a potential source of hepatic oval cells. Science 1999; 284:1168-1170.

Phinney DG, Prockop DJ: Concise review: mesenchymal stem/ multipotent stromal cells: the state of transdifferentiation and modes of tissue repair –current views. Stem Cells 2007; 25:2896-2902.

Piras AM, Dessy A, Chiellini F, Chiellini E, Farina C, Ramelli M, Della Valle E: Polymeric nanoparticles for hemoglobin-based oxygen carriers. Biochim Biophys Acta 2008; 1784:1454-61.

Pirilä E, Korpi JT, Korkiamäki T, Jahkola T, Gutierrez-Fernández A, Lopez-Otin C, Saarialho-Kere U, Salo T, Sorsa T: Collagenase-2 (MMP-8) and matrilysin-2 (MMP-26) expression in human wound of different etiologies. Wound Repair Regen. 2007; 15(1): 47-57.

Pittenger MF, Mackay AM, Beck SG, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR: Multilineage potential of adult human mesenchymal stem cells. Science 1999; 284:143-147.

Priya SG, Jungvid H, Kumar A: Skin tissue engineering for tissue repair and regeneration. Tissue Engineering 2008; Part B 14:105-118.

Prockop DJ: Marrow stromal cells as stem cells for nonhematopoietic tissues**.** Science 1997; 276:71-74.

Proksch E, Brandner JM, Jensen JM: The skin: an indispensable barrier. Exp Dermatol 2008; 17(12): 1063-1072.

Puolakkainen PA, Reed MJ, Gombotz WR, Twardzik DR, Abrass IB, Sage EH: Acceleration of wound healing in aged rats by topical application of transforming growth factor-beta (1). Wound Repair Regen. 1995; 3(3): 330-339.

Puolakkainen PA, Bradshaw AD, Brekken RA, Reed MJ, Kyriakides T, Funk SE, Gooden ME, Vernon RB, Wight TN, Bornstein P, Sage EH: SPARC-thrombospondin-2-double-null mice exhibit enhanced cutaneous wound healing and increased fibrovascular invasion of subcutaneous polyvinyl alcohol sponges. J Histochem Cytochem 2005; 53(5): 571-578.

Puzanowska-Tarasiewicz H, Kuzmicka L, Tarasiewicz M: Biological functions of some elements and their compounds. IV. Silicon, silicon acids, silicones. Pol Mekur Lekarski 2009; 27:423-426.

Qiu C, Hanson E, Olivier E, Inada M, Kaufman DS, Gupta S, Bouhassira E: Differentiation of human embryonic stem cells into hematopoietic cells by coculture with human fetal liver cells recapitulates the globin switch that occurs early in development. Exp Hematol 2005; 33:1450-1458.

Raekallio J, Viljanto J: Regeneration of subcutaneous connective tissue in children. A histological study with application of the CELLSTIC device. J Cutan Pathol 1975; 2:191-197.

Raffaghello L, Bianchi G, Bertoletto M, Montecucco F, Busca A, Dallegri F, Ottonello L, Pistoia V: Human mesenchymal stem cells inhibit neutrophil apoptosis: a model for neutrophil preservation in the bone marrow niche. Stem Cells 2008; 26:51-162.

Raghow R: The role of extracellular matrix in postinflammatory wound healing and fibrosis. FASEB J. 1994; 8:823-831.

Ramundo J, Gray M: Enzymatic wound debridement. J Wound Ostomy Continence Nurs. 2008; 35(3): 273-280.

Rappolee DA, Mark D, Banda MJ, Werb Z: Wound macrophages express TGF-α and other growth factors *in vivo*: Analysis by mRNA phenotyping. Science 1988; 241: 708-712.

Rapraeger AC, Krufka A, Olwin BB: Requirement of heparin sulfate for FGF-mediated fibroblast growth and myoblast differentiation. Science 1991; 252: 1705-1708.

Rasmusson I, Le Blanc K, Sundberg B, Ringden O: Mesenchymal stem cells stimulate antibody secretion in human B cells. Scand. J Immunol 2007; 65:336-343.

Rattner B, Hoffman A, Schoen FJ, Lemons JE: Biomaterials science: A multidisciplinary endeavor. In: Biomaterials science: an introduction to materials in medicine, 2nd ed, pp 1-10. Ed. Ratner B. Elsivier Academic Press, San Diego, London 2004.

Reed MJ, Puolakkainen P, Lane TF, Dickerson D, Bornstein P, Sage EH: Differential expression of SPARC and thrombospondin 1 in wound repair: immunolocalization and in situ hydridization. J Histochem Cytochem 1993; 41(10): 1467-1477.

