Siim Suutre

The role of TGF-β isoforms and
osteoprogenitor cells in the pathogenesis
of heterotopic ossification.
An experimental and clinical study
of hip arthroplasty

Department of Anatomy, University of Tartu, Estonia
Department of Molecular Biology, University of Umeå, Sweden

Dissertation is accepted for the commencement of the degree of Doctor of Medical Sciences on November 18, 2009 by the Council of the Faculty of Medicine, University of Tartu, Estonia

Supervisors: Andres Arend, MD, PhD, Professor of Histology,
               Department of Anatomy, University of Tartu, Estonia

               Gunnar Selstam, MD, PhD, Professor of Molecular Biology,
               Department of Molecular Biology, University of Umeå, Sweden.

               Alar Toom, MD, PhD, Senior Registrar of Orthopaedic Surgery,
               Tartu University Clinics and Research Fellow, University of
               Tartu, Department of Traumatology and Orthopaedics.

               Mihkel Zilmer, PhD (med), Professor of Biochemistry,
               Department of Biochemistry, University of Tartu, Estonia

Reviewers: Docent Aleks Lenzner, MD, PhD, Department of Traumatology
               and Orthopaedics, University of Tartu, Tartu, Estonia

               Senior Research Fellow Anti Kalda, MD, PhD
               Department of Pharmacology, University of Tartu, Tartu, Estonia

Opponent: Prof. Petri Lehenkari MD, PhD
               Clinical Research Centre, Department of Surgery and Cell
               Biology, University of Oulu, Oulu, Finland

Commencement: February 26, 2010

Publication of this dissertation is granted by the University of Tartu.

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

I.   Suutre S, Toom A, Arend A, Selstam G. Bone tissue content of TGF-β_2 changes with time in human heterotopic ossification after total hip arthroplasty. Growth Factors. 2009; 27(2): 114–120.

II. Toom A, Arend A, Gunnarsson D, Ulfsparre R, Suutre S, Haviko T, Selstam G. Bone Formation Zones in Heterotopic Ossifications: Histologic Findings and Increased Expression of Bone Morphogenetic Protein 2 and Transforming Growth Factors β_2 and β_3. Calcif Tissue Int. 2007; 80(4): 259–267.

III. Suutre S, Toom A, Arend A, Selstam G. BMP-2, TGF-β_2 and TGF-β_3 signaling is involved in initial and early stages of heterotopic ossification in a rat experimental model. Accepted for publication in Scand J Lab Anim Sci.

IV. Toom A, Suutre S, Märtson A, Haviko T, Selstam G, Arend A. Lack of a central role for osteprogenitor cells from the femoral canal in heterotopic ossification of the hip: an experimental study in a rat model. Accepted for publication in J Bone Joint Surg Br.

Author's contribution:

I.   The author conducted immunohistochemical experiments, analyzed the data and was the main person responsible for writing the manuscript.
II. The author conducted immunohistochemical experiments and participated in writing the manuscript.
III. The author assisted in the animal experiment and sample harvesting, conducted immunohistochemical experiments and mRNA *in situ* hybridization and was the main person responsible for analyzing the data and writing the manuscript.
IV. The author assisted in the animal experiment and sample harvesting, conducted immunohistochemical experiments and participated in writing the manuscript.

ABBREVIATIONS

ActR	activin receptor
ALK	anaplastic lymphoma kinase
AMH	Anti-Müllerian Hormone
AMHR	Anti-Müllerian Hormone receptor
AP	alkaline phosphatase
BMP	bone morphogenetic protein
BMPR	bone morphogenetic protein receptor
BV/TV	ratio of bone volume to total sample volume
CNS	central nervous system
COX	cyclooxygenase
DABM	demineralized allogenic bone matrix
DIG	digoxigenin
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EMT	epithelial-mesenchymal cell transformation
ES/BS	ratio of eroded surface to bone surface
GDF	growth/differentiation factor
GDNF	Glial cell line-derived neurotrophic factor
GTP	Guanosine triphosphate
Gy	Gray (radiation unit)
HO	heterotopic ossification
IHC	immunohistochemistry
ISH	*in situ* hybridization
IL	interleukin
Md.V/TV	mineralized volume ratio to total sample volume
MIS/AMH	Müllerian Inhibiting Substance/Anti-Müllerian Inhibiting Substance
mRNA	messenger ribonucleic acid
NBT/BCIP	Nitro blue tetrazolium chloride/5-Bromo-4-chloro-3-indolyl phosphate
NSAIDs	Non-steroidal anti-inflammatory drugs
Ob.S/BS	ratio of osteoblast surface to bone surface
OS/BS	ratio of osteoid surface to bone surface
OS/BV	ratio of osteoid surface to bone volume
OS/Es	ratio of osteoid surface to endostal surface
OS/Ps	ratio of osteoid surface to periostal surface
OV/BV	ratio of osteoid volume to bone volume
PBS	phosphate buffered saline
PBT	phosphate buffered saline containing 0.1% Tween 20
PFA	paraformaldehyde
PGE	prostaglandin E
rhBMP	recombinant human bone morphogenetic protein
SD	standard deviation

SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SSC	sodium chloride-sodium citrate
TGF	transforming growth factor
THA	total hip arthroplasty

INTRODUCTION

Bone metabolism stays active throughout life, i.e. bone is a dynamic tissue, being formed and resorbed continually under the control of hormones, cytokines, growth factors and physical forces. Remodeling of bone requires coordinated actions of osteoclasts to remove bone, and osteoblasts to replace it. In this way microfractures are repaired and bone structures are adapted to stress and other biomechanical forces. Bone turnover is normally low in adults but its basal level can increase in the case of different processes like fracture healing or heterotopic ossification (HO), which is defined as non-malignant abnormal formation of mature, lamellar bone (often containing bone marrow) in soft tissue structures where bone normally does not exist (Spry *et al.*, 1995). HO, which was first described already in 1692 by Patin (Geschickter and Maseritz, 1938), has many causes and it can be found in different locations. Most frequently, it has been described in the hip following total hip arthroplasty (THA). HO has also been described to be caused by trauma of muscles, spinal cord or brain or bone fracture (Sawyer *et al.*, 1991; Stołtny *et al.*, 2007). The exact reasons for this phenomenon are still unknown. It has been suggested that HO may originate from the mesenchymal stem cells and/or particles of the bone/bone matrix that are left in the operation wound after surgery (Bosse, 1997). Due to active cellular communication and active metabolism of bone as a vascular tissue, many systemic and local effectors, e.g. cytokines, hormones, growth factors and prostaglandins/leukotriens and their antagonists have a significant impact on formation and function of normal bone and HO. The growth factor type mainly studied in connection with bone is the bone morphogenetic protein (BMP). This thesis focus on the role of transforming growth factor beta (TGF-β) isoforms in HO as, although they are considered to be the key players in bone formation and functioning, a few studies have focused on their role in HO formation. One direct indication of the role of TGF-β is that the content of TGF-β has been found to be 6.8 times higher in HO than in normal bone (Sawyer *et al*., 1991).

As TGF-β isoforms have been detected in osteoblasts, chondrocytes and also in the bone matrix (Joyce *et al.*, 1990b) and as different levels of expression of TGF-β isoforms and their receptors have been described in the human during bone formation (Horner *et al*., 1998), TGF-β isoforms are of interest in the context of HO.

Because HO after THA is the most common HO to cause clinical problems and because relatively little is known about TGF-β isoforms in HO, the aim of this study was to monitor changes of TGF-β isoforms in HO after THA, as well to describe the histomorphology of developing HOs and to find out progenitor cells for HO formation. To address these questions, in addition to studying patients' HO samples, a rat HO exprerimental model was developed mimicking the situation after THA.

A better knowledge of the dynamics of HO formation and specific changes of growth factors in HO are important in understanding the pathogenesis of HO and rapid bone formation.

REVIEW OF LITERATURE

1. TGF-β superfamily

The transforming growth factor β (TGF-β) superfamily is a large family of structurally similar proteins that regulate a wide range of cellular functions, including tissue differentiation, cellular migration, morphogenesis and proliferation. The superfamily was named after its first member, TGF-β_1, originally described in 1983 (Assoian *et al.*, 1983). The TGF-β-family members comprise about 30 members in the mammalian system and can be divided into the TGF-β/activin group and the BMP group (bone morphogenetic protein).

