Annina Sipola

EFFECTS OF VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF-A) AND ENDOSTATIN ON BONE
ANALYSIS OF BONE MECHANICAL PROPERTIES USING COMPLEX MICROCT IMAGING TECHNIQUES

Academic dissertation to be presented with the assent of the Faculty of Medicine of the University of Oulu for public defence in Auditorium A101 of the Department of Anatomy and Cell Biology (Aapistie 7 A), on 4 December 2009, at 2 p.m.

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Oulu, Finland

**Abstract**

Angiogenesis is essential for the replacement of cartilage by bone during skeletal growth and regeneration. Vascular endothelial growth factor-A (VEGF-A) is a key regulator of angiogenesis whereas endostatin, a potent inhibitor of endothelial cell proliferation and migration, is a natural antagonist of VEGF-A. The regulatory roles of these peptides in angiogenesis, bone formation and bone cells were investigated in this study.

In the present work we studied the effects of VEGF-A, delivered with an adenoviral vector, on the recovery of bone drilling defects in rat femur. Our data confirm the important role of VEGF in bone healing and that adenoviral VEGF gene transfer may modify bone defect healing in a rodent model.

We studied the effects of VEGF-A and endostatin on bone resorption activity. It was found that VEGF-A is a potent stimulator of bone resorption and osteoclast differentiation *in vitro* and endostatin can antagonize this stimulatory effect when acting directly on bone cells. This suggests that endostatin is indeed a regulator of bone resorption, but not a critical one.

In the present study we induced ectopic bone formation in the hamstring muscles of adult mice. The effects of VEGF-A and endostatin in the ectopic bone formation assay were evaluated by the simultaneous delivery of both peptides with recombinant adenoviral vectors. It was found that endostatin retards the cartilage phase in endochondral ossification that subsequently reduces bone formation. We conclude that bone growth and healing, which share features with ectopic bone formation, may be regulated by endostatin.

To confirm *in vivo* effects on bone formation we further investigated the effects of endostatin and VEGF-A on mouse pre-osteoblastic cells *in vitro*. Finally the effects of endostatin on bone were studied in transgenic mouse lines overexpressing endostatin, and mice lacking collagen XVIII.

**Keywords:** bone regeneration, endostatin, osteogenesis, VEGF-A
To my family
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Oulu, November 2009
**Abbreviations**

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>α-MEM</td>
<td>α-modified minimum essential medium</td>
</tr>
<tr>
<td>α/β3</td>
<td>Vitronectin receptor</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>BMU</td>
<td>Bone multicellular unit</td>
</tr>
<tr>
<td>BRU</td>
<td>Bone remodeling unit</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BSP</td>
<td>Bone sialoprotein</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony-forming unit</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>E</td>
<td>Embryonic day</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cell</td>
</tr>
<tr>
<td>ENDO</td>
<td>Endostatin</td>
</tr>
<tr>
<td>ES</td>
<td>Endostatin</td>
</tr>
<tr>
<td>F-actin</td>
<td>Filamentous actin</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FSD</td>
<td>Functional secretory domain</td>
</tr>
<tr>
<td>HSPG</td>
<td>Heparan sulfate</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>µM</td>
<td>Micromolar, 10–6 g/mol</td>
</tr>
<tr>
<td>NCP</td>
<td>Non-collagen protein</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cell</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>OCN</td>
<td>Osteocalcin</td>
</tr>
<tr>
<td>OPG</td>
<td>Osteoprotegerin</td>
</tr>
<tr>
<td>OPN</td>
<td>Osteopontin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Osx</td>
<td>Osterix</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>pQCT</td>
<td>Peripheral quantitative computed tomography</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>RANK</td>
<td>Receptor activator of NF-κB</td>
</tr>
<tr>
<td>RANKL</td>
<td>Receptor activator of NF-κB ligand</td>
</tr>
<tr>
<td>RGD</td>
<td>Arginine-glycine-aspartic acid</td>
</tr>
<tr>
<td>Runx2</td>
<td>Runt-related transcription factor 2</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TRACP</td>
<td>Tartrate-resistant acid phosphatase</td>
</tr>
<tr>
<td>SZ</td>
<td>Sealing zone</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WGA</td>
<td>Wheat germ agglutinin</td>
</tr>
<tr>
<td>1,25(OH)₂D₃</td>
<td>1,25-dihydroxyvitamin D₃</td>
</tr>
</tbody>
</table>
List of original publications

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1 Introduction

Throughout life, bones continuously alter their internal structure by remodeling unlike other durable structures, such as teeth, tendons, and cartilage. Bone uses this process to adapt to mechanical stress, repair damaged bone and maintain ion homeostasis.

During bone growth, development, and remodeling, angiogenesis as well as osteogenesis are closely associated processes. Physiological bone angiogenesis occurs primarily during development and fracture healing. The majority of bones in the vertebrate skeleton are formed by endochondral ossification, and blood vessel invasion into cartilage is a crucial first step in the process whereby cartilage is replaced by bone. This vasculature is also required for the transport of nutrients and precursor cells, such as precursors of chondrocytes and osteoclasts to the renewing bone tissue. Vascular endothelial growth factor, VEGF, is a key mediator of physiological and pathophysiological angiogenesis. Bone remodeling is intimately associated with vascular sinusoids and VEGF has an important role in mediating interactions between the bone and the vasculature. Bone endothelium has been found to signal in unique ways to other bone cells, including bone-resorbing osteoclasts, bone forming osteoblasts, and their progenitors.

Endostatin, a C-terminal fragment of collagen XVIII that is known to antagonize VEGF-A, is found in basal membranes and is expressed by human cartilage and fibrocartilage. Endostatin is capable of neutralizing the endothelial cell migration, neovascularization, and vascular permeability induced by VEGF. Resorbing osteoclasts express $\alpha_v$-integrins, which are receptors for endostatin, thus providing a potential mechanism for endostatin to control of osteoclast function. Angiogenic and antiangiogenic factors play important roles in ossification. It is hypothesized that endostatin expression by mature cartilage may suppress osteoclasts while endostatin-deficient ossifying cartilage allows osteoclastic activity and endochondral ossification.

The aim of this study was to explore the roles of VEGF-A and endostatin in bone and also in processes that are not directly related to angiogenesis. We approached these issues by using in vitro cell cultures and in vivo experiments. We studied the role of VEGF, delivered with a first generation adenoviral vector, in bone healing of drilling defects in rat femur. We also studied the effects of VEGF-A and endostatin on bone resorption activity with a rat pit formation assay and mouse osteoclast differentiation assay. Furthermore, we employed an
endochondral ossification model in the mouse hind muscle pouch stimulated by BMPs, and overexpressed endostatin with or without VEGF-A using recombinant adenoviral vectors. Finally, we investigated the influences of endostatin and VEGF-A on mouse pre-osteoblastic cells (MC3T3-E1). The role of endostatin in bone formation in vivo was also studied on transgenic mouse lines overexpressing endostatin (ES-tg), and mice lacking collagen XVIII (Col18a1−/−).
2 Review of the literature

2.1 Bone

Bone has properties that make it ideal for its functions. It is mechanically strong having very high tensile and compressive strength, but at the same time being relatively light and elastic. Bone tissue has three main functions: First, it has a mechanical function giving shape and rigidity to the body and providing attachment sites for muscles as simple mechanical lever systems to produce body movement. Second, bones provide a rigid framework that supports and protects the soft organs of the body. The skull, for example, protects the brain. Third, it serves as a metabolic tissue maintaining calcium and phosphate homeostasis. Bone also provides an environment for hematopoiesis.

Anatomically, bones in the skeleton can be divided into flat bones, such as skull bones, mandible and scapula and long bones, like humerus, femur and tibia. In long bones, the shaft, composed mainly of compact bone, is called the diaphysis and the region between the growth plate and the expanded end of the bone, the epiphysis. The metaphysis is the junctional region between the growth plate and the diaphysis. There are two morphologically distinct types of bone. Cortical (compact) bone forms the outer thick and protective layer whereas trabecular (cancellous) bone, found inside the bone, has a sponge-like structure that serves a metabolic function due to its large surface area for remodeling. (Marks & Odgren 2002)

Based on bone matrix arrangement, bone tissue can be classified as lamellar or woven. Mature and cortical bone are typically lamellar in that with collagen fibers are arranged in lamellae. In cortical bone, they are concentrically organized around a neurovascular canal, termed a Haversian canal, commonly referred to as osteons. Adjacent osteons are linked together by lateral canals. In trabecular bone lamellae are arranged differently (parallel to each other) compared to those in the denser compact bone. Woven bone, observed in the developing or immature bone, is the type initially laid down during osteogenesis. Bones have an outer fibrous sheath called the periosteum, and an inner surface, referred to as the endosteum, which is in direct contact with the marrow. The periosteum covers all bone surfaces except at the joints and is anchored to the bone by strong, collagenous fibers called Sharpeys’ fibers that penetrate into the bone tissue. The endosteum is a membranous sheath that lines the marrow cavity and also envelopes the surface.
of cancellous bone and lines the blood vessel canals (Volkman’s canals) that run through the bone. They both contain an abundant supply of blood vessels, nerves, nerve endings and osteoprogenitor cells. Bone has a rich vascular supply, receiving 10–20% of the cardiac output. Blood vessels not only supply oxygen and nutrition to forming and growing bones, but they play an active role in bone formation. (Marks & Odgren 2002)

2.1.1 Bone formation

Bone formation can occur through two mechanisms, by intramembranous or endochondral ossification. In either case, bone develops by the replacement of a preexisting connective tissue. The skeleton is formed by mesenchymal cells, derived from cranial neural crest, somites and lateral plate mesoderm. These cells condense and form the future skeletal elements. (Hall & Miyaki 1992, Zelzer & Olsen 2005)

Intramembranous ossification is the process by which a mesenchymal template is replaced by bone. It takes place mainly during the development of some flat bones of the skull and facial bones, when mesenchymal precursor cells differentiate directly into bone-forming osteoblasts and begin to secrete bone matrix. Endochondral ossification is responsible for the formation of bones of the extremities and vertebral column. Bone tissue replaces a preexisting hyaline cartilage, the anlagen of the future bone.

2.1.2 Endochondral bone formation

Endochondral ossification is initiated when mesenchymal progenitor cells condense and differentiate into chondrocytes to form the calcified cartilaginous scaffold for new bone formation. Cells in the connective tissue surrounding the cartilage (perichondrium) differentiate into osteoblasts that deposit a mineralized bone matrix and give rise to the bone collar, the cortical bone. Capillaries invade the perichondrium that surrounds the future diaphysis and transform it into the periosteum.

Hypertrophic chondrocytes in the central region of the cartilage secrete VEGF to induce the invasion of blood vessels. Vascularization of the calcified cartilage brings osteoclasts that resorb the cartilage, osteoblasts that deposit bone matrix and also other mesenchymal cells into the cartilage from the perichondrium (Carlevero et al. 2000, Zelzer et al. 2001, Colnot et al. 2004). This
invasion results in the formation of a primary ossification center in the midshaft, and cartilage is eroded and replaced by bone marrow and trabecular bone in the ossification center. The process of endochondral ossification is seen in figure 1.

Later, secondary ossification centers of ossification appear within the cartilaginous epiphysis by a mechanism that is similar to the formation of the primary center (Karaplis 2002). Discrete layers of residual chondrocytes form growth plates between the epiphyseal and metaphyseal bone centers to support further postnatal longitudinal bone growth (Maes & Carmeliet 2008). Endochondral ossification occurs during development as well as in pathological osteogenesis such as fracture repair and ectopic ossification. The association of angiogenesis, VEGF and endochondral bone formation will be discussed more in chapter 2.5.2.

Fig. 1. A schematic presentation of an endochondral process leading to the formation of the mouse tibia, where the differentiation of chondrocytes from mesenchymal cells results in the formation of an avascular cartilage model of the future bone. ED = embryonic day. Modified from (Zelzer & Olsen 2005).

2.1.3 Bone matrix

Bone tissue consists of mineralized extracellular matrix (ECM) and bone cells, including bone-forming osteoblasts, bone-lining cells, osteocytes and bone-resorbing osteoclasts. The ECM can be divided to organic and inorganic components, which constitutes 35% and inorganic which constitutes 65% of bone tissue, respectively. Inorganic bone matrix consists mainly of hydroxyapatite. The
association of these substances gives bone its hardness and resistance. The organic matrix is composed primarily of the most abundant protein in the human body, type I collagen, and several non-collagenous proteins. Type I collagen is the major protein in bone matrix, representing about 90% of the organic matrix. Type I collagen is a triple-helical structure, which contains two \( \alpha_1 \) chains and one \( \alpha_2 \) chain. These assemble into fibrils, which are then arranged in layers. The network of type I collagen fibers provides the structure on which bone mineral is deposited. The remaining 10–15% of the bone total protein is composed of non-collagenous proteins (NCPs), which can be divided into cell-attachment proteins, proteoglycans and gamma-carboxylated proteins.

The interaction of cells with the extracellular matrix is essential for their anchorage, proliferation, migration and differentiation. Virtually all of the bone matrix proteins are modified post-translationally to contain either N- or O-linked oligosaccharides, being glycoproteins. In bone matrix, there are multiple glycoproteins, including fibronectin, thrombospondin, vitronectin, osteoadherin, osteopontin (OPN) and bone sialoprotein (BSP) that contain the integrin-binding amino acid sequence Arg-Gly-Asp (RGD) and may therefore mediate cell attachment to the bone surface (Robey 2002).

2.2 Bone cells

2.2.1 Osteoblasts

Osteoblasts are bone-forming cells located on the surface of bone or osteoid. Morphologically, osteoblasts are cuboidal and columnar in shape and have an extremely well developed rough endoplasmic reticulum, a large nucleus, and an enlarged Golgi complex due to the high level of protein synthesis (Marks & Ogden 2002). There are four maturational stages of osteoblast development: preosteoblast, osteoblast, osteocyte and bone-lining cell (Aubin & Liu 1996).

Osteoblasts are polarized cells: they have an architecture in which different structures are located at each end, based on the cell’s activity. Osteoblasts are responsible for synthesizing the organic components of bone matrix, type I collagen and non-collagenous proteins and regulate the formation of hydroxyapatite crystals in the newly formed bone. The production of type I collagen by active osteoblasts occurs after osteoblast precursor proliferation, followed by alkaline phosphatase (ALP) expression that is needed for
mineralization of bone matrix. Several non-collagenous proteins are deposited in
the matrix, like bone sialoprotein (Bsp), osteopontin (OPN), osteocalcin (OCN)
and also proteoglycans and glycoproteins.