Ribatti D, Vacca A, Nico B, Roncali L, Dammacco F: Postnatal vasculogenesis. Mech Dev 2001; 100:157-163.

Riedel GE, Valentin-Opran A: Clinical evaluation of rhBMP-2/ACS in orthopaedic trauma: a progress report. Orthopaedics 1999; 22:663-665.

Robson MC, Phillips LG, Lawrence WT, Bishop JB, Youngerman JS, Hayward PG, Broemeling LD, Heggers JP. The safety and effect of topically applied recombinant basic fibroblast growth factor on the healing of chronic pressure sores. Ann Surg 1992; 216:401-406; discussion 406-408.

Robson MC. Wound infection: a failure of wound healing caused by an imbalance of bacteria. Surg Clin North Am 1997; 77:637-650.

Ruberto E, Espinola R, Brusco J, Bacchiocchi M, Zoppi J, Paz R: Idiopathic myelofibrosis with extramedullary hematopoiesis foci in the skin and testicles. Report of a case. Sangre (Barc) 1995; 40:157-160.

Rusche B: The 3Rs and animal welfare –conflict or the way forward? ALTEX 2003; 20 (Suppl 1): 63-67.

Saarialho-Kere UK, Kovacs SO, Pentland AP, Olened JE, Welgus HG, Parks WC: Cell-matrix interactions modulate interstitial collagenase expression by human keratinocytes actively involved in wound healing. J Clin Invest 1993; 92(6): 2858-2866.

Saaristo A, Tammela T, Färkkila A, Kärkkäinen M, Suominen E, Ylä-Herttuala, Alitalo K: Vascular endothelial growth factor-C accelerates diabetic wound healing. Am J Path 2006; 169:1080-1087.

Sadlon TJ, Dell'Oso T, Surinya KH, May BK: Regulation of erythoid 5-aminolevulinate synthase expression during erythropoiesis. Int J Biochem Cell Biol 1999; 31:1153-1167.

Sage EH, Reed M, Funk SE, Truong T, Steadele M, Puolakkainen P, Maurice DH, Bassuk JA: Cleavage of the matricellular protein SPARC by matrix metalloproteinase 3 produces polypeptides that influence angiogenesis. J Biol Chem 2003; 278(39): 37849-37857.

Sarkar PK, Ballantyne S: Management of leg ulcers. Postgrad Med J 2000; 76:674-682.

Sartoris DJ, Holmes RE, Tencer AF, Mooney V, Resnick D: Coralline hydroxyapatite bone graft substitutes in a canine metaphyseal defect model: radiographic-biomechanical correlation. Skeletal Radiol 1986; 15(8): 635-641.

Satoh H, Fujii H, Okazaki T: Molecular cloning and sequence analysis of two rat major globin cDNAs. Biochem Biophys Res Commun 1987; 146:618-624.

Schantz J-T, Chim H, Whiteman M: Cell guidance in tissue engineering: SDF-1 mediates site-directed homing of mesenchymal stem cells within three-dimensional polycaprolactone scaffolds. Tissue Eng 2007; 13:2615-2624.

Schatteman GC, Hanlon HD, Jiao C, Dodds SG, Christy BA: Blood-derived angioblasts accelerate blood-flow restoration in diabetic mice. J Clin Invest 2000; 106:571-578.

Schatteman GC, Ma N: Old bone marrow cells inhibit skin wound vascularisation. Stem Cells 2006; 24:717-721.

Schmitz JP, Schwarz Z, Hollinger JO, Boyan BD: Characterization of rat calvarial non-union defects. Acta Anat Basel 1990; 138:185-192.

Schulz C, von Andrian UH, Massberg S: Hematopoietic stem and progenitor cells: their mobilization and homing to bone marrow and peripheral tissue. Immuno. l Res. 2009; 44:160-168.

Schwentker A, Vodovotz Y, Weller R, Billiar TR: Nitric oxide and wound repair: role of cytokines? Nitric Oxide 2002; 7:1-10.

Schönherr E and Hausser H-J: Extracellular matrix and cytokines: A functional unit. Developmental Immunology 2000; 7:89-101.

Sequiera Lopez MLS, Cherñavvsky DR, Nomasa T, Wall L, Yanagisawa M, Gomez RA: The embryo makes red blood cell progenitors in every tissue simultaneously with blood vessel morphogenesis. Am J Physiol Regul Integr Comp Physiol. 2003; 284:R1126-R1137.