TGF-β superfamily ligands are:
– activins (activin A, activin B, activin AB, activin C),
– BMPs (Bone Morphogenetic Proteins) (BMP-2, BMP-3, BMP-4, BMP-5, BMP-6, BMP-7, BMP-8, BMP-9, BMP-10, BMP-3b/GDF-10, BMP-15/GDF-9B, decapentaplegic),
– Growth/Differentiation Factors (GDFs) (GDF-1, GDF-3, GDF-5, GDF-6, GDF-7, GDF-8, GDF-9, GDF-11, GDF-15),
– GDNF Family Ligands (Artemin, Neurturin, GDNF, Persephin),
– TGF-β family (TGF-β_1, TGF-β_2, TGF-β_3, TGF-β_4, TGF-β_5),
– Other TGF-β Superfamily Ligands (Lefty, MIS/AMH, Lefty-1, Nodal, Lefty-A) (Chang *et al.*, 2002).

All TGF-β superfamily members have roles in skeletal morphogenesis, their receptors and receptor binding proteins affect skeletal differentiation and function. Mutations in genes encoding these proteins result in a variety of skeletal malformations (Chang *et al.*, 2002).

1.1 TGF-β signaling

TGF-βs regulate cellular processes by binding to three high-affinity cell-surface receptors known as types I, II and III (Blobe *et al.*, 2000). The receptors directly involved in signaling of TGF-β superfamily members are the type I and type II serine/threonine kinase receptors. Five type II receptors (BMPR-II, ActR-II, ActR-IIB, TGF-βR-II and AMHR-II) and seven type I receptors (ALK 1–7), also termed receptor-like kinases (ALKs), have been identified (Shi and Massagué, 2003). The type II receptor is a constitutively active kinase which, upon ligand-mediated heteromeric complex formation, phosphorylates particularly serine and threonine residues in the type I receptor (Arsura *et al.*, 2003). For BMP signal transduction, ALK-2 is the most important receptor.

In the extracellular space TGF-βs bind either the type III TGF-β receptor (RIII) (characteristic of TGF-β_2), which then presents it to the type II receptor

(RII) or directly to RII on the cell membrane (characteristic of TGF-β_1 and TGF-β_3). The binding of TGF-β to RII leads to binding of the type I receptor (RI) to the complex and to phosphorylation of RI. This phosphorylation activates the RI protein kinase, which then phosphorylates the transcription factor Smad2 or Smad3. The TGF-β receptors transduce their signals via Smad proteins. Phosphorylated Smad2 or Smad3 binds to Smad4, and the resulting complex moves into the nucleus. In the nucleus the Smad complex interacts with various other transcription factors in a cell-specific manner to regulate the transcription of TGF-β-responsive genes and to mediate the effects of TGF-β at the cellular level (Blobe *et al.*, 2000) (see Figure 1).
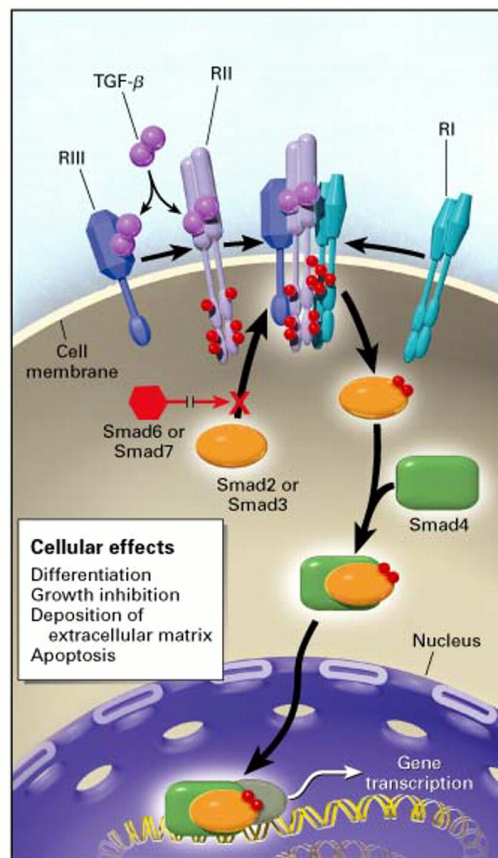
Figure 1. TGF-β-signaling. For all the TGF-β isoforms, TGF-β signaling begins with high-affinity binding to a type II Ser/Thr kinase receptor called TGF-β RII. The TGF-β_2 also binds to a complex of the accessory receptor β-glycan (also known as TGF-β RIII). The receptor TGF-β RII then phosphorylates and activates a second Ser/Thr kinase receptor, TGF-β RI (also called activin receptor-like kinase (ALK) -5), or alternatively, ALK-1. This complex phosphorylates and activates Smad proteins that regulate transcription (Wahl *et al.*, 2006; Zúñiga *et al.*, 2005; de Caestecker, 2004). From the article by Blobe and co-workers (Blobe *et al.*, 2000; Reproduced with permission).