2.2.2 Differentiation of osteoblasts

Osteoblasts arise from the same mesenchymal stem cells (MSC) as chondroblasts,
adipocytes, myoblasts and fibroblasts (Bennet et al. 1991, Grigoriadis et al. 1988,
Yamaguchi et al. 1991). The proliferation and differentiation of cells of the
osteoblast lineage occur under the influence of a number of transcription
factors, growth factors and hormones (Aubin & Triffit 2002).

The transcription factors, Runx2 (runt related transcription factor, also known
as core binding factor 1, CBFA1) and Osterix (Osx), which functions downstream
from Runx2 regulate osteoblast differentiation (Karsenty & Wagner 2002,
Karsenty 2003, Nakashima et al. 2002). In the absence of either Runx2 or osterix,
no osteoblasts are formed. Runx2 null mice have an almost perfectly patterned
skeleton composed of cartilage, but no bones (Komori et al. 1997, Otto et al.
1997). Also chondrocyte differentiation to hypertrophy is diminished, except in
the distal appendicular skeleton (tibia-fibula, radius-ulna), where chondrocytes do
differentiate to hypertrophy. In addition, there is no blood vessel invasion into
hypertrophic cartilage in Runx2 null mice (Inada et al. 1999, Kim et al. 1999,
Zelzer & Olsen 2005). Runx2 can be phosphorylated and activated by the
mitogen-activated protein kinase (MAPK) pathway. This pathway can be
stimulated by a variety of signals, including bone morphogenic proteins (BMPs).
Bone morphogenetic proteins (BMPs), members of a family of secreted growth
factors, provide important tissue-specific signals to preosteoblasts that are
essential for full osteogenic differentiation (Chen et al. 2004, Mundy 2002). The
MAPK pathway can be also stimulated by signals initiated by the extracellular
matrix (ECM), fibroblast growth factor (FGF-2), mechanical loading and
hormones such as parathyroid hormone (PTH) (Franceschi & Xiao 2003).

Many studies have highlighted the important role of Wnt signaling in bone
formation. The formation and proliferation of preosteoblast cells requires
signaling through the Wnt-frizzled-Lrp5 (low density lipoprotein receptor-related
protein) β-catenin signaling pathway (He et al. 2004).

A number of factors support the growth and differentiation of
osteoprogenitors and the commitment of mesenchymal stem cells to an osteoblast
lineage. This regulation is a complex phenomenon taking place in multiple points
in the maturational sequence. The hormones, growth factors and cytokines participating in this process may have biphasic or opposite activities at different times or on different bone compartments (Aubin et al. 2006). FGFs, TGFβ, and BMPs are potent osteoinductive factors at the early stages of osteoprogenitor differentiation and proliferation. In addition, PTH, PTHrP, IGFs, glucocorticoids, vitamin D, and estrogen are important regulators of osteoblast differentiation and maturation (Aubin & Triffitt 2002, Aubin et al. 2006, Hill 1998).

### 2.2.3 Wnt-signaling and bone

Wnts are a family of 19 secreted proteins that participate in the development and maintenance of many organs and tissues, including bone (Wodarz & Nusse 1998, Logan & Nusse 2004, Moon et al. 2004). Wnt binds to membrane receptor complexes including low-density lipoprotein receptor-related protein (LRP)-5/6 as well as frizzled proteins (Logan & Nusse 2004, Moon et al. 2004). The formation of this ligand-receptor complex initiates a number of signaling cascades that include the canonical/beta-catenin pathway as well as several noncanonical pathways. Canonical Wnt signaling has been reported to play a significant role in the control of bone formation.

Briefly, the canonical pathway is transduced through stabilization of the beta-catenin protein, which is the key mediator of the Wnt pathway. Upon Wnt activation it cannot be phosphorylated by GSK-3 leading to its cytoplasmic accumulation and translocation into the nucleus where it triggers gene expression. The absence of Wnt expression or inhibition of binding to its membrane receptors leads to degradation of beta-catenin and inactivation of the signaling cascade (Liu et al. 2002).

Wnt signaling regulates bone formation through a number of mechanisms and through different mechanisms at different stages of life (Krishnan et al. 2006). It controls mesenchymal stem cells and it regulates most aspects of osteoblast physiology (Reya & Clevers 2005). It dictates osteoblast specification from osteo-/chondroprogenitors and stimulates osteoblast proliferation. In addition it enhances osteoblast and osteocyte survival (Khosla et al. 2008).

beta-catenin is required for the early and terminal differentiation proliferation and function of osteoblasts (Hill et al. 2005, Rodda & McMahon 2006). The Wnt pathway regulates the expression of certain transcription factors, such as Runx2, Osterix and dlx5, and strongly stimulates osteoblastogenesis through the
interaction of various components (Bennett et al. 2005, Yavropoulou & Yovos 2007).

β-catenin has been shown to be essential for determining whether mesenchymal progenitors become chondrocytes or osteoblasts. Precursors lacking β-catenin develop into chondrocytes (Hill et al. 2005, Day et al. 2005) but the maturation of chondrocytes requires the presence of Wnt signaling (Church et al. 2002, Chimal-Monroy et al. 2002). Wnt signaling has a role in both embryonic cartilage development and postnatal endochondral bone formation (Yang 2008). Wnt signaling regulates osteoclast formation and bone resorption. β-catenin regulates osteoclastogenesis through effects on the expression of osteoprotegerin and RANKL (Glass et al. 2005, Holmen et al. 2005).

In clinical cases, mutations have been found in the Wnt genes, receptors and inhibitors of the Wnt signaling pathway that are associated with changes in bone formation and turnover. Wnt signaling also plays a role during bone regeneration following fracture (Yavropoulou & Yovos 2007).

![Fig. 2. A schematic presentation of the role of canonical Wnt signaling in skeletal development. Plus signs indicate positive effects of Wnt; minus signs indicate inhibitory effects of physiological canonical Wnt signaling. Wnt signaling facilitates osteoblast specification from mesenchymal progenitors at the expense of adipogenesis. It cooperates with Runx2 and osterix to maintain and promote osteoblast maturation. Wnt signaling influences osteoclast maturation by regulating RANKL levels in osteoblasts (Khosla et al. 2008). Modified from (Liu et al. 2008).](image-url)
2.2.4 Osteocytes

Osteocytes are terminally differentiated bone-forming cells that comprises the majority, 90–95% of bone cells (Bonewald 2008). When osteoblasts have completed their matrix-forming function, approximately 50–70% die by apoptosis, and the remainder are either incorporated into the newly formed osteoid matrix and become osteocytes or remain on the surface as bone lining cells (Dempster 2006). Bone lining cells can revert back to osteoblasts under certain circumstances, for example after stimulation by PTH or mechanical force (Dobnig & Turner 1995). Osteocytes have a very particular location in bone, they are spaced throughout the mineralized matrix. Dendritic-shaped osteocytes are highly branched cells that occupy small spaces between lamellae, called lacunae. From these cell bodies, long cytoplasmic processes radiate in all directions. Osteocytes form a unique three-dimensional organization, that is a metabolically and electrically active network via cytoplasmic processes that pass through the bone matrix via small channels, the canaliculi, which course through the lamellae and interconnect neighboring lacunae and the lining cells on the bone (Nijweide et al. 2002, Knothe Tate et al. 2004).

Several functions of osteocytes and their possible roles in the process of bone remodeling have been proposed (Noble 2008). Based on the morphological characteristics of osteocytes, the sensation of mechanical stress loaded onto bone is suspected to be one of their functions. The flow of extracellular fluid in response to mechanical forces throughout the canaliculi induces shear stress and deformation of the cell membranes. It has been suggested that primary cilia mediate mechanosensing in bone cells (Malone et al. 2007, Xiao et al. 2006). It has also been hypothesized, that osteocytes have a role in regulating local bone remodeling. A recent finding, that osteocytes are the source of sclerostin, a molecule that regulates osteoblast function, supports this hypothesis (Noble 2008). In addition, osteocytes participate in calcium homeostasis (Nijweide et al. 2002).

The life span of osteocytes is probably largely determined by bone turnover: it is thought that osteocytes are phagocytozed by osteoclasts during bone resorption (Nijweide et al. 2002).
2.2.5 Osteoclasts

Osteoclasts are cells of hematopoietic origin (Suda et al. 1992). They function in bone resorption during bone growth for the resorption of calcified cartilage and modeling of growing bone. Resorption is also needed during fracture healing, as well as tooth eruption.

Osteoclasts are giant (20–100 µm) cells, enabling them to cover a relatively large matrix area and thus operate more efficiently (Bar-Shavit 2007). They are multinuclear cells having an average of 4–20 nuclei, but around 100 nuclei are found in osteoclasts in Paget's disease patients (Roodman & Windle 2005). Osteoclasts are also characterized by numerous mitochondria that are needed to provide the energy required for the resorption process (Mundy 1990). Osteoclasts cycle between resorbing and nonresorbing phases. To resorb bone, they attach to the bone matrix, their cytoskeleton reorganizes and they assume a highly polarized morphology containing several different plasma membrane domains (Baron 1989). The characteristics of osteoclasts also include the expression of calcitonin receptors, vitronectin receptors and tartrate resistant acid phosphatase (TRACP) (Takahashi et al. 2002)

2.2.6 Osteoclast differentiation

Osteoclasts are multinucleated bone resorbing cells derived from hematopoietic cells of the monocyte-macrophage progenitor cell lineage in the bone marrow (Suda et al. 1992, Takahashi et al. 2002). After proliferating in the bone marrow, mononuclear preosteoclasts fuse into multinucleated cells. They reach the bone surface through the blood circulation. Mononuclear precursors of osteoclasts also circulate in the peripheral blood (Väänänen 2005). Under physiological conditions, osteoclast formation is supported by cell-to-cell contact by osteoblast/stromal cells, which express the receptor activator of nuclear factor kappa B (NF-κB) ligand, RANKL, as a membrane bound factor (Suda et al. 1999). The differentiation of osteoclasts is dependent on the presence of RANKL and macrophage colony-stimulating factor (M-CSF) that are expressed by osteoblasts and stromal cells (Yasuda et al. 1998). RANKL together with M-CSF, induces osteoclast differentiation without the need for supportive cells. RANKL also increases the bone-resorbing activity of mature osteoclasts and enhances their survival (Yasuda et al. 1998). M-CSF is crucial for the proliferation and survival of precursor cells of osteoclasts as well as macrophages. Signals are
transmitted through the cFMS, which belongs to the receptor tyrosine kinase superfamily. The op/op mouse lacks functional M-CSF and has osteoclast-deficient osteopetrosis (Yoshida et al. 1990).

RANKL and RANK are members of the tumor necrosis factor (TNF) and TNF receptor superfamilies. RANKL is a type II transmembrane protein found on the surface of expressing cells and as a proteolytically released soluble form (Lacey et al. 1998, Wong et al. 1997, Anderson et al. 1997). RANK is a type I homotrimeric transmembrane protein.

Osteoprotegerin (OPG), also released by osteoblasts, but not exclusively, is a soluble decoy receptor for RANKL. It blocks RANK-RANKL interaction and thus protects the skeleton from excessive bone resorption (Simonet et al. 1997, Lacey et al. 1998). OPG expression is regulated by most of the factors that induce RANKL expression by osteoblasts. In general, upregulation of RANKL is associated with downregulation of OPG. A recent finding suggests, that OPG expression is regulated by Wnt/β-catenin signaling in osteoblasts, the same pathway that regulates osteoblastic bone formation (Boyce et al. 2005, Glass et al. 2005, Holmen et al. 2005).

Systemic and local factors control the recruitment, differentiation and activity of osteoclasts, mainly by affecting the RANK-RANKL-OPG system through stromal cells and osteoblasts. Disorders in this system seem to affect the rate of bone resorption in a several pathological states (Hofbauer & Heufelder 2001). Parathyroid hormone (PTH), 1,25 dihydroxyvitamin D3, the biologically active form of vitamin D3 tumor necrosis factor-α (TNF-α), and interleukins (ILs) have been implicated as factors that enhance osteoclast formation and resorption (Alsina et al. 1996). Estrogen, TGF-β, calcitonin, IGF and ILs have been shown to have inhibitory effects (Roodman 2001). Recently, it was found that estrogen prevents bone loss via the estrogen receptor α and induction of the Fas ligand (Nakamura et al. 2007). Bone resorption is also regulated by the immune system, where T-cell expression of RANKL may contribute to pathological conditions such as periodontitis and autoimmune arthritis (Katagiri & Takahashi 2002, Schett et al. 2005).

### 2.2.7 Osteoclastic bone resorption

After osteoclasts migrate to the resorption site, they initiate a multistep process called the resorption cycle (Väänänen & Zhao 2002). First, mononuclear precursors fuse to form a polykaryon and move to the site of resorption. Then,
Osteoclasts attach tightly to the mineralized bone matrix and generate an isolated resorption site. The part of osteoclast cell membrane, the sealing zone, seals the cell onto the bone matrix (Väänänen & Horton 1995). The primary adhesion mediating structures of osteoclasts are actin-rich structures known as podosomes (Lakkakorpi et al. 1991, Jurdic et al. 2006). αvß3 integrin activation has an important role in the osteoclast attachment leading to podosome formation (Väänänen 2005). The sealing zone surrounds the ruffled border, which is a resorbing organelle. Bone demineralization involves acidification of the isolated extracellular environment. pH ~4.5 is created by fusion of acidic vesicles with the ruffled border and by an electrogenic proton pump (H⁺-ATPase) coupled to a Cl⁻ channel (Blair et al. 1989, Väänänen et al. 1990). In addition to these domains, osteoclasts also have a basolateral membrane facing the bone marrow and a fourth functional secretory domain (FSD) which is located in the upper part of the cell opposite to the ruffled border (Bar-Shavit 2007).

Osteoclasts degrade both the mineral and organic component of bone matrix in resorption lacunae under ruffled border membranes. The acidic milieu first dissolve bone mineral (hydroxyapatite), exposing the demineralized organic component of bone for lysosomal proteases. It seems, that cathepsin K is the main bone matrix-degrading enzyme (Gowen et al. 1999). Metalloproteinases, especially MMP9s, are also released into resorption lacuna to degrade collagen and noncollagen proteins (Everts et al. 1998, Xia et al. 1999).

Bone degradation products are endocytosed, endocytic vesicles are transported to the functional secretory domain and degradation products are released at the cell’s antiresorptive surface (Nesbitt & Horton 1997, Salo et al. 1997). TRACP has been shown to be localized in transcytotic vesicles and able to destroy collagen and other proteins (Halleen et al. 1999). Finally, the osteoclast detaches from the bone and loses its polarized structure. Then, the osteoclast either relocates to a new resorption site or undergoes apoptosis (Bar-Shavit 2007).