Setton-Avruj CP, Musolino PL, Salis C, Alló M, Bizzozero O, Villar MJ, Soto EF, Pasquini JM: Presence of α-globin mRNA and migration of bone marrow cells after sciatic nerve injury suggests their participation in the degeneration/regeneration process. Exp Neurol 2007; 203:568-578.

Shah A, Brugnano J, Sun S, Vase A, Orwin E: The development of a tissue-engineered cornea:

Biomaterials and culture methods. Ped atr Res 2008; 63:535-544.

Shi C, Zhu Y, Su Y, Cheng T: Stem cells and their applications in skin-cell therapy. Trends Biotechnol 2006; 24:48-52.

Shi HP, Efron DT, Most D, Barbul A: The role of iNOS in wound healing. Surgery 2001; DOI:10.1067/msy.2001.115837.

Simon DA, Dix FP, McCollum CN: Management of venous leg ulcers. BMJ 2004; 328:1358-1362.

Singer AJ, Clark RA: Cutaneous wound healing. N Engl J Med. 1999; 341(10): 738-746. Review.

Sipponen A, Jokinen JJ, Sipponen P, Papp A, Sarna S, Lohi J: Beneficial effect of resin salve in treatment of severe pressure ulcers: a prospective, randomized and controlled multicentre trial. Br J Dermatol. 2008; 158(5): 1055-1062.

Sivan-Loukianova E, Awad OA, Stepanovic V, Bickenbach J, Schatteman GC: CD34+ blood cells accelerate vascularization and healing in diabetic mouse skin wounds. J Vasc Res 2003; 40:368-377.

Smith AJ, Lumley PJ, Tomson PL, Cooper PR: Dental regeneration and materials – a partnership. Clin Oral Invest 2008; 12:103-108.

Soballe K, Hansen ES, Brockstedt-Rasmussen H, Hjortkal VE, Juhl GI, Pedersen CM, Hvid I, Bünger C: Gap healing enhanced by hydroxyapatite coating in dogs. Clin Orthop Relat Res. 1991; 272: 300-307.

Song B, Estrada KD, Lyons KM: Smad signaling in skeletal development and regeneration. Cytokine Growth Factor Rev. 2009; 20:379-388.

Stadelmann WK, Digenis AG, Tobin GR: Physiology and healing dynamics of chronic cutaneous wounds. Am J Surg 1998; 176 (Suppl 2A):26S-38S.

Stagg J, Pommey S, Eliopoulos N, Galipeau J. Interferon-γ-stimulated marrow stromal cells: a new type of nonhematopoietic antigen-presenting cell. Blood 2006; 107:2570-2577.

Stanley A, Osler T: Senescence and the healing rates of venous ulcers. J Vasc Surg 2001; 33:1206-1211.

Steed DL: The role of growth factors in wound healing. Surg Clin North Am. 1997; 77:575-586.

Steele RH, Wilhelm DL: The inflammatory reaction in chemical injury. 3. Leucocytosis and other histological changes induced by superficial injury. Br J Exp Pathol 1970; 51(3): 265-279.

Stoddart MJ, Grad S, Eglin D, Alini M. Cells and biomaterials in cartilage tissue engineering. 2009; 4 :81-98.

Strupe P, Sentuerk U, Riha T, Kasper K, Mueller M, Kasper G, Matziolis G, Duela GN, Perka C: Influence of age and mechanical stability on bone defect healing: Age reverses mechanical effects. Bone 2008; 42:758-764.

Sunderland K, Barratt J, Pidcock M: Extramedullary hematopoiesis arising in the gut mimicking carcinoma of the cecum. Pathology 1994; 26:62-64.

Säämänen AM, Salminen HJ, Rantakokko AJ, Heinegård D, Vuorio EI: Murine fibromodulin: cDNA and genomic structure, and age-related expression and distribution in the knee joint. Biochem J 2001; 355:577-585.

Tachi M, Hirabayashi S, Yonehara Y, Suzuki Y, Bowler P: Development of an experimental model of infected skin ulcer. Int Wound J 2004; 1(1): 49-55.

Tachibana K, Hirota S, Iizasa H, Yoshida H, Kawabata K, Kataoka Y, Kitamura Y, Matsushima K, Yoshida N, Nishikawa S, Kishimoto T, Nagasawa T: The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. Nature 1998; 393:591-594.

Takahashi PY, Kiemele LJ, Chandra A, Chas S, Targonski PV: A retrospective cohort study of factors that affect healing in long-term care residents with chronic wounds. Ostomy Wound Manage 2009; 55:32-37.