Eight different Smad proteins are known today. The functional classes of the Smad proteins are:

I   – the receptor activated R-Smads (Smads 1, 2, 3, 5, and 8).
II   – the co-mediator Co-Smad (Smad 4).
III – the inhibitory I-Smads (Smads 6 and 7).

In non-active cells, R-Smads are generally localized in the cytoplasm, Co-Smads are equally distributed in the cytoplasm and in the nucleus and I-Smads are generally located in the nucleus. When the receptors of the TGF-β super-family are stimulated, R-Smads become phosphorylated and activated and will undergo dimerization and form heterotrimers with Co-Smads. The formed complexes then translocate to the nucleus where they affect transcriptional regulation (Lebrin *et al.*, 2005). Transcription factors (like AP-1) facilitate binding of Smads to DNA.

The TGF-βs are stored to different degrees in the bone matrix and are released in their activated form during bone remodeling but also under several pathophysiologic conditions like ischemia/reperfusion. It is likely that TGF-βs are important in normal bone remodeling and normal injury repair (Mundy, 1991; Poncelet and Schnaper, 2001). Enhanced expression of TGF-β responsive genes, like collagen, is also mediated by Smads and AP-1 (Poncelet and Schnaper, 2001).

1.2 TGF-β subfamily

In higher vertebrates five TGF-β isoforms (TGF-$β_{1-5}$) with close amino acid sequence homologies have been characterized. The TGF-$β_1$, TGF-$β_2$ and TGF-$β_3$ are found in human and murine tissues while TGF-$β_4$ and TGF-$β_5$ are found in chicken and Xenopus, respectively, and in 1998 the first fish TGF-β gene (rainbow trout TGF-β – related mostly to TGF-$β_5$ and TGF-$β_4$) was charac-terized (Hardie *et al.*, 1998). Five isoforms share approximately 75% of se-quence identity but exert different biological activities in normal bone for-mation although there occurs some overlapping (Mittl *et al.*, 1996).

Transforming growth factor β (TGF-β) isoforms are involved in early embryonal development, in tissue repair as well as in remodeling and haemato-poiesis (Herpin *et al.*, 2004), but also in development of early inflammatory reaction. They recruit inflammatory cells, primarily neutrophils and macro-phages, to the site of inflammation, as they have been reported to be powerful chemoattractants (Faler *et al.*, 2006).

1.3 Examples of the functions of TGF-β isoforms in the body

TGF-$β_1$ is a multifunctional protein that controls proliferation, differentiation, and several other functions in many cell types. Many cells synthesize TGF-$β_1$

and essentially all cells have specific receptors for TGF-β_1. Examination of TGF-β_1 mRNA levels in adult murine tissues indicates that expression of TGF-β_1 is predominant in the spleen, lung and placenta (Miller *et al.*, 1989). TGF-β_1 has a part in controlling the immune system, and it has different effects on different cell types, or on cells at different developmental stages. The TGF-β_1 increases the expression of certain cytokines in T cells and promotes their proliferation. The TGF-β_1 is also secreted by most immune cells (or leukocytes) (Letterio and Roberts, 1998). The TGF-β_1 is known to induce severe and progressive fibrosis (Bonniaud *et al.*, 2003). In carcinogenesis, TGF-β_1 plays a complex role, having both tumor suppressor and oncogenic actions (de Caestecker *et al.*, 2000).

The TGF-β_2 mediates activation and separation of endothelial cells and it is the only ligand involved in EMT (epithelial-mesenchymal cell transformation) (Mercado-Pimentel and Runyan, 2007). The TGF-β_2 is also expressed in the myocardium and bronchial epithelia (McCartney-Francis and Wahl, 1994). The TGF-β_2 regulates the excitability of the basal forebrain cholinergic neurons through an atypical signaling mechanism (Williams *et al.*, 2002). Cardiac, lung, craniofacial and urogenital defects have been noted in mice lacking TGF-β_2 (Sanford *et al.*, 1997).