2.3 Bone remodeling

Bones continually alter their internal structure by remodeling. The remodeling rate varies in different types of bones: trabecular bone is remodeled at a higher rate than cortical bone in a healthy adult (Parfitt 2002). At any one time, approximately 20% of the cancellous bone surface is undergoing remodeling, and at any one surface location, remodeling will occur on average every 2-years (Parfitt et al. 1997). The bone remodeling processes involve the resorption of the
old bone and the formation of new bone, which are coupled to one another and are strictly controlled. Most adult skeletal diseases are due to an excess of osteoclastic activity, leading to an imbalance in bone remodeling which favors resorption. Such diseases include osteoporosis, periodontal diseases, rheumatoid arthritis, multiple myeloma, and metastatic cancers (Boyle et al. 2003). Osteoporotic bone loss is the result of high bone turnover in which bone resorption outpaces bone deposition (Rodan & Martin 2000, Teitelbaum 2007) while osteopetrosis, which is a metabolic bone disease characterized by decreased bone resorption, leads to the accumulation of excessive amounts of bone. Although having a higher bone mass, osteoprotic bones are easier to fracture (Tuukkanen et al. 2000).

Bone remodeling occurs in discrete locations and involves a group of different kinds of cells. The basic multicellular unit (BMU), which comprises osteoblasts and osteoclasts within the bone-remodeling cavity, is located in close proximity to a blood vessel, as seen in figure 3. In cortical bone, the capillary grows along the excavated tunnel, and on trabecular surfaces, small capillaries are frequently seen adjacent to osteoblasts (Ott 2000). Another model proposed for the relationship of osteoblasts, osteoclasts, and capillaries, involves bone remodeling compartments (BRCs). A BRC comprises the BMU, as well as the layer of flat lining or stromal cells positive for osteoblast markers associated with the capillary (Hauge et al. 2001, Parfitt 2000), as seen in figure 4. They are connected to the osteocyte network through gap junctions. It has been thought that BRCs may be structures through which mechanosensory signals from the osteocyte network are transmitted to osteoclasts and osteoblasts so that they can change their activity on trabecular surfaces (Eriksen et al. 2007).

A BMU is not a permanent structure. It forms in response to a signal or stimulus, performs its function and disbands. The skeleton contains millions of BMUs, all at different stages and they all are geographically and chronologically separated from other BMUs (Hill 1998). The bone remodeling cycle involves a complex series of steps that are highly regulated and always follows certain phases. It starts with activation of osteoclast precursors, and osteoclastic resorption. Then on the reversal phase it continues with apoptosis of osteoclasts and smoothing of the erosion cavities by mononuclear cells. Finally, mesenchymal cells differentiate into functional osteoblasts that lay down matrix mineralization (Frost 1973, Frost 1986).
2.3.1 Bone remodeling cycle

Activation is a continuous process in BMUs. When a BMU spreads, new surfaces undergo activation. Activation involves the recruitment of osteoclast precursors to the bone and their differentiation and fusion into functional osteoclasts. It is not clear what attracts the resorptive cells to the remodeling site. It has been suggested that osteoclast precursors (monocytes) are mobilized by chemotaxis, and the chemoattractants responsible for this activity are derived from the bone matrix, or in the case of collagen and osteocalcin, directly from the osteoblasts that produce them (Malone et al. 1982, Bar-Shavit 2007). MMPs produced by osteoclast lineages have been found to be important for the migration of precursor cells (Delaisse et al. 2003).

Heino and colleagues have suggested that healthy osteocytes inhibit resorption, and when these signals cease, the osteoclast precursors migrate toward the site (Heino et al. 2002). Alternatively, it has been proposed that apoptotic osteocytes may secrete regulatory factors that induce osteoclast differentiation and initiate bone repair (Hedgecock et al. 2007).

During the resorption phase, osteoclasts remove both mineral and organic components of the bone matrix (Blair et al. 1986). Multinucleated osteoclasts are active for approximately 12 days (Parfitt et al. 1996), and after the osteoclasts...
have finished resorption, the phase ends with osteoclast apoptosis followed by a reversal phase, which lasts approximately 9 days (Reddy 2004).

During the reversal phase osteoblast-like cells remove the remaining organic matrix from the resorption lacuna and it is populated by osteoblast precursors that proliferate and differentiate into osteoblasts (Everts et al. 2002, Mulari et al. 2004). During reversal, all-important coupling signals are also sent out to osteoblasts to replace the bone that has been removed (Olsen 2006).

After that, the osteoblasts begin to synthesize the osteoid and finally 15 days after the organic matrix is secreted, it begins to mineralize. Osteoblasts secrete small membrane bound vesicles, called matrix vesicles that establish suitable conditions for initial mineral deposition by the concentration of calcium and phosphate ions (Anderson 2003).

**Fig. 4. Remodeling cycle.** A) Activation of osteoclastic precursors. B) Resorption by activated multinuclear osteoclasts. C) Reversal, when osteoblast like cells remove the remaining organic matrix from the bottom of the resorption lacuna before depositing new collagen fibrils. Osteoblast precursors proliferate and differentiate into osteoblasts and then migrate into the resorption lacuna. D) Formation and mineralization. Osteoblasts continue to deposit new bone matrix and mineralize osteoid until the cavity is filled. E) Resting. Once osteoblasts are embedded in osteoid, they mature into terminally differentiated osteocytes. Modified from (Matsuo & Irie 2008).
2.4 Vascularization in bone

Bone is a highly vascularized tissue. The significance of capillary growth in bone modeling and remodeling is well established in bone development and fracture healing (Stevens & Williams 1999). The vasculature is required for the transport of oxygen, nutrients, growth enhancing molecules and precursor cells, such as precursors of chondroclasts and osteoclasts, for the renewing bone tissue.

The majority of bones in the vertebrate skeleton are formed by endochondral ossification, and blood vessel invasion into the cartilage is an important phenomenon in endochondral ossification. In embryonic skeletal tissue, osteogenesis and angiogenesis are temporally related and a lack of VEGF activity arrests the bone development (Gerber et al. 1999, Ensig et al. 2000). There are numerous skeletal pathologies such as osteoporosis, osteopetrosis and inflammatory bone loss that are related to modifications in blood supply (Brandi & Collin-Osdoby 2006).

Angiogenesis is thought to depend on a finely tuned balance between endogenous stimulators and inhibitors (Polverini 1995). Molecules that serve as positive regulators of angiogenesis include fibroblast growth factor (FGF), transforming growth factor (TGF)-α, TGF-β, hepatocyte growth factor (HGF), tumor necrosis factor (TNF)-α, angiogenin, interleukin (IL)-8, and the angiopoietins and particularly members of the vascular endothelial growth factor (VEGF) family (Folkman & Shing 1992, Yancopoulos et al. 2000, Ferrara et al. 2003, Dai & Rabie 2007).

2.4.1 Formation and structure of blood vessels

The formation of a vascular network is implemented by both vasculogenesis and angiogenesis. Vasculogenesis is a process whereby vessels are formed de novo from endothelial cell (EC) precursors, known as angioblasts (Gonzalez-Crussi 1971). Angiogenesis is defined as the growth of new capillaries from a pre-existing vascular network (Risau 1997). The blood vessel system is hierarchically organized to deliver oxygen, soluble factors, and various types of cells to all tissues in a carefully regulated manner. The inside of a blood vessel is lined with endothelial cells, adhering to each other and to specific matrix molecules. The endothelium functions as a barrier that limits the movement of cells and molecules between the circulation and tissues (Risau 1995). The endothelial layer is attached to the basement membrane (BM), which is composed of collagen IV,
XV, XVIII, laminins, heparan sulfate proteoglycans, nidogen-entactin and other matrix components (Timpl 1996). Angiogenesis is an invasive process that requires controlled activity of extracellular proteases and their inhibitors (Liotta et al. 1991). As a result the BM dissolves causing the EC to come in contact with proteolytic fragments of the constituents of the basement-membrane.

Specialized smooth muscular cells called pericytes envelop the surface of the vascular tube. The interaction between pericytes and the EC is important for the maturation, remodeling and maintenance of the vascular system. Gap junctions provide direct connections between the cytoplasm of pericytes and endothelial cells, and they enable the exchange of ions and small molecules (Bergers & Song 2005). Pericytes and supporting mural cells sense the external environment through integrin receptors that promote cell survival, growth, migration and tube formation (Hall et al. 2006). Integrins have an important role in the angiogenic activity of endothelial cells because their proliferation is anchorage-dependent. Inhibitors of various integrins have all demonstrated anti-angiogenic effects. The endothelium signals between surrounding cells through a host of humoral and growth factors, cytokines and chemokines, reactive metabolites, and polarized surface-associated molecules (Cleaver & Melton 2003). Pericytes have VEGF receptors and are capable of differentiating to chondrocytes and osteoblasts (Doherty & Canfied 1999). Thus they provide a pool of stem cells in endochondral ossification and bone healing.

**2.4.2 Bone endothelium**

Bone endothelium has been found to signal to other bone cells, including osteoclasts, osteoblasts, and their progenitors. Bone remodeling sites associate intimately with vascular sinusoids, and histological findings suggest that osteoblasts and osteoprogenitor cells develop concomitantly with endothelial cells in blood vessels adjacent to sites where new bone is formed (Dai & Rabie 2007). Osteoclasts, which are derived from hematopoietic precursors are present in both the bone marrow and the peripheral circulation (Roodman 1999). They must migrate through the endothelium to reach future sites of bone resorption. It has been shown, that endothelial cells can stimulate the formation of osteoclasts by several mechanisms, including RANKL expression, and also promote bone resorption in vitro (Formigli et al. 1995, Collin-Osdoby et al. 2001) There is also some evidence, that osteoclasts stimulate angiogenesis (Tanaka et al. 2007). It has
been shown that osteoblast lineage cells are also present in the circulation (Eghbali-Fatourechi et al. 2005).

Vascular endothelial cells synthesize and secrete many factors known to have an effect on bone cells, including M-CSF, RANKL, various growth factors, cytokines, chemokines, prostanoids, free radicals, small peptides, adhesion molecules, and matrix constituents. Additionally, bone endothelial cells are also able to respond to bone modulators, including PTH, sex steroids, and proinflammatory cytokines (Brandi et al. 1993, Brandi & Collin-Osdoby 2006).

Two key molecules that have well defined roles are VEGF and angiopoietins. The receptors for both these ligands are predominantly expressed on ECs and they regulate the proliferation and survival of these cells. For example, angiopoietin-1 (Ang-1), expressed predominantly upon ECs, regulates the proliferation and survival of endothelial cells. Ang-1 is expressed also in osteoblasts. It binds to the tyrosine kinase receptor, Tie-2, which is expressed in endothelial cells and also in hematopoietic stem cells (HSC) (Huang & Bao 2004, Huang et al. 2007). Tie-2 positive HSC are localized to the bone surface where they are in contact with Ang-1 expressing osteoblasts. It was recently discovered that a subset of osteoblasts function as a key component of the HSC niche, controlling HSC numbers. In addition to osteoblasts, HSC interact with other stromal cells too, including ECs (Yin & Li 2006).

2.5 VEGF

Vascular endothelial growth factor, VEGF, generally referred to as VEGF-A is an angiogenic factor that is important for vascular development and maintenance in all mammalian organs and therefore a key mediator of physiological angiogenesis, for example during embryogenesis, reproductive functions and skeletal growth. In addition, VEGF-A has a crucial role in pathophysiological angiogenesis that is associated with numerous malignant, ischemic, inflammatory, infectious and immune disorders (Carmeliet 2005). Numerous different strategies that target VEGF receptor signal transduction pathways have been designed to inhibit pathological angiogenesis in several malignancies (Ferrara et al. 2003). Additionally, there is also clinical interest in promoting angiogenesis in conditions such as stroke and ischemia (Olsson et al. 2006).

VEGF is also called the vascular permeability factor (VPF), because in 1983 it was first discovered as a permeability factor based on its ability to induce vascular leakage (Senger et al. 1983). In the late 1980s VEGF was discovered as
an angiogenic factor that was found to stimulate endothelial cells to proliferate, migrate, and survive in a serum poor environment (Leung et al. 1989).

The importance of VEGF-A during angiogenesis has been demonstrated in transgenic mouse gene deletion studies. Mice lacking a single Vegf allele resulted in embryonic lethality, due to deficient and abnormal vascular development (Carmeliet et al. 1996). Similar results were obtained with mice carrying deletions of the VEGF receptor allele VEGFR-1 (Fong et al. 1995) or VEGFR-2 (Shalaby et al. 1995).

The VEGF family members are secreted dimeric glycoproteins, which belong to a family of proteins that include VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placenta-like growth factor (PLGF). The predominant form, VEGF-A, is alternatively spliced into at least five different isoforms in humans. VEGF121, VEGF 145, VEGF 165, VEGF 189, and VEGF 206, that have different biological activities such as different abilities to interact with the VEGF co-receptors heparan sulfate proteoglycans (HSPGs) and neuropilin (Houck et al. 1991, Poltorak et al. 2000). VEGF164 and VEGF188 show increased binding to heparin sulfate containing proteoglycans present on the cell surface and extracellular matrix. The VEGF120 splice form is completely soluble and unable to bind heparin (Ferrara & Davis-Smyth 1997, Hall et al. 2006). Murine VEGF-As are shorter than the corresponding human isoforms of 121, 145, 165, 189, and 206 amino acids (termed VEGF120, VEGF164, etc.)(Tamayose et al. 1996). Proteolytic processing of VEGF-A splice variants affects their ability to interact with the VEGF co-receptors (Lee et al. 2005).

VEGF165, used in this study, is physiologically the most abundant isoform of VEGF-A (Tischer et al. 1991). VEGF120 is diffusible, VEGF188 binds to the cell surface and extracellular matrix and VEGF164 combines these properties. VEGF165 exists in both a diffusible form and as an extracellular matrix bound form, which can be released or activated by plasmin, lactate (Kumar et al. 2007), or matrix metalloproteinases (MMPs) (Lee et al. 2005, Breen 2007). Many major growth factors including epidermal growth factor, TGF-a, TGF-ß, keratinocyte growth factor, insulin-like growth factor-1, FGF, and platelet-derived growth factor upregulate VEGF mRNA expression, suggesting that paracrine or autocrine release of such factors cooperates with local hypoxia in regulating VEGF release in the microenvironment (Ferrara et al. 2003).
2.5.1 VEGF receptors

The biological functions of VEGF are mediated through the binding to specific receptors and co-receptors that are primarily expressed in the vascular and lymph-vessel endothelial cells, but are also located on non-endothelial cell types including monocytes and macrophages, hematopoietic stem cells, epithelial cells, fibroblasts, smooth muscle cells, and myogenic precursor cells (satellite cells) (Olsson et al. 2006, Breen 2007).