Tao H and Ma DDF: Evidence for transdifferentiation of human bone marrow-derived stem cells: recent progress and controversies. Pathology 2003; 35:6-13.

Tárnok A, Ulrich H, Bosci j: Phenotypes of stem cells from diverse origin Cytometry Part A 2010: 77A: 6-10.

Tarnuzzer RW, Schultz GS: Biochemical analysis of acute and chronic wound environments. Wound Rep Reg 1996; 4: 321-325.

Tat PA, Sumer H, Jones KL, Upton K, Verma PJ: The efficient generation of induced pluripotent stem (iPS) cells from adult mouse adipose tissue-derived and neural stem cells. Cell Transplant. 2010, Feb 8 (Epub ahead of print).

Thomas MV, Puleo DA, Al-Sabbagh M. Bioactive glasses three decades on. J Long term Eff Med Implants. 2005; 15:585-97.

Toriseva MJ, Ala-Aho R, Karvinen J, Baker AH, Marjomäki VS, Heino J, Kähäri V-M: Collagenase-3 (MMP-13) enhances remodeling of three-dimensional collagen and promotes survival of human skin fibroblasts. J Invest Dermatol 2007; 127:49-59.

Toriseva M, Kähäri V-M: Proteinases in cutaneous wound healing. Cell Mol Life Sci 2009; 66:203-224.

Trengove NJ, Langton SR, Stacey MC: Biochemical analysis of wound fluid from nonhealing and healing leg ulcers. Wound Rep Reg 1996; 4:234-239.

Tsiftsoglou AS, Vizirianakis IS, Strouboulis J: Erythropoiesis: model systems, molecular regulators, and developmental programs. IUBMB Life 2009; 61:800-830.

Tsiridis E, Upadhyay N, Giannoudis P: Molecular aspects of fracture healing: Which are the important molecules? Injury 2007; 38S1: S11-S25.

Tunaci M, Tunaci A, Engin G, Ozkormaz B, Dincol G, Acunas G, Acunas B: Imaging features of thalassemia. Eur Radiol 1999: 9:1804-1809.

Tögel F, Hu Z, Weiss K, Isaac J, Lange C, Westenfelder C: Administered mesenchymal stem cells protect against ischemic acute renal failure through differentation-independent mechanism. Am J Physiol Renal Physiol 2005; 289:F31-42.

Urist MR: Physiological basis of bone-graft surgery, with special reference to the theory of induction. Clin Orthop 1953; 1:207-216.

Valtieri M, Sorrentino A: The mesenchymal stromal cell contribution to homeostasis. J Cell Physiol 2008; 7:296-300.

van der Rest M and Garrone R: Collagen family of proteins. FASEB J 1991; 5:2814-2823.

Varani J, Schuger L, Dame MK, Leonard C, Fligiel SE, Kang S, Fisher GJ, Voorhees JJ.: Reduced fibroblast interaction with intact collagen as a mechanism for depressed collagen synthesis in photodamaged skin. J Invest Dermatol 2004; 122:1471-1479.

Vaughn BK, Lambardi AV Jr, Mallory TH: Clinical and radiographic experience with a hydroxyapatite-coated titanium plasma-sprayed porous implant. Semin Arthroplasty 1991; 2(4): 309-316.

Veeramachaneni NK, Harken AH, Cairns CB: Clinical implications of hemoglobin as a nitric oxide carrier. Arch Surg 1999; 134:434-437.

Velasquez OC: Angiogenesis and vasculogenesis: inducing the growth of new blood vessels and wound healing by stimulation of bone marrow-derived progenitor cell mobilization and homing. J. Vasc. Surg. 2007; 45(Suppl. A):A39-A47.

Viguet-Carrin S, Garnero P, Delmas PD: The role of collagen in bone strenght. Osteoporos Int. 2006; 17(3):319-336.

Viljanto J, Kulonen E: Correlation of tensile strength and chemical composition in experimental granuloma. Acta Pathol Microbiol Scand 1962; 52:120-126.

Viljanto J: Eine neue Methode zur Behandlung offener Wundflächen. Ann Chir Gynaecol Fenn 1972; 60:94-100.

Viljanto J, Jääskeläinen A: Stimulation of granulation tissue in burns. Ann Chir Gyn Fenniae 1973; 62:18-24.

Viljanto J, Raekallio J: Local hyperalimentation of open wounds. Br J Surg 1976 (A); 63:427-430.

Viljanto J, Raekallio J: Wound healing in children as assessed by the CELLSTIC method. J Ped Surg 1976 (B); 11(1):43-49.