VEGF ligands bind to three receptor tyrosine kinases (RTKs) denoted VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1), and VEGFR-3 (Flt-4) as well as to co-receptors. Co-receptors, including heparan sulfate proteoglycans (HSPGs) and neuropilins (NRP), lack tyrosine kinase activity but may modify the binding to tyrosine kinase containing VEGFRs or bind VEGF directly to signal a cellular response (Breen 2007). All isoforms bind VEGFR1 and VEGFR2, whereas only VEGF164 has been shown to bind NRP-1 (Soker et al. 1998, Neufeld et al. 2002).

The deletion of a single VEGF allele or receptor gene allele, VEGFR-1 or VEGFR-2, has been shown to result in fatal vascular defects in the embryo (Fong et al. 1995, Shalaby et al. 1995, Carmeliet et al. 1996, Ferrara et al. 1996). VEGFR1 is expressed on many types of endothelial cells, monocytes (Barleon et al. 1996) and dendritic progenitors (Wu et al. 1996). VEGFR1 ligands are chemoattractive for monocytes and osteoclasts (Ensig et al. 2000, Clauss et al. 1990). VEGFR1 acts as a decoy through its ability to bind VEGF-A and regulate the level of VEGF available to bind to VEGFR2 and initiate vessel formation during embryogenesis. A soluble form of VEGFR1 also acts as a natural VEGF trap since it is expressed through alternative transcription and binds to VEGF-A to prevent angiogenesis (Maynard et al. 2003, Breen 2007). The expression of VEGFR2 is almost exclusively limited to vascular endothelial cells and their progenitors (Asahara et al. 1997). VEGFR2 is associated with the integrin-dependent migration of endothelial cells, since it can form a complex with integrin αvβ3 and induce of endothelial cell proliferation, migration, and in vivo angiogenesis (Hutchings et al. 2003, Dai & Rabie 2007). VEGFR3 has been shown to be important for lymphatic-endothelial cell development and function (Kaipainen et al. 1995).
2.5.2 VEGF in endochondral bone formation

Over 40 years ago Trueta and others concluded that blood vessels have an active role in bone formation (Trueta & Amato 1960, Trueta & Buhr 1963, Trueta & Trias 1961). Skeleton formation starts with mesenchymal condensation and the transcription factor SOX9 has been found to be an important regulator in this process (Bi et al. 1999). Inactivation of SOX9 results in complete absence of mesenchymal condensation leading to failure of cartilage formation. Recent findings provide evidence for the involvement of SOX9 in the regulation of VEGF expression in condensing mesenchyme, whereas VEGF expression has been found to regulate limb vasculature via a direct long-range mechanism (Eshkar-Oren et al. 2009).

Harada and others were first to document the expression of VEGF in bone tissue and osteoblasts (Harada et al. 1994). Vessel invasion has a key role in endochondral bone formation and VEGF plays an important role in this phenomenon. VEGF may mediate interactions between endothelial cells and bone cells. Endothelial cells, chondrocytes (Carlevaro et al. 2000), and osteoblasts (Wang et al. 1997) secrete endogenous VEGF that may stimulate chondrocytes (Carlevaro et al. 2000), osteoblasts (Mayr-Wohlwart et al. 2002, Deckers et al. 2000) and osteoclasts (Nakagawa et al. 2000). In endochondral ossification, an avascular cartilage model is first formed. Immature and proliferating chondrocytes secrete and express angiogenic inhibitors (Moses et al. 1999). Chondrocytes in the cartilage template and later in the growth plate first proliferate and then differentiate into mature hypertrophic cells. They express high levels of VEGF (Gerber et al. 1999).

VEGF expression in the hypertrophic cartilage causes the upregulation of VEGFR1 and VEGFR2 expression in the perichondral endothelium (Colnot et al. 2001, Zelzer et al. 2001, Zelzer et al. 2002) and thereupon induces vessels to invade the hypertrophic cartilage from the perichondrium (Colnot et al. 2004). Zelzer and Olsen have speculated that high levels of VEGF near hypertrophic chondrocytes maintain endothelial cells in a sprouting mode. This has been explained by the fact, that sprouting growth plate capillaries contain no basement membranes or pericytes but, in the region where bone matrix is being synthesized, endothelial cells are surrounded by BMs and pericytes (Zelzer & Olsen 2005).

VEGF upregulation is maintained in the sprouting vessels under the growth plates during all stages of bone development (Gerber et al. 1999). Concurrent
with vascular invasion, the hypertrophic cartilage matrix is degraded by invading osteoclasts/chondroclasts. Osteoblasts and marrow cells start to populate and ossify two eventual centers of ossification: the primary ossification center on midshaft and a secondary center on the epiphysis (Maes & Carmeliet 2008). It has been shown, that MMP-9 specifically regulates proteolysis of nonmineralized cartilage and the release of ECM-bound VEGF, which has a direct chemotactic affect on osteoclasts (Engsig et al. 2000).

A study by Gerber and colleagues showed that blocking the VEGF receptors inhibited vascular invasion of the cartilage and bone formation and resorption in juvenile mice. In their study, blood vessel invasion was almost completely suppressed, concomitant with impaired trabecular bone formation and expansion of the hypertrophic chondrocyte zone (Gerber et al. 1999). In addition, transgenic mice having chondrocyte specific inactivation of VEGF-A, show embryonic lethality, disturbance in the blood vessel development and aberrant endochondral bone formation (Haigh et al. 2000). The specific contributions of the VEGF isoforms have been studied by using models in mice expressing only one of the major isoforms. These studies demonstrate a significant role of VEGF at both an early and late stage of cartilage vascularization. The matrix-binding isoforms mediate metaphyseal angiogenesis and thereby regulate both trabecular bone formation and growth plate morphogenesis during endochondral bone formation. The soluble isoforms diffuse to the perichondrium and are required for epiphyseal vascularization and secondary ossification in growing bone (Zelzer et al. 2002, Maes et al. 2002, Maes et al. 2004).

2.5.3 VEGF and chondrocytes

VEGF is expressed at high levels in hypertrophic chondrocytes and low levels in maturing chondrocytes in rats (Carlevaro et al. 2000). VEGF-A secretion by hypertrophic chondrocytes is not only a paracrine process that attracts and stimulates proliferation of endothelial cells, but is also an autocrine process that is needed for chondrocyte survival (Dai & Rabie 2007). Pufe and others have shown that VEGF affects the proliferation of chondrocytes in a dose dependent manner with the C28/12 immortalized chondrocyte cell line (Pufe et al. 2004a).

It has been suggested, that Runx2 is involved in the regulation of VEGF in hypertrophic chondrocytes. There is no blood vessel invasion into hypertrophic cartilage in Runx2 null mice, since the upregulated expression of VEGF is absent in the hypertrophic cartilage of Runx2-deficient mice (Zelzer 2001). The culture
of chondrocytes under hypoxic conditions up-regulates VEGF-A expression via HIF-1α (Pfander et al. 2003). VEGF expression via HIF-1α is also induced by mechanical overload of cartilage discs (Pufe et al. 2004b).

2.5.4 VEGF and osteoclasts/chondroclasts

Osteoclasts share a common hematopoietic precursor with monocytes and macrophages, and, like them, osteoclasts express FLT1 as their main VEGF receptor (Barleon et al. 1996, Niida et al. 1999). However, the expression of KDR by cultured osteoclasts has also been reported (Nakagawa et al. 2000, Tombran-Tink & Barnstable 2004). VEGF induces osteoclast differentiation in combination with RANK from spleen-or bone marrow-derived precursors in culture and further, a single injection of recombinant human VEGF-A (rhVEGF-A) into osteopetrotic op/op mice (lack of MCSF-1) induced osteoclast recruitment and survival, while stimulating osteoclastic bone resorption (Niida et al. 1999). VEGF has been shown to stimulate the resorption activity of osteoclasts in vitro (Nakagawa et al. 2000) and to be a chemoattractant for osteoclasts invading into developing long bones (Ensig et al. 2000). VEGF and RANK stimulate the migration of the osteoclasts into hypertrophic cartilage through the extracellular signal-regulated kinases 1 and 2 (ERK1/2) pathway (Henriksen et al. 2003, Min et al. 2003). VEGF also enhances dose- and time-dependent osteoclastic bone resorption in a culture of highly purified rabbit mature osteoclasts partially by enhancing the survival of the cells (Nakagawa et al. 2000).

2.5.5 VEGF and osteoblasts

VEGF has been implicated in various aspects of osteoblast function. Osteoblasts have been found to express VEGF and its receptors in vitro with the highest expression levels being found at the late differentiation stages (Harada et al. 1994, Deckers et al. 2000, Harper et al. 2001).

VEGF is a central molecule in osteoblast-stimulated angiogenesis, having both autocrine and paracrine mechanisms of action (Grellier et al. 2009). Several factors with important roles in regulating bone formation also induce the expression of VEGF by osteoblasts. Prostaglandins E1 and E2, BMP-4, BMP-6, BMP-7, FGF-2, TGF-β, endothelin-1, IGF-1, and vitamin D3 can all induce VEGF expression in osteoblasts by activating a variety of signaling pathways, but
VEGF expression is inhibited by bone catabolic factors such as glucocorticoids (Zelzer and Olsen 2005).

VEGF acts as a potent chemoattractant for rat (Nakagawa et al. 2000) and human (Mayr-Wohlwart et al. 2002) osteoblasts, as well as bone-marrow-derived mesenchymal progenitor cells (Fiedler et al. 2005). A number of studies indicate, that VEGF induces the differentiation of osteoblastic cells (Midy & Plouet 1994, Deckers et al. 2000, Street et al. 2002). Zelzer and coworkers showed that VEGF has a stimulatory effect on bone formation. During calvaria organ culture, treatment with VEGF led to an increase in the thickness of parietal bone (Zelzer et al. 2002).

Furthermore, it has been reported that adenovirus-mediated VEGF gene transfer induced bone formation by increasing osteoblast number and osteoid forming activity in vivo (Hiltunen et al. 2003). VEGFR-1 signaling has been shown to be critical for osteoblast activity during bone formation through participation in osteoclastogenesis, as well as in the maintenance of a bone marrow hematopoiesis supportive microenvironment. Mice that were deficient in flt-1 signaling were shown to have very depleted numbers of osteoclasts and osteoblasts, and also a deficiency in bone marrow cavity formation (Niida et al. 2005). Recently, also VEGFR3 has been found to be important for osteoblast differentiation (Orlandini et al. 2006).

2.6 XVIII collagen and endostatin

2.6.1 XVIII collagen

Type XVIII collagen has been localized in basement membrane (BM) prominently in vascular and epithelial BM throughout the body (Muragi et al. 1995). Collagen XVIII is a heparan sulfate proteoglycan (HSPG) (Halfter et al. 1998). HSPGs are composed of a core protein and heparin sulfate (HS) side chains. Collagen XVIII belongs to the multiplexin (multiple triple-helix domains and interruptions) subclass of the non-fibrillar collagen family, having ten interrupted triple-helical (COL) domains that vary in length, separated by 11 non-collagenous (NC) regions, flanked by N- and C-terminal non-collagenous domains (Erickson & Couchman 2000). Collagen XVIII is expressed as three N-terminal variants. These are transcribed from two different promoters, promoter 1 being responsible for the production of the short variant, and promoter
2 for the two longer variants. The middle variant is generated from the long variant by alternative splicing (Muragi et al. 1995, Rehn & Pihlajaniemi 1995, Saarela et al. 1998). The C terminus includes a trimerization region, a hinge region, which provides protease-sensitive sites, and an anti-angiogenic fragment, endostatin.

### 2.6.2 Endostatin

Endostatin is a 20kDa carboxy-terminal fragment of the non-collagenous domain of the collagen XVIII α1 chain. It was originally identified from murine hemangioendothelioma cells (EOMA) (O’Reilly et al. 1997).

Structurally, endostatin is characterized by compact globular folding with surface exposed arginine-rich patches (Hohenester et al. 1998). They are involved in binding to heparin sulfate, which in turn may be important for endostatin’s anti-angiogenic function (Kreuger et al. 2002, Sasaki et al. 1999, Dixelius et al. 2003).

Endostatin is liberated from the non-collagenous domain by the action of various proteases, including matrix metalloproteinases (MMP) Cathepsin L, and the serine protease elastase (Wen et al. 1999, Felbor et al. 2000). The released forms can be found in the vessel wall, in platelets and freely circulating. According to a number of studies, the normal mean circulating endostatin plasma level is in the range of 10–50ng/ml (Dixelius et al. 2003).
Fig. 5. A schematic presentation of the structure of collagen XVIII. Ten collagenous domains are interrupted and flanked by 11 non-collagenous domains. These are numbered from the carboxy-to the amino terminus, NC1- through NC-11. Collagen XVIII is expressed as three N-terminal variants. These are transcribed from two different promoters. Modified from Dixelius et al. 2003, Bix et al. 2005).

2.6.3 Collagen XVIII /Endostatin inhibits angiogenesis

The effects of endostatin are broad, since in endothelial cells it inhibits endothelial cell proliferation (O’Reilly et al. 1997), migration (Yamaguchi et al. 1999), proteinase activity (Wickström et al. 2001) and vessel stabilization (Dixelius et al. 2000). Furthermore, endostatin induces endothelial cell apoptosis (Dhanabal al. 1999, Dixelius et al. 2000). Endostatin also affects tumor angiogenesis indirectly by suppressing the endogenous expression of VEGF (Hajitou et al. 2002).

Collagen XVIII deficient mice survive and do not display obvious vascular abnormalities or altered tumor growth (Fukai et al. 2002). They suffer from eye abnormalities, having defects in almost all ocular structures that express collagen XVIII. These abnormalities are similar to those observed in patients with Knobloch’s syndrome, which is caused by an inactivating mutation in the collagen α1(XVIII) gene (Fukai et al. 2002).
2.6.4 Endostatin, bone and cartilage

Only little is known regarding endostatin’s role in bone and cartilage. Pufe and others have shown that endostatin/collagen XVIII is expressed in human cartilage and fibrocartilage and they suggest it might reflect the importance of endostatin for the development and maintenance of avascular zones. According to their hypothesis, endostatin expression by mature cartilage may suppress osteoclasts while endostatin-deficient ossifying cartilage allows osteoclastic activity and endochondral ossification (Pufe et al. 2004c). Lau and others found that endostatin may have a role in the anabolic effect of mechanical stimuli on bone since endostatin can prevent the effect of fluid shear stress on osteoblasts (Lau et al. 2006). There is evidence that endostatin inhibits Wnt-signaling, acting possibly at the level of β-catenin in the signaling pathway (Hanai et al. 2002).

2.6.5 Mechanism of endostatin action

The exact mechanism of endostatin mediated inhibition has not yet been elucidated. Endostatin binds to a number of cell surface molecules, but the signal transduction events following binding to these receptors that result in the inhibition of angiogenesis have so far been poorly characterized (Wickström et al. 2005, Delaney et al. 2006). Endostatin modulates many intracellular pathways.

Endostatin has been shown to be a heparin binding protein (Hohenester et al. 1998, Sasaki et al. 1999). This suggests that endostatin may competitively inhibit the binding of angiogenic growth factors to heparan sulfate proteoglycans, which are known to act as co-receptors for a number of cytokines (Hohenester et al. 1998). Dixelius and colleagues showed that heparin binding is needed for the anti-angiogenic effects of endostatin. Endostatin mutants lacking heparin-binding ability failed to block growth factor induced angiogenesis, in contrast to wild – type endostatin in the chicken chorioallantoic membrane assay (Dixelius 2000). Endostatin binding with low affinity to the heparan sulfate proteoglycans glypican-1 and glypican-4 or with a high affinity to an as yet unidentified receptor has been reported (Karumanchi et al. 2001).

It has been shown, that endostatin’s anti-angiogenic effects are mediated at least in part, by interactions with integrins. Integrins play an essential role in the angiogenic activity of endothelial cells because their proliferation is anchorage-dependent. Inhibitors of various integrins have all demonstrated anti-angiogenic effects (Bix & Iozzo 2005). Inhibitory action by endostatin has been proposed to
involve binding to the receptor α5β1 (Sudhakar et al. 2003). According to studies of Wickström and colleagues, endostatin interacts with endothelial cells α5β1 integrin and perhaps concomitantly, with cell surface, heparan sulfate proteoglycans and caveolin (Wickström et al. 2002, Wickström et al. 2003), which leads to complex signaling cascades (Wickström et al. 2002, Bix & Iozzo 2005).

Interestingly, endostatin blocks both the VEGFR1 and VEGFR2 receptors of VEGF and by that mechanism inhibits VEGF signaling (Kim et al. 2002). Recent evidence shows that endostatin can also bind to VEGFR3 (Kojima et al. 2008). In addition, endostatin’s inhibitory action is involved with several signaling pathways, including Wnt/β-catenin (Hanai et al. 2002) and ERK1/2 signaling (Schmidt et al. 2006). The anti-angiogenic activity of endostatin also requires the presence of endothelial cell E-selectin, an endothelial cell-specific membrane glycoprotein that is important for leukocyte attachment (Yu et al. 2004). Furthermore, endostatin binds tropomyosin 3, a member of a large family of actin-interacting proteins (MacDonald et al. 2001).

2.7 Gene therapy as a method of Growth Factor Delivery

VEGF has been used to promote angiogenesis of ischemic muscle tissues and accelerate fracture healing, whereas endostatin has been found to inhibit the growth of primary tumors and their metastases in a number of animal models and human subjects in clinical trials (Spector et al. 2000, Folkman 2006, Keramaris et al. 2008, Uchida & Haas 2009).

The application of exogenous proteins may be limited by their short biological half-lives and because of difficulties associated with obtaining sufficient quantities for optimal therapeutic benefit. A single dose of recombinant protein may not be sufficient to increase or decrease angiogenesis or osteogenesis (Spector et al. 2000). A better strategy for protein delivery may be gene therapy, which involves the transfer of genetic information to cells, and can be completed in vivo or ex vivo. For in-vivo gene therapy, a vector carrying the therapeutic DNA is directly implanted or injected into the target tissue so that host cells are transfected and express the protein. For ex-vivo therapy, gene transfer can be delivered through transfection of cultured cells, which are then implanted or injected into the target tissue. The duration of protein synthesis after gene therapy depends on the techniques used to deliver the gene to the cell. (Kofron & Laurencin 2006)
Vectors are agents that enhance the entry and expression of DNA in a target cell. Ideal vectors have high transfection efficiency, low toxicity, and consistent gene expression. They may be of viral and nonviral origin. Currently, the most common types of vectors are viruses that have been genetically altered to carry non-viral DNA. Viruses have evolved specialized molecular mechanisms to efficiently transport their genomes inside the cells they infect. Some of the different types of viruses used as gene therapy vectors are for example, adenoviruses, retroviruses, adeno-associated viruses and lentiviruses. Adenoviruses can be produced in high titer and can transfect dividing and non-dividing cells. Adenoviral DNA does not integrate into the genome and is not replicated during cell division, with the result that transfected cells are gradually diluted out of the population. The primary disadvantage of adenoviral vectors is a potential host immune response, which targets the virus and cells transfected by the virus. (Lieberman et al. 2002, Franceschi 2005, Kofron & Laurencin 2006)
3  Aims of the study

Angiogenesis is a fundamental part of osteogenesis as well as bone remodeling. Since there is an intimate relationship between the vasculature and bone cells within the bone remodeling site, the role of the basement membranes of the vascular endothelium in the recruitment of bone cells was studied. One component of basement membrane is collagen XVIII, a proteoglycan containing heparan sulfate, and endostatin is a fragment of collagen XVIII, therefore, the hypothesis was that endostatin could have a role in the activation of bone cells. The effects of the major angiogenic factor, VEGF, on bone cells was studied.

The aims of the present study were:

1. to investigate the feasibility of a first-generation adenoviral vector to deliver VEGF overexpression locally at the site of bone injury in order to alter the course of bone regeneration in the rat femur.
2. to study the possible role of endostatin and VEGF-A on osteoclasts in vitro by using a classical resorption pit assay, and to investigate osteoclast mediated bone resorption and osteoclastogenesis in a differentiation assay using bone marrow hematopoietic stem cells.
3. to follow the bone formation cascade in vivo in an endochondral ossification model where ectopic bone formation is stimulated by BMPs in the mouse hind muscle pouch, in the presence of overexpressed endostatin with or without VEGF-A using a recombinant adenoviral vector.
4. to further investigate the influences of endostatin on osteoblastic behavior in vitro. The role of endostatin in bone was studied in vivo using a transgenic mouse line overexpressing endostatin (ES-tg) and knockout mice lacking collagen XVIII (Col118a1−/−).
4 Materials and methods

4.1 Reagents (I-IV)

4.1.1 Recombinant proteins (II, IV)

VEGF-A
recombinant human VEGF165 produced in Spodoptera frugiperda (Calbiochem) was diluted in sterile PBS containing ≥ 0.1% BSA.

Endostatin
recombinant human endostatin polypeptide produced in Pichia pastoris (Calbiochem) was diluted in sterile PBS.

4.1.2 Recombinant adenoviruses (I, III)

Recombinant E1-E3-deleted adenoviral vectors encoding VEGF164 (AdVEGF-A) (Breier et al. 1992) and nuclear targeted β-galactosidase (AdlacZ) under the CMV promoter were constructed and produced in 293 human embryonic kidney cells as previously described (Laitinen et al. 1998). Adenoviruses were analyzed to be free from helper viruses, lipopolysaccharide and bacteriological contaminants (Laitinen et al. 1998, Puomalainen et al. 1998).

To create a recombinant adenovirus expressing rat endostatin, the expression cassette was cloned into the pQBI-AdCMV5-IREs-GFP vector (Quantum Biotechnologies, Montreal, Quebec, Canada). The vector and the plasmid pJM17 (Microbix Biosystems, Toronto, Ontario, Canada) were then cotransfected into 293 cells, and the recombinant adenovirus was isolated, amplified, and purified according to standard procedures (Pulkkanen et al. 2002).

4.2 Experimental animals in vivo (I, III, IV)

4.2.1 Bone defect healing model (I)

Male Sprague-Dawley rats (350–410 g) were anesthetized by intraperitoneal injection of fentanyl citrate 0.315 mg/ml, midazolam 5 mg/ml, and fluanisone 10 mg/ml (1:1:1). A cylindrical 3-mm osteoperiosteal defect was drilled in distal
femurs approximately 5 mm from the epiphyseal plate. The surrounding muscle was closed around the lesion and 2 × 10^8 plaque-forming units (pfu) of adenoviral vector encoding vascular endothelial growth factor (AdVEGF-A) were injected into the muscle in a 10-µl volume. The contralateral femurs served as controls and received equal amounts of β-galactosidase adenoviral vector (AdlacZ). The animals were sacrificed 1, 2, or 4 weeks (at least seven animals at each time point) following the procedure and the femurs were collected and fixed in 4% paraformaldehyde in 0.15 M sodium phosphate (pH 7.2) for further analysis.

4.2.2 Endochondral ossification animal model (III)

Balb/c mice, age 10–12 weeks, were anesthetized by intraperitoneal injection of ketamine midazolam (0.08 ml/10 g body weight). Ectopic bone formation was induced by implanting native reindeer (Rangifer tarandus) BMP extract combined with gelatine gel (5 mg of each) in 0.9% saline and recombinant adenoviral vectors designed to overexpress endostatin (AdEndostatin), VEGF-A (AdVEGF-A), or nuclear targeted β-galactosidase (AdlacZ). The mixture (100 µl) was injected (1 ml syringe, 20 G needle) into both thigh muscle pouches in the bilateral hind legs. The mice used in this study (total of 76) were divided into four groups. The control group received BMP and gelatin gel only (n = 16) while the second group received BMP combined with the gelatin gel and 1.2 × 10^9 pfu of AdVEGF-A (n = 19). The third and the fourth groups were injected with BMP combined with gelatin gel and 1.5 × 10^9 pfu Ad-endostatin only (n = 20) or in conjugation with 1.2 × 10^9 pfu AdVEGF-A (n = 20), respectively. In addition, four animals served as controls and received equal amounts, 1.6 × 10^9 pfu of AdlacZ in BMP combined with gelatine gel. The animals were sacrificed in a chamber with carbon dioxide 1, 2 or 3 weeks after the operation and the hind legs were collected for further analysis. The animal tests were performed after approval by the National Laboratory Animal Center. All aspects of animal care complied with the Animal Welfare Act and the recommendations of the NIH-PHS Guide for the Care and Use of Laboratory animals.

4.2.3 Transgenic mice and knockout mice (IV)

J4 mice overexpressing monomeric endostatin in the keratinocytes under the keratin-14 promoter (ES-tg) with age- and sex-matched FVB/N wild type
controls, and knock-out mice deficient in collagen XVIII (Col18a1−/−) with age- and sex-matched C57BL/6J wild type controls were used to study the effects of collagen XVIII/endostatin on bone development in vivo. The generation of collagen XVIII knock-out and J4 mice has been described in previous reports (Fukai et al. 2002, Elamaa et al. 2005). Groups of 6–10 mice containing equal numbers of males and females were used for the experiments, and the mice were sacrificed at the age of 14 or 30 days. All the experiments were approved by the Animal Care and Use Committee of the University of Oulu, and in the case of the Col18a1−/− mice also by the State Provincial Office in Oulu.

4.3 Cell Cultures

4.3.1 Osteoclast isolation and culture (II)

The procedure for the isolation and culture of osteoclasts described earlier by Boyde et al. and by Chambers et al. was modified slightly and has been described in detail previously (Boyde et al. 1984, Chambers et al. 1984). Briefly, mechanically harvested osteoclasts from the long bones of 1- or 2-day-old Sprague-Dawley rat pups were allowed to attach to ultrasonicated bovine cortical bone slices (0.125 cm² or 0.5 cm²). After 30 minutes, the nonattached cells were rinsed away, and the remaining cells on the bone slices were cultured in Dulbecco’s modified Eagle’s medium (α-MEM) buffered with 20 mM HEPES and containing 0.84 g of sodium bicarbonate/liter, 2 mM L-glutamine, 100 IU of penicillin/ml, 100 µg of streptomycin/ml and 7–10% heat-inactivated fetal calf serum (FCS), at +37°C (5% CO₂ and 95% air). The cells were divided into ten groups: the control group had α-MEM-FCS as their culture medium with no added substances (later referred to as control), the VEGF-A-treated cells had 100 ng/ml, 50 ng/ml or 10 ng/ml VEGF-A in α-MEM-FCS (later referred to as VEGF). The endostatin groups had 0.02, 0.2 or 2 µg/ml endostatin in α-MEM-FCS (later referred to as ENDO) and the last groups had both VEGF-A and endostatin added into the complete culture media (later referred to as VEGF+ENDO). The last groups had 100 ng/ml VEGF-A and 2 µg/ml endostatin, 50 ng/ml VEGF-A and 0.2 µg/ml endostatin or 10 ng/ml VEGF-A and 0.02 µg/ml endostatin. The cells were allowed to grow for 48 h, after which the bone slices were fixed with freshly made refrigerated 3% paraformaldehyde (PFA) and 2% sucrose in phosphate-buffered saline (PBS) for 10 minutes at room temperature.
The data shown were gathered from three independent experiments, each of which gave identical results.

4.3.2 Osteoclast differentiation assay (II)

The procedure for osteoclast differentiation described earlier by Takahashi et al. was slightly modified (Takahashi et al. 1988). Mouse (C57BL/6, 8–12 weeks) bone marrow cells were isolated from mouse long bones using a syringe and transferred to a petri dish. After incubation at +37 °C for 2 hours, non-attached cells were collected from petri dish and seeded in 24-well plates containing sonicated cortical bovine bone slices (0.125–0.5 cm²) at a concentration of 1 × 10⁶ cells/well. Cells on the bone slices were cultured in four groups: control, VEGF (100ng/ml), ENDO (2µg/ml) and VEGF + ENDO respectively. The control group was cultured in α-MEM medium (Sigma, UK) with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 IU/ml penicillin, 10 µg/ml streptomycin and 20 mM Hepes, a medium later referred to as α-MEM-FCS. The cells were cultured in 500 µl α-MEM containing 30 ng/ml RANKL (Peprotech Ec., UK) and 10 ng/ml M-CSF (R&D Systems). Half of the medium was replaced every 3rd day. The cells were cultured at +37 °C (5% CO2, 95% air) for 7 days, after which the cultures were stopped by fixing the cells with 3% paraformaldehyde (PFA) / 2% sucrose in PBS.

4.3.3 Osteoblast MC3T3 culture (IV)

MC3T3-E1 cells are pre-osteoblasts derived from murine calvarias (American Type Culture Collection, ATCC, USA). They were maintained in α-MEM supplemented with 10% FCS, 2mM L-glutamine, 100 IU 7ml penicillin and 10 µg/ml streptomycin (α-MEM-FCS), and passaged every 2–3 days using standard techniques. After pre-culturing the cells were seeded at a density of 10.000 cells / cm² in the presence of α-MEM with 50 µg/ml ascorbic acid and 10 mM β-glycerophosphate (mineralization medium). The cells were cultured in six groups and the medium was supplemented as follows: 1) control group (α-MEM-FCS as a culture medium with no added substances), 2) VEGF-A 100 ng/ml (recombinant human VEGF165 produced in Spodoptera frugiperda, Calbiochem), 3) endostatin 2 µg/ml (recombinant human endostatin polypeptide produced in Pichia pastoris, Calbiochem), 4) endostatin 20 µg/ml, 5) VEGF-A + endostatin 2 µg/ml and 6) VEGF-A + endostatin 20 µg/ml. The cells were cultured at +37 °C (5% CO2,
95% air) and the medium freshly supplemented with VEGF-A, endostatin or VEGF-A and endostatin together was replaced after 1, 3, 7 and 10 days of culture. Each experiment was repeated at least twice.