Viljanto J: A Cellstick device for wound healing research. In: Wound Healing and Skin Physiology. pp. 513-522. Ed. Altmeyer *et al*. Springer-Verlag, 1995.

Voigt B, Serikawa T: Pluripotent stem cells and other technologies will eventually open the door for straightforward gene targeting in the rat. Dis Model Mech. 2009; 2:341-343.

Välimäki V-V, Aro HT: Molecular basis for action of bioactive glasses as bone graft substitute. Scand J Surg 2006; 95:95-102.

Wall IB, Moseley R, Baird DM, Kipling D, Giles P, Laffafian I, Price PE, Thomas DW, Stephens P: Fibroblast dysfunction is a key factor in the non-healing of chronic venous leg ulcers. J Invest Dermatol 2008; 128:2526-2540.

Wallace HJ, Stacey MC: Levels of tumor necrosis factor-alpha (TNF-alpha) and the soluble TNF receptors in chronic venous leg ulcers – correlations to healing status. J Invest Dermatol 1998; 110:292-296.

Weller R: Nitric oxide: a key mediator in cutaneous physiology. Experimental dermatology 2003; 28:511-514.

Werner S and Grose R: Regulation of wound healing by growth factors and cytokines. Physiol Rev 2003; 83:835-870.

Whitby DJ, Ferguson MW: Immunohistochemical localization of growth factors in fetal wound healing. Dev. Biol. 1991 A; 147: 207-215.

Whitby DJ, Ferguson MW: The extracellular matrix of lip wounds in fetal, neonatal and adult mice. Development 1991 B; 112: 651-668.

Whitmont K, Reid I, Tritton S, March L, Xue M, Lee M, Fulcher G, Sambrook P, Slobedman E, Cooper A, Jackson C: Treatment of chronic leg ulcers with topical activated protein C. Arch Dermatol 2008; 144(11): 1479-1483.

Wong PM, Chung SW, Chui DH, Eaves CJ: Properties of the earliest clonogenic hemopoietic precursors

to appear in the developing murine yolk sac. Proc Natl Acad Sci USA 1986; 83:3851-3854.

Wood D, Southgate J: Current status of tissue engineering in urology. Curr Opin Urol2008; 18:564-569.

Woodward J: Regulation of haematopoietic progenitor cell proliferation and survival: The involvement of the osteoblast. Cell Adh Migr 2009; 4:

Wu Q, Shao H, Darwin ED, Li J, Li J, Yang B, Webster KA, Yu H. Extracellular calcium increases CXCR4 expression on bone marrow-derived cells and enhances pro-angiogenesis therapy. J Cell Mol Med 2009; 13:3764-3773.

Xiao YQ, Malcom K, Worthen GS, Gardai S, Schiemann WP, Fadok VA, Bratton DL, Henson PM: Cross-talk between ERK and p38 MAPK mediates selective suppression of pro-inflammatory cytokines by transforming growth factor-beta. J Biol Chem 2002; 277:14884-14893.

Yager DR, Chen SM, Ward SI, Olutoye OO, Diegelmann RF, Cohen IK: Ability of chronic wounds to degrade peptide growth factors is associated with increased levels of elastase activity and diminished levels of proteinase inhibitors. Wound Rep Reg 1997; 5:23-32.

Yamaguchi Y, Kubo T, Murakami T, Takahashi M, Hakamata Y, Kobayashi E, Yoshida S, Hosokawa K, Yoshikawa K, Itami S: Bone marrow cells differentiate into wound myofibroblasts and accelerate the healing of wounds with exposed bones when combined with an occlusive dressing. Br J Dermatol. 2005; 152: 616-622.

Yayon A, Klagsbrun M, Esko JD, Leder E, Ornitz DM: Cell surface heparin molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. Cell 1991; 64: 841-848.

Yusen RD and Kollef MH: Acute respiratory failure due to extramedullary hematopoiesis. Chest 1995; 108:1170-1172.

Zhang G, Ezura Y, Chervoneva I, Robinson PS, Beason DP, Carine ET, Susluwsky LJ, Iozzo RV, Birk DE: Decorin regulates assembly of collagen fibrils and acquisition of biomechanical properties during tissue development. J Cell Biochem 2006; 98:1436-1449.

Zhang Y, Zhang G, Wei M: Controlling the biodegradation rate of magnesium using biomimetic apatite coating. J Biomed Mater Res Part B. Appl Biomater 2009; 89B:408-414.

Zhong Y, Bellamkonda RV. Biomaterials for the central nervous system. J R Soc Interface 2008; 5:957-975.