4.4 Histology, histomorphometry and immunohistochemistry (I, III, IV)

For histological analysis to evaluate bone defect healing, the femurs were fixed in phosphate-buffered 4% paraformaldehyde and decalcified in 10% ethylenediaminetetraacetic acid (EDTA) (I). For evaluation of ectopic new bone, the collected specimens were fixed in 10% neutral formalin solution and decalcified in 5% formic acid. After decalcification, the bones were embedded in paraffin (III). When studying transgenic mouse lines (IV), the bone specimens were fixed in 70% ethanol, decalcified and embedded in paraffin.

4.4.1 Haematoxylin-eosin (HE) staining (I, III, IV)

The decalcified bones were cut into 5-µm tissue sections and stained with hematoxylin and eosin. Morphological analysis was performed with a digital image analysis system MCIDM4 (Imaging Research Inc., St Catharines, Canada) using a Nikon Optiphot II microscope and a Sony DXC-930P color camera.

Study (I): The histology of the tissue was analyzed in order to demonstrate differences in healing between the VEGF-treated and control femurs. The proportion of fibrous reparative tissue was measured histomorphometrically from the cross sections of the defect area 1, 2 or 4 weeks after the procedure. The amount of cartilage at the periosteal rim of the defect was also measured 1 and 2 weeks after the procedure.

Study (III): Evaluation of ectopic new bone was based on the proportional area of remaining cartilage in the ossicle 2 and 3 weeks after the injection determined with morphometrical analysis. The ossicle was defined as the tissue surrounded by fibrous capsule and containing a cortical outer bone shell and developing bone marrow around the remaining endochondral cartilage.

Study (IV): Femur samples were studied for the analysis of secondary ossification centers in 2 and 4 week old Col18a1−/−, C57BL/6J, ES-tg and FVB/N mice.
4.4.2 Immunostaining of capillaries by FVIII (I, III)

The VEGF (I, III) and endostatin (III) effect in bone was demonstrated by immunostaining of capillaries using a polyclonal rabbit anti-human antibody to FVIII-related antigen (Dako, Dakopatts, Denmark). The primary antibody was applied on the slides for 1 h (dilution of 1 : 50) followed by a secondary anti-rabbit antibody (Dako), after which the ABC-complex was applied. The color was developed with diaminobenzidine. In study (I) the vascular organization was evaluated and density was estimated as the number of positively stained blood vessels per high power field (HPF). In each section, a minimum of five HPFs was analyzed. In study (III) morphometrical analyses of the factor VIII positive blood vessels were done from three microscopic fields (digital image analysis system MCID M4 using a Nikon Optiphot II microscope and a Sony DXC- 930P color camera) of the highest vessel density per section in a continuous fashion. The numbers of the positively stained blood vessels were counted as proportional area per microscopic field.

4.4.3 Collagen XVIII antibodies (IV)

Paraffin-embedded 5 μm sections were dewaxed, washed in PBS and stained with collagen XVIII antibodies using a tyramide signal amplification (TSA) kit (PerkinElmer). The following antibodies were used in immunohistochemistry: a polyclonal rabbit-anti mouse antibody recognizing the N-terminal part of type XVIII collagen (anti-all moXVIII) and a polyclonal rabbit-anti mouse antibody recognizing the N-terminal part of the two longer variants of type XVIII collagen (anti-long moXVIII) (Saarela et al., unpublished data) Heat-induced epitope retrieval was carried out in citrate buffer (10mM sodium citrate, 0.05% Tween, pH 6.0) for 10 min in a microwave oven. The slides were then incubated overnight at + 4 °C with either anti-all moXVIII (4 μg/ml) or anti-long moXVIII (10 μg/ml) antibody, diluted in TNB buffer (0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl, 0.5% Blocking Buffer). This was followed by a 30-minute incubation with a biotinylated secondary anti-rabbit antibody (Vector Laboratories) at room temperature. Counterstaining was performed with haematoxylin.
4.4.4 TRACP staining for identifying osteoclasts (II, III)

The cells were stained for tartrate-resistant acid phosphatase (TRACP), a commonly accepted marker of osteoclasts (Minkin, calcify tissue int 1982) using a Sigma TRACP kit (no. 386-A, Sigma Chemicals, St.Louis, MO) according to the manufacturer’s instructions. To visualize the nuclei, the cells were incubated with the DNA-binding fluorochrome Hoechst 33258 (1 mg/ml stock diluted 1:800 in PBS, Sigma Chemical Co, St. Louis, MO) for 10 minutes at room temperature.

Study (II): The numbers of multinucleated TRACP-positive cells on each bone slice were counted, using a Nikon Eclipse 600 microscope and Nikon Plan Fluor 20x/0.50 objective with an appropriate filterset.

Study (III): The numbers of the multinucleated TRACP-positive cells in contact with cartilage or newly formed bone trabeculae were counted from five microscope fields using a Nikon Eclipse 600 microscope with a 40x/0.50 objective with an appropriate filterset.

4.4.5 Area Measurement of Excavated Pits (II)

Prior to the staining of the pits, total detachment of the cells from the bone slices was ensured by wiping the cellular surface of the slices with a soft brush. The pits were stained with peroxidase-conjugated wheat germ agglutinin-lectin (WGA; 20 μg/ml, Sigma Chemical Co., St.Louis, MO) for 20 minutes, washed with PBS, and incubated for 5 minutes in diaminobenzidine (0.5 mg/ml) +0.03% H2O2. Resorption pits were visualized as brown-colored areas after staining with WGA. Morphometric analysis of the resorption pits was performed with an MCID image analyzer, utilizing M2 software (Imaging Research Inc., Brock University, Ontario, Canada) to the quantify areas of the resorption lacunae.

4.5 Biological assays

4.5.1 Determination of cell proliferation (WST-1) (IV)

MC3T3-E1 cell proliferation was measured by a WST-1 assay (Roche, Germany), which quantifies the reduction of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells. Analyses were performed 3, 7, 10 and 14 days after the cells were plated. WST-1 reagent (20μl) and α-MEM with ascorbic and β-glycerophosphate (200 μl) were added to the cells and the culture incubated at
+37°C (5% CO2, 95% air) for 1 hour. The absorbance of the reaction product was measured with a 1420 Victor2 Multilabel counter (Wallac, Turku, Finland) at a wavelength of 450 nm.

4.5.2 Alizarin red S staining and quantification of calcium deposition (IV)

The mineralization of matrix and nodules was determined using Alizarin red S staining. Briefly, MC3T3-E1 cell cultures were fixed at culture day 14 with 3% PFA/2% sucrose in PBS for 10 minutes at room temperature, washed with PBS and stained for 30 min with a 1% solution of Alizarin red S (Amresco, USA) pH 4.2 at room temperature with rotation. The cultures were washed three times with deionized water and once with 70% ethanol before the addition of 100 nM cetylpyridinium chloride (Sigma) for 1 hour to solubilize and release calcium-bound Alizarin red into the solution. The solution was collected and the Alizarin red S concentration was determined by absorbance measurements at 570 nm using an Alizarin red S standard curve in the same solution.

4.6 Radiographical analysis

4.6.1 Radiographic evaluation of bone formation (III)

Standard lateral position radiographs (100 mA, 20 kV, 0.08 s/exp; Mamex de Maq, Soredex, Orion, Finland) were taken from all the hind legs of the mice. Calibration of the equipment for the measurement of optical density was performed using an aluminum wedge. New bone formation was evaluated as the area (in mm²) of calcified tissue visible in the radiographs with a digital image analysis system MCID M4 (Imaging Research Inc., St Catharines, Canada). The radiographs were digitized on a light table with a ccd camera (Dage MTI 72E, Dage-MTI, Inc., Michigan City, IN, USA) using a Micro Nikkor 55mm objective (Nikon, Tokyo, Japan).
4.6.2 Peripheral quantitative computed tomography (pQCT) (I, IV)

Bones were scanned with a Stratec XCT 960 A peripheral quantitative computed tomography (pQCT) system with software version 5.21 (Norland Stratec Medizintechnic GmbH, Birkenfeld, Germany).

Study (I): The bone mineral content of the femurs treated with AdVEGFAor AdlacZ was analyzed at 1, 2, and 4 weeks after the operation. Distal femurs were scanned for three to five consecutive cross-sectional images with slice distances of 0.5 mm. Bones were scanned with a voxel size of $0.148 \times 0.148 \times 1.25 \text{ mm}^3$. A slice representing the central area of the defect was chosen for analysis, and the total bone mineral content (BMC, mg/mm) was recorded.

Study (IV): To evaluate the effects of endostatin in vivo, a voxel size of $0.148 \times 0.148 \times 1.25 \text{ mm}^3$ (transgenic mice overexpressing endostatin and FVB/N control mice) and $0.092 \times 0.092 \times 1.25 \text{ mm}^3$ (mice deficient in collagen XVIII (Col18a1/-) and C57BL/6J control mice) were used for the measurements. The scout view of the pQCT system determined the middle point of each bone, from which one cross-sectional slice was scanned and the total mineral content and density measured.

4.6.3 Faxitron X-ray imaging (IV)

Radiographs of the dissected bones were taken in anteroposterior (AP) and lateral projection on a Faxitron Specimen Radiography System (Model MX-20, Wheeling, IL, USA) with a high-resolution algorithm. The imaging parameters were identical for all the specimens and the following settings were used: 6 s, 30 kV for the 30 day old mice and 20 kV for the 14 day old mice, pixel matrix $1024 \times 1054$; resolution 200 pixels mm$^{-1}$. The images were transferred to a personal computer for analysis. The lengths and areas of tibias and femurs were measured from the radiographs. As the weight of the mice varied from litter to litter in both mutant and control mice, we normalized the bone density data by the length and area of the mouse bones measured from the Faxitron images.

4.7 Statistical analysis (I-IV)

The values are the mean ± SEM. Significance was calculated using one-way ANOVA followed by Student’s t-test with the statistical package Origin 6.0 or 7.5. (Microcal Software Inc., Northampton, MA)
5 Results

5.1 Bone defect healing model (I)

5.1.1 Bone mineral content evaluated with peripheral quantitative computed tomography (pQCT) (I)

The bone mineral content (BMC) at the injury site was analyzed by peripheral quantitative computed tomography (pQCT) revealing a significant increase in BMC in the VEGF-treated femurs (13.9 mg/mm vs. 12.1 mg/mm, difference 14.9%, $p < 0.001$) 2 weeks after the operation. At the 1-week and 4 week time points, there was no statistically significant difference between the two sides.

5.1.2 Induction of vascularization (I)

The number of capillaries was significantly increased at the 1-week time point only (5.5 in control group vs. 7.5 in VEGF group, 36.4% difference, $p < 0.01$), after which the difference was no longer significant.

5.1.3 Histological evaluation (I)

The histology of the tissue sections was analyzed in order to demonstrate differences in healing between the VEGF-treated and the control femurs. One week after the procedure (Figures 6A and 6B), both sides still had large hematomas with surrounding seams of mesenchymal stem cells. The morphological difference in the amount of non-ossified callus is most obviously seen at the 2-week time point (Figures 6C and 6D), indicating amplified ossification in the VEGF-treated femurs. The proportion of fibrous tissue representing residual defect was significantly smaller in the VEGF group 2 weeks after the operation (19.5% in VEGF group vs. 31.0% in control group, 59.0% difference, $p < 0.05$). At the 4-week time point (Figures 6E and 6F), the defect was difficult to localize due to almost complete healing and little difference was seen between the VEGF overexpression and the control sides.
Fig. 6. Example of histology of bone regeneration in the drilling defects in the VEGF-treated (right panel) and the control femurs (left panel) 1 (A, B), 2 (C, D), and 4 weeks (E, F) after the operation. HE-stained decalcified sections. Scale bar: 1mm.

Endochondral ossification took place at the periosteal rim of the drilling defect whereas direct ossification occurred at the central part of the healing metaphyseal defect. The amount of hyaline cartilage was high at the early phases of healing,
but the VEGF treatment stimulated rapid replacement of the cartilage with mature bone. At 2 weeks, the amount of cartilage was significantly lower in the VEGF group compared with the control (0.015 mm² vs. 0.065 mm², 3.3-fold difference, \( p < 0.01 \)). At 4 weeks, no cartilage was detected in the defect area. The data support the hypothesis that VEGF overexpression significantly enhanced the endochondral ossification (Figure 7).

Fig. 7. Examples of cartilage at the periosteal defect side. The control defect 1 week after the operation (A) had a lower amount of cartilage compared with the VEGF-treated femur (B). At 2 weeks, the amount of cartilage was significantly higher in the control (C) compared with the VEGF group (D). Hematoxylin-eosin staining of paraffin-embedded tissue. Scale bar 50 µm.
5.2 Effects of VEGF-A and endostatin on osteoclastic bone resorption and differentiation \textit{in vitro} (II)

5.2.1 Bone resorption assay (II)

The number of osteoclasts in each sample group was counted by analyzing multinuclear TRACP positive cells in the resorption assay. No significant differences in the number osteoclasts were found between the different groups. The total resorbed area was significantly increased in response to VEGF-A treatment and the effect was dose dependent. Endostatin alone had no inhibitory effect on the resorbed area when compared to the basal level of osteoclastic bone resorption. When both endostatin and VEGF-A were supplemented, the stimulatory effect by VEGF-A on bone resorption was limited to the basal level. The resorption activity was even below basal level with the highest concentrations ($p < 0.05$ in ctrl vs. VEGF100 and endostatin). Thus, endostatin could block the VEGF-A-induced stimulatory effect on osteoclastic bone resorption.

5.2.2 Osteoclast differentiation (II)

VEGF-A was added to the medium containing M-CSF and RANKL in order to achieve the maximal differentiation stimulus. Endostatin inhibited the maximal osteoclastogenesis alone or in combination with VEGF.

5.3 Endochondral ossification model (III)

5.3.1 Radiographic evaluation of bone formation (III)

The effect of the recombinant adenoviral endostatin gene transfer with or without VEGF-A in bone formation was first evaluated from radiographs. X-ray images of the dissected legs displayed significant bone formation in all treatment groups at the 2- and 3-week time points. Two weeks after the injection, there were no significant differences between the study groups. At the 3-week time point, however, we could see retardation of bone formation in radiographs of the endostatin-treated animals compared to the VEGFA- treated and the control
groups. In addition, the retardation was clearly visible in the animals that received a combination of VEGF-A and endostatin.

5.3.2 Induction of vascularization (III)

The samples were processed for immunohistochemistry in order to demonstrate vascular structures positive for the FVIII-related antigen. At the 1-week time point, previously existing vessels (Figures 8A and 8G) were easily identified due to the well-defined endothelium. At all time points, endothelial staining at the bone formation site was increased in the VEGF-A group compared to the control. After 2 and 3 weeks, endostatin blunted the VEGF-A effect. Endostatin alone, however, did not decrease the basal neovascularization (Figure 8). At the 2- and 3-week time points, staining for the factor VIII-related antigen indicated intense and partially premature vascularization in the VEGF-A-stimulated ossification. The new vessels were morphologically more mature in the endostatin group compared to the VEGF-A stimulated angiogenesis.
Fig. 8. Bones were decalcified, cut into tissue sections, and immunostained for FVIII-related antigen. The figure shows examples of BMP control (A–C), VEGF-A (D–F), endostatin (G–I) and VEGF-A+endostatin (J–L) treated ossicles 1 week (A, D, G, J), 2 weeks (B, E, H, K) and 3 weeks (C, F, I, L) after the injection. Pre-existing vessels were nicely stained all over the induction site. Examples of these old vessels are seen in (A) and (C). Scale bar: 100 μm.

5.3.3 Bone histology (III)

The histology of the tissue sections was analyzed in order to demonstrate differences in endochondral bone development. At the 2-week time point, there was no difference in the proportional ossification of the cartilage phase. Significant differences were seen in the area of cartilage remnants of the bone nodules in different test groups 3 weeks after the injection. We could demonstrate that endostatin inhibited cartilage resorption compared to control and VEGF-A-
treated animals. The proportional area of bone was lower in the endostatin group compared to the control (Figure 9).

Fig. 9. (A) Example of new bone formation induced by BMP injection. Ectopic new bone was evaluated as a proportional area of bone tissue/osseous nodule 2 and 3 weeks after the injection. (Arrow indicates the cortical shell of the ossicle and * indicates cartilage). (B) At the 3-week time point endostatin inhibited the proportional area of bone tissue. At 1 or 2 weeks, no difference in proportional area of bone tissue/ossicle was seen. **p < 0.01. Scale bar: 200 μm.
5.3.4 The number of cartilage-resorbing chondroclasts (III)

Cartilage resorption was studied by identifying the number of TRACP-positive chondroclasts/osteoclasts. VEGF-A alone stimulated osteoclasts as demonstrated by the increased number of TRACP-positive cells 3 weeks after the operation. Endostatin gene transfer decreased the amount of TRACP-positive cells compared to the VEGF-A group at the 2- and 3-week time points. Endostatin also decreased the VEGF-A stimulated osteoclastogenesis at the 2-week time point.

5.4 The effects of endostatin on the MC3T3-E1 osteoblastic cell line

5.4.1 Osteoblast proliferation in vitro

MC3T3-E1 cell proliferation was measured by a WST-1 assay. On days 3–10 there were no statistical differences between the groups. On day 14 of culture endostatin 20 μg/ml decreased osteoblast proliferation compared to the basal control level ($p < 0.05$) and VEGF-A group ($p < 0.05$). Endostatin 2 μg/ml also decreased osteoblast proliferation, but not significantly. Endostatin 2 μg/ml together with VEGF-A decreased osteoblast proliferation compared to the basal level ($p < 0.001$), VEGF-A ($p < 0.001$) and endostatin 2 μg/ml ($p < 0.05$). Interestingly, VEGF-A together with the higher dose of endostatin, 20 μg/ml did not significantly affect proliferation.

5.4.2 Matrix mineralization in vitro

The immature osteoblast cell line, MC3T3-E1, up-regulates osteoblast-specific genes and produces mineralized nodules undergoing the developmental stages associated with differentiating osteoblasts. The process of mineralization is initiated around day 10. A low dose of endostatin 2 μg/ml together with VEGF-A decreased the mineralisation compared either to the basal control level, VEGF-A or endostatin alone ($p < 0.05$). Endostatin 20 μg/ml together with VEGF-A does not significantly affect mineralization when compared to the basal control level, but if compared to treatment with VEGF-A alone, a decrease in matrix mineralization can be observed ($p < 0.05$).
5.5 Transgenic mice and knockout mice

5.5.1 pQCT and faxitron results

The bone mineral content or density, as analyzed with pQCT, was not altered in ES-tg or Col18a1⁺⁻ mice as compared to their FVB/N or C57BL/6J controls. Moreover, there were no significant differences in bone sizes between the Col18a1⁻⁻ mice and their controls. The lengths of tibias and femurs were measured from radiographs. In the ES-tg mice the lengths of femurs and tibias were smaller at the age of 14 days, compared to FVB/N controls ($p < 0.01$). This delay of growth was transient, as no difference was seen at 30 days of age.

5.5.2 The formation of secondary ossification centers

In the bones of 14-day-old Col18a1⁻⁻ mice we detected a delay in the formation of secondary ossification centers. In all C57BL/6J mice the secondary ossification centers were already clearly visible in the epiphyseal areas of femurs. However, in Col18a1⁻⁻ mice the secondary ossification centers were either missing, or very small (Figure 10). At the age of one month, no clear differences could be observed between the knock-out and control mice: in both, the ossification of the epiphyses had occurred. The growth plate was still visible in both mouse lines at this age. In ES-tg mice we could see no difference in the ossification of the epiphyseal area as compared to their FVB/N controls.
In 2-week-old C57BL/6J control mouse femurs (A) the formation of secondary ossification centers is clearly visible in the epiphyseal areas, whereas in the Col18a1-/- mutants (B) the secondary ossification centers are either very small, or, as in the case of some samples, totally absent at this timepoint. In 2-week-old FVB/N (C) and ES-tg (D) mice the secondary ossification centers are well developed, and no obvious differences can be detected between these two mouse lines.

5.5.3 Localization of collagen XVIII

Immunohistochemistry was used to study the localization of collagen XVIII in bone tissue. Collagen XVIII was localised in the blood vessels of bone marrow: staining was seen with the antibody against all three variants (anti-all moXVIII). The short variant of collagen XVIII was responsible for this immunosignal, as the antibody that stains the two longer variants of collagen XVIII (anti-long moXVIII) did not stain the vessels (Figure 11). Staining with collagen XVIII antibodies also gave a signal in the epiphyseal cartilage, surrounding the chondrocytes, but this signal proved to be unspecific as similar staining could be seen in cartilage of mice lacking collagen XVIII.
Fig. 11. Collagen XVIII was localized in the central vessels and some smaller vessels of the bone marrow (A and B) and vessels of the periosteum (C) and at the skeletal muscle attachment site (D). As expected, the collagen XVIII KO used as negative controls showed no staining (E and F). The samples are from 14-day-old mice.
6 Discussion

6.1 Adenoviral VEGF-A gene transfer induces angiogenesis and promotes bone formation in healing osseous tissues (I)

Angiogenesis has a crucial role in bone healing. VEGF is expressed within the first weeks of bone healing (Uchia et al. 2003) and it may enhance osteoblast differentiation (Deckers et al. 2000). Pufe and colleagues have shown that VEGF is expressed in fracture callus at the beginning of fracture healing after which expression decreases by day 5 (Pufe et al. 2002). We studied the effect of VEGF-A on bone healing and the feasibility of first–generation adenoviral vector to deliver VEGF overexpression at the bone injury site.

The exogenous VEGF protein is an unstable, short-lived protein in vivo and moreover, costly to produce (Keramaris et al. 2008). The use of gene therapy for bone regeneration enables delivery of VEGF at physiological levels using natural cellular mechanisms. In order to achieve fracture healing, the required therapeutic level of VEGF must be maintained for a period of time. A first-generation adenoviral vector typically produces expression that lasts for 1–2 weeks in vivo, after which the virus may be eliminated by the immune response (Cao et al. 2002, Ylä-Herttuala et al. 2000, Okubo et al. 2001). The rodent model is useful for studying bone healing properties, since rodent bone healing is a very rapid process. Bone regeneration occurs within 4 weeks even in the absence of interventions (Uusitalo et al. 2001).

In this study, the defects were healed finally in both groups, but the healing process was accelerated in the VEGF treated group. In order to monitor bone recovery, we first measured the bone mineral content (BMC) using peripheral quantitative computed tomography (pQCT). A number of studies have used pQCT in mice and rats to observe cortical bone geometry along with cortical and trabecular bone mineral density (BMD). This imaging method has been used to monitor such changes after pharmacologic or mechanical interventions and to monitor fracture healing (Breen et al. 1998, Armamento-Villareal et al. 2005, Gasser et al. 2008, McCann et al. 2008). Locally VEGF-A overexpression increased the bone mineral content measured with (pQCT) 2 weeks after the operation. At the 1-week time point, there was as yet no statistically significant difference between the two sides and at the 4-week time point, the mineral
content of both VEGF-treated and control bones was decreased due to the reorganization of the callus at the injury location.

Endochondral ossification took place at the periosteal rim of the drilling defect whereas intramembranous ossification occurred at the central part of the healing metaphyseal defect. The importance of angiogenesis and VEGF on endochondral ossification has been pointed out in the study of Gerber and colleagues (Gerber et al. 1999). Increased vasculature stimulates intramembranous ossification as well (Collin-Osdoby 1994, Zelzer et al. 2002, Horner et al. 1999).

Factor VIII expression is normally restricted to vascular endothelial cells and therefore Factor VIII immunostaining is considered to be a reliable method to detect blood vessels in dense connective tissue. In the present study, the number of FVIII-related capillaries was significantly increased at the 1-week time point providing increased blood supply to the healing bone and accelerated healing of the defect. At 2 or 4 weeks, the difference could not be seen anymore. This could be due to VEGF production at the early phases of bone healing and viral expression may shut down soon after the virus delivery. Furthermore, skeletal muscle is not an ideal target tissue for the recombinant adenoviral vector due to the low number of viral receptors, the quiescence of myoblasts in mature muscle, and physical barriers (Cao et al. 2002, Nalbantoglu et al. 2001). Our results thus support previous results that increased vasculature also stimulates intramembranous ossification (Collin-Osdoby 1994, Zelzer et al. 2002). The capillaries were more disorganized in the VEGF-A stimulated condition compared with the control. The induction of disorganized vascularization indicates that the viral gene transfer resulted in increased VEGF-A activity. It could be possible to adjust VEGF dosing with other growth factors to reduce this phenomenon.

In this study, a first-generation adenoviral vector produced VEGF concentrations with biological effects on bone metabolism and was able to modify the histological features of bone healing. The morphological difference in the amount of non-ossified callus was most obviously seen at the 2-week time point, where the proportion of fibrous reparative tissue was more efficiently cleared and replaced with bone in the VEGF-A group, indicating amplified ossification in the VEGF-A treated femurs. We also measured the amount of hyaline cartilage at the periosteal rim, and at the early phases of healing the amount of cartilage was high. However, VEGF treatment stimulated rapid replacement of the cartilage with mature bone. At the 4-weeks time point the
defect was difficult to localize due to almost complete healing, and only little differences were seen between groups.

6.2 Endostatin inhibits VEGF-A induced osteoclastic bone resorption in vitro (II)

The intimate contact of the bone remodeling site with vascular sinusoids indicates interplay between the vascular endothelium and the bone cells. A continuous supply of bone-degrading osteoclasts is needed during bone development. Osteoclasts are derived from hematopoietic precursors present in both the bone marrow and the peripheral circulation (Roodman 1999). Osteoclast precursors need to adhere to and migrate through endothelium to reach future sites of bone resorption. It has been reported that VEGF is potentially a monocyte chemoattractant (Clauss et al. 1996). Monocytes have been shown to express VEGFR1, but not VEGFR2 (Barleon et al. 1996). Soluble Flt-1 blocks invasion of osteoclasts into the marrow cavity of developing long bones in vivo (Niida et al. 1999). Mature osteoclasts express VEGFR1 and VEGFR2 on the cell surface (Gerber et al. 1999, Niida et al. 1999, Nakagawa et al. 2000). Endostatin is a known antagonist of VEGF-mediated endothelial cell migration and proliferation (Yamaguchi et al. 1999, Taddei et al. 1999). Interestingly, the recruitment and invasion of osteoclast precursors can be disturbed by endostatin in the metatarsal model, in which fusion and migration of osteoclast precursors is required for osteoclastic bone resorption (Henriksen et al. 2003).

Only little is known about the effects of endostatin on other cells and thus we investigated the possibility that VEGF and endostatin may control osteoclastogenesis and osteoclastic bone resorption in vitro. Endostatin has been shown to bind αv- and α5-integrins (Rehn et al. 2001), and resorbing osteoclasts express at least αv-integrins (Chuntharapai et al. 1993, Duong et al. 2000). Therefore it is possible that endostatin could control osteoclast function.

We used a classical resorption pit assay and we analysed the number of osteoclasts by counting the multinuclear TRACP-positive cells. However significant differences between the different groups were not detected. Next we measured osteoclastic bone resorption. In response to VEGF-A treatment the total resorbed area was significantly increased and the response was dose dependent. Endostatin alone had no inhibitory effect on the resorbed area when compared to the basal level of osteoclastic bone resorption, but when both endostatin and VEGF-A were present, the stimulatory effect by VEGF-A was reduced to the
basal level. Our finding concerning the increased resorption achieved by VEGF is consistent with the result of Nakagawa and colleagues (Nakagawa et al. 2000). However, they demonstrated that VEGF-A enhanced the survival of pure rabbit osteoclasts, although in our resorption pit assay containing osteoblasts and osteoclasts, the number of TRACP-positive cells remained unchanged. The data show that the increased total resorbed area in our model is not due to enhanced survival rate. The observed effect is more likely a result of a direct VEGF-A stimulation of osteoclastic bone resorption.

Since the basal resorption was not affected by endostatin in the pit assay, we investigated whether endostatin could inhibit the differentiation of osteoclasts. Bone marrow hematopoietic cells differentiate into osteoclasts in the presence of RANKL and M-CSF. When VEGF-A was added to the medium containing M-CSF and RANKL, endostatin inhibited osteoclastogenesis compared to the VEGF induced maximal stimulus. Interestingly, we could see the inhibitory effect also in the absence of VEGF-A.

It seems, however, that the regulatory effect of endostatin is not critical since endostatin alone cannot modify the basal bone resorption. In the study of Henriksen and colleagues, endostatin completely inhibited VEGF mediated osteoclast invasion but had no effect on RANKL or M-CSF induced migration. In cultures of purified osteoclasts both RANKL and VEGF induced phosphorylation of ERK1/2 MAP kinase (Henriksen et al. 2003). It is possible that endostatin may not only limit the migration of osteoclast precursors, it may also inhibit VEGF-A-induced osteoclast activation. Pufe and colleagues have detected a similar influence of endostatin on hyaline articular cartilage. Endostatin alone had no effect on the basal levels of the phosphorylation of ERK1/2, whereas co-incubation with endostatin blocked VEGF-induced ERK1/2 phosphorylation in immortalized human chondrocytes (C28/I2) (Pufe et al. 2004c, Pufè et al. 2004d).

6.3 Endostatin inhibits endochondral ossification (III)

Angiogenesis is essential for the replacement of cartilage by bone during skeletal growth and regeneration as well as on ectopic ossification. The function of endostatins during the angiogenesis process of bone formation is quite unknown. In our previous studies we observed that VEGF-A stimulates osteoclasts and osteoclastogenesis thus regulating the duration of the cartilage phase in this process. In addition, endostatin inhibited osteoclastogenesis and osteoclast
function in vitro indicating that endostatin may coordinate angiogenesis and ossification in vivo.

It is hypothesized that endostatin expression by mature cartilage may suppress osteoclasts while endostatin-deficient ossifying cartilage allows osteoclastic activity and endochondral ossification (Pufe et al. 2004c). Moreover, it has been suggested that endostatin has a homeostatic function in cartilage metabolism (Feng et al. 2005). Recently, endostatin has been found to repress the development and volume of tissue grafts in mice with rheumatoid arthritis, which is characterized by an inflammatory erosive synovitis, where invasion of inflammatory cells into the joint synovium is accompanied by excessive angiogenesis causing destruction of the cartilage and fibrous tissue (Matsuno et al. 2002, Yin et al. 2002).

Since the role of endostatin in the presence of existing stem cells in vivo requires further exploration, we employed an endochondral ossification model in the mouse hind muscle pouch stimulated by BMPs. Endostatin was overexpressed with or without VEGF-A using a recombinant adenoviral vector. BMPs have been used to heal bone defects in experimental animal tests, but also in clinical settings, mainly in orthopedic and oral surgery (Pekkarinen et al. 2005).

The muscle pouch model of mouse has been a standard method for studying the activity of BMP since its discovery and has been shown to be an appropriate animal model (Sampath & Reddi 1981). In this study, we used injectable BMP implants, which require only a minimally invasive technique. Previously, they have been investigated in many animal experiments with promising results (Forslund et al. 1998, Bax et al. 1999, Muschik et al. 2000, Blokhuis et al. 2001, Li et al. 2002). The native reindeer BMP extract we used (Pekkarinen et al. 2003) contains a cocktail of purified proteins consisting of multiple BMPs and other non-BMP proteins combined with gelatin gel (Aldinger et al. 1991, Iwata et al. 2002, Jortikka et al. 1993a, Jortikka et al. 1993b). Although individual rhBMPs are able to induce ectopic new bone formation, purified BMP extract from human matrix has been demonstrated to produce new bone more efficiently than rhBMP-2 (Bessho et al. 1999). This suggests that the effect of native BMP extract represents synergistic activity between different BMP proteins.

We observed strong ectopic bone formation in all our study groups that was induced with BMP extract combined with gelatin gel in conjugation with the viruses. First we evaluated the new bone formation from the X-ray images taken from the dissected legs at 2- and 3-week time points. At the 1-week time point, the mineralization of cartilage had not yet started. At 2 weeks, the difference
between the study groups was not significant, but we observed less bone formation in endostatin-treated animals at the 3 weeks time point. Contrary to our previous finding with the bone drilling defect model, VEGF overexpression did not enhance bone formation. This may indicate that the surrounding tissue may have regulatory properties that control ossification.

Peng and others have described a synergistic enhancement of VEGF and BMP-4 or BMP-2 on bone formation (Peng et al. 2002, Peng et al. 2005). However, VEGF alone did not promote bone regeneration when using a model where the healing of cortical defects in the parietal bones of mice was studied. They also reported that the beneficial effect of VEGF on bone healing elicited by BMP-4 depends critically on the ratio of VEGF to BMP4, with an improper ratio leading to unfavorable effects on bone healing, while disruption of the BMP-2/VEGF ratio has less negative effects on bone formation (Peng et al. 2002).

Furthermore, Kakudo and others reported an increased amount of VEGF induced ectopic bone formation seen in X-rays. They implanted human recombinant BMP-2 in rat calf muscle using a disc implant (Kakudo et al. 2006). Thus it is possible that species differences and the mode of application of the BMP and VEGF/BMP ratio influence bone formation. Moreover, Murphy and colleagues suggested that the increased vascularization achieved with VEGF enhances mineralized tissue generation, but not necessarily osteoid formation when using VEGF with the bio-mineralized scaffold compared to mineralized scaffold alone (Murphy et al. 2004).

In this study, we could not see increased endochondral bone formation caused by VEGF-A overexpression in the mesenchyme of the muscular space indicating that the amount of BMP determines the quantity of bone to be formed at the ectopic site. VEGF-A however, stimulated the number of cartilage resorbing osteoclasts and the number of FVIII-related antigen-positive blood vessels thus accelerating the endochondral phase. Endostatin overexpression alone or in combination with VEGF-A decreased the number of TRACP-positive cells compared to animals with VEGF-A overexpression at 2- and 3-week time points suggesting that cartilage resorption by chondroclasts is decreased in response to endostatin even in the presence of VEGF-A. These results are in line with our previous results showing that endostatin inhibits VEGF-A-stimulated osteoclastic bone resorption in vitro independent of VEGF-A. In addition, as expected, endostatin prevented the formation of vessels and thus inhibition of vascular development. Endostatin could reduce the amount of BMP-induced bone
formation, since cartilage resorption and osteoblast function are dependent on blood vessel formation.

6.4 Endostatin inhibits proliferation of MC3T3-E1 osteoblasts and together with VEGF-A suppress mineralization (IV)

We investigated how endostatin affects the behaviour of osteoblasts. A number of studies have pointed out the significant role of canonical Wnt signaling in the control of osteoblastogenesis and bone formation (Yavropoulou & Yovos 2007) and recently it was reported that endostatin is a potent inhibitor of Wnt signaling (Hanai et al. 2002). Thus, endostatin may have a role in the anabolic effect of bone. Mechanical loading generates interstitial fluid flow that exerts a shear stress at surfaces of osteoblasts and osteocytes. The canonical Wnt pathway is one of the pathways that is up-regulated in response to the fluid shear stress, thus translating fluid shear signals into biological effects in bone cells. Endostatin can prevent the effect of fluid shear stress on osteoblasts completely (Lau et al. 2006).

In the present study we used MC3T3-E1 cells, which behave as immature, committed osteoblastic cells and go on to differentiate in response to intracellular and extracellular cues. We measured MC3T3-E1 cell proliferation using a WST-1 assay and the mineralization of matrix and bone nodules was determined using Alizarin red S staining. VEGF alone had no effect on MC3T3-E1 cell proliferation, nor a significant effect on mineralization, which is consistent with previous findings (Kitagawa et al. 2005). Proliferative effects of VEGF, at least in endothelial cells, are mediated by Flk-1/KDR (Guo et al. 1995, Quinn et al. 1993), that is not expressed by MC3T3-E1 cells, which could explain why VEGF did not have a proliferative effect on these cells. Kitagawa and others suggested that since VEGF has earlier been shown to induce markers of osteoblast differentiation, e.g. alkaline phosphatase and osteocalcin expression, but not to increase mineralization, other factors are needed in addition to VEGF for the induction of mineralization (Kitagawa et al. 2005). However, treatment of cells with endostatin alone decreased the proliferation of osteoblasts. Endostatin 20 μg/ml decreased osteoblast proliferation on day 14 of culture, and endostatin 2 μg/ml also slightly decreased osteoblast proliferation but this difference was not statistically significant. The addition of endostatin alone did not affect mineralization either.

Interestingly, again we could see the highest inhibitory effect of endostatin when it was used together with VEGF. A low dose of endostatin (2 μg/ml)
together with VEGF decreased osteoblast proliferation compared to the basal level, VEGF-A, and endostatin 2 μg/ml groups. Likewise, matrix mineralization was inhibited when a low dose endostatin (2 μg/ml) added together with VEGF-A was used compared to control, VEGF-A, or endostatin groups. With a higher concentration of endostatin (20 μg/ml) combined together with VEGF-A, this inhibitory effect could not be seen. It is not known why the inhibitory effect of endostatin is highest when it is combined with VEGF. The concentration of endostatin may be crucial for obtaining a certain effect. A recent report indicates that the doses of endostatin required for meaningful effects in vitro and in animal models vary over a wide scale (Sun et al. 2009). It has also been shown, that endostatin’s effects on angiogenesis and tumor growth are biphasic, operating over a U-shaped curve (Celik et al. 2005).

Endostatin has been shown to block the interaction of VEGF with both Flt-1 and Flk-1/KDR receptors (Kim et al. 2002). Recently endostatin has been found to bind to VEGFR3 (Kojima et al. 2008), which has a role in osteoblast differentiation (Orlandini et al. 2006). Furthermore, endostatin has been shown to affect the VEGF-mediated signaling pathway (Kim et al. 2002) and also ERK1/2 signaling (Schmidt et al. 2006). Since MC3T3-E1 cells do not express Flk-1/KDR receptors, endostatin can only interact with the Flt-1 or VEGFR3, and VEGF-A only with the Flt-1 receptor and therefore more endostatin peptides may be available for binding to VEGFR3.

6.5 The role of collagen XVIII/endostatin on bone development in vivo in transgenic mice overexpressing endostatin and mice lacking collagen XVIII

Our previous studies and those of others, have suggested that endostatin seems to have a number of effects on bone and therefore, we wanted to further explore its role in bone development in vivo using transgenic mice overexpressing endostatin (ES-tg) and mice lacking collagen XVIII (Col18a1−/−). However, it has been reported that collagen XVIII/endostatin does not have a role as a critical negative regulator of angiogenesis during development and postnatal growth, since a lack of collagen XVIII and endostatin in mice and humans does not increase angiogenesis in major organs (Marneros & Olsen 2005).

In this study, we detected a delay in the formation of secondary ossification centers in the bones of 14-day-old Col18a1−/− mice. At the age of one month the ossification of the epiphyses had already occurred and clear differences were not
observed. Delayed ossification was also not detected at embryonic stages E15.5 and E18.5. In ES-tg mice no difference in the ossification of the epiphyseal area could not be seen. Moreover, the bone mineral content or density, as analyzed with pQCT was not altered and there were no significant differences in bone sizes between the Col18a1−/− mice and their controls.

The possible involvement of endostatin in Wnt signaling may explain the delay in the formation of the ossification centers in Col18a1−/− mice. The longest variant of collagen XVIII contains a Frizzled-module, which has been shown to be able to inhibit Wnt/beta-catenin signaling by binding to Wnt-molecules and hence inhibiting their binding to cell-membrane receptors. The lack of the longest variant could result in altered Wnt signalling (Quelard et al. 2008). In mice that constitutively overexpress active beta-catenin in cartilage, growth plates were totally disorganized, and the maturation of chondrocytes and endochondral ossification were inhibited. The activation of Wnt/β-catenin in mature chondrocytes stimulates hypertrophy, matrix mineralization, and the expression of VEGF, MMP-13, and several other MMPs among others (Tamamura et al. 2005). It has been found, that the phenotype of collagen XVIII deficient mice is similar to mice expressing the VEGF120 and VEGF188 isoforms only. They have been shown to have differences in solubility and matrix binding properties and also in their ability to bind to heparin and heparan sulfate proteoglycans. Since collagen XVIII is a heparan sulfate proteoglycans it has proposed that collagen XVIII may be involved in VEGF function and is required for the binding of to its receptor (Hurskainen et al. 2005).

In ES-tg mice we observed a delay in the growth of bone length: femurs and tibias were smaller at the age of 14 days. This difference could not be seen in 30-day-old mice suggesting that this delay was temporary. Endostatin overexpression takes place in the keratinocytes of ES-tg mice, but they show a seven-fold increase in the concentration of endostatin in their circulation. It is possible that the endostatin concentration in the bones is not high enough to cause any longer lasting or permanent defects, but it only leads to a slight delay in the growth rate. We used immunohistochemistry to determine the localization of collagen XVIII in bone tissue. Collagen XVIII was detected surrounding the vessel structures in bone marrow and periosteum, and at the skeletal muscle attachment site by the antibody against all three variants. The short variant of collagen XVIII was shown to be responsible for this immunosignal. Pufe and colleagues reported that collagen XVIII/endostatin was expressed in human cartilage and fibrocartilage (Pufe et al. 2004c). In present study, there was a signal in the cartilage, but this
proved to be unspecific as staining was also seen in the mice lacking collagen XVIII. However, this can result from unspecific attachment of our primary antibodies to the matrix surrounding the chondrocytes and thus it is still possible that collagen XVIII is expressed in mouse cartilage.

Although mild delays in both mutant mouse lines were detected in the bone development process, collagen XVIII/endostatin does not seem to have a crucial role in skeletal development. Endostatin is one of the several regulators of angiogenesis and it is likely that other factors may compensate for the loss of collagen/endostatin in most tissues.
7 Conclusions

Our results confirmed the important role of VEGF in bone healing. VEGF overexpression was delivered locally to the bone injury site using adenoviral VEGF-A gene transfer, which was able to modify the histological features of bone healing in a rodent model. The endochondral phase was completed earlier, and also bone mineral content was enhanced in animals overexpressing VEGF.

The data from pit assay studies showed that VEGF-A induces bone resorption and endostatin can suppress this VEGF-A induced resorption to the basal level. In addition, osteoclastogenesis can be inhibited by endostatin. However, our findings suggest that the regulatory effect of endostatin is not critical, since endostatin alone does not modify the basal bone resorption.

The results from the endochondral ossification model, where the mouse hind muscle pouch was stimulated by BMPs and endostatin was overexpressed with or without VEGF-A using a recombinant adenoviral vector, showed that endostatin retards the cartilage phase and subsequently reduces bone formation.

Endostatin has minor effects on the behavior of osteoblasts in cell culture conditions. These effects are enhanced when cells are treated together with VEGF.

Finally, we investigated the role of endostatin on bone development in vivo using transgenic mice overexpressing endostatin and mice lacking collagen XVIII. Mild delays in both mutant mouse lines were detected in the bone development process. The formation of secondary ossification centers in the bones of mice lacking collagen XVIII was delayed, as well as the growth of bone length in ES-tg mice. On the basis of these results, we conclude that collagen XVIII/endostatin does not seem to have crucial role in skeletal development.

As an overall conclusion, VEGF-A overexpression accelerates bone defect healing. Endostatin retards bone formation and seems to inhibit VEGF-A stimulated effects on bone cells. However, collagen XVIII/endostatin does not seem to have a crucial role in skeletal development.
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EFFECTS OF VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF-A) AND ENDOSTATIN ON BONE