The TGF-β_3 mediates endothelial cell invasion into the extracellular matrix (Mercado-Pimentel and Runyan, 2007). It has also been noted to be expressed in the developing lung, in the bronchial epithelia and in the mouse embryonic heart as well as TGF-β_2 (Mercado-Pimentel and Runyan, 2007). The TGF-β_3 is a cytokine which is involved in cellular differentiation, e.g. in skeletal muscle progenitor cells (Schabort *et al.*, 2009), in embryogenesis, e.g. in a wide variety of mesenchymal tissues including areas of chondrogenic activity (Gatherer *et al.*, 1990), and in embryonic development, e.g. during umbilical cord development (Copland *et al.*, 2002; Herpin *et al.*, 2004). Mice lacking TGF-β_3 have been noted to have cleft palates (Proetzel *et al.*, 1995).

2. TGF-βs in bone

In normal bone TGF-β isoforms are, besides some bone morphogenetic proteins (BMPs), the most important growth factors of the TGF-β superfamily, that affect bone growth, repair and regeneration. TGF-β isoforms have been found, for example, in proliferating mesenchymal cells, in osteoblasts lining forming bone, in young and mature chondrocytes and in the bone matrix (Joyce *et al.*, 1990b). TGF-β isoforms stimulate bone formation but do not possess any osteoinductive properties, either in human or in rodent cells (Matsaba *et al.*, 2001). Instead, addition of TGF-β to forming bone accelerates the bone formation process (Joyce *et al.*, 1990a). However, TGF-β isoforms have been noted to have osteogenetic properties in the non-human primate *Papio ursinus* (Ripamonti *et al.*, 2009). TGF-β isoforms have been suggested to have bone stimulatory effects in the early phases of osteoblast differentiation (Bonewald, 2002).

In healing fractures in a chick model, TGF-β isoform expressions have been observed, with more prominent expressions of the TGF-β_2 and TGF-β_3 isoforms (Rosier *et al.*, 1998).

The TGF-β_1 is a polypeptide that is most abundant in the bone matrix of the three isoforms, with 200 μg/kg of TGF-β_1 being present in bone (Seyedin *et al.*, 1985). It is produced by bone cells, and it modulates proliferation and differentiation of osteoblastic cells in vitro (Pfeilschifter *et al.*, 1988). The TGF-β_1 plays an important role in bone remodeling as a potent stimulator of osteo-blastic bone formation, causing chemotaxis, proliferation and differentiation of committed osteoblasts (Mundy, 1991). The TGF-β_1 mRNA appears to be more highly expressed by differentiated osteoblasts as well as by bone resorbing osteoclasts, compared to other isoforms. In bone and cartilage, mRNA of TGF-β_1 can be detected more readily at later developmental stages and it can also be detected in ossification centers (Centrella *et al.*, 1994).

The TGF-β_2 takes part in endochondral ossification and chondrogenesis (Joyce *et al.*, 1990b; Opperman *et al.*, 2000) and is, for example, involved in regulation of cell proliferation and apoptosis in cranial suture formation (Opperman *et al.*, 2000). The TGF-β_2 mRNA is mainly localized in the mesenchymal components of tissues such as bone, cartilage and blood vessels (Pelton *et al.*, 1989). During bone formation TGF-β_2 expression is high in the preosteoblasts and in the osteoblast-rich cambial layers (Zhang *et al.*, 1999), as well as in the perichondrium, in precartilagineous regions and in the growth zones of long bones (Centrella *et al.*, 1994). In transgenic mice over-expressing TGF-β_2, there were noted increased activities of osteoblasts and osteoclasts but impaired matrix mineralization by osteoblasts (Erlebacher and Derynck, 1996).

The TGF-β_3 regulates many functions associated with bone formation, primarily collagen synthesis, mitogenesis, and alkaline phosphatase activity and it is, like TGF-β_2, involved in regulation of cell proliferation and apoptosis in suture formation (ten Dijke *et al.*, 1990; Opperman *et al.*, 2000). With mRNA *in situ* hybridization, TGF-β_3 mRNA appears to be strongly expressed in the perichondrium and in the less differentiated mesenchyme close to sites of ossification of the intramembranous bone (Millan *et al.*, 1991). The expression of TGF-β_3 has been shown to be decreased in osteoporotic mice (Orlic *et al.*, 2007). It has also been shown that exogenous TGF-β_3 inhibited the osteogenic differentiation of mesenchymal stem cells and significantly reduced alkaline phosphatase activity and mineral deposition (Moioli *et al.*, 2007). The TGF-β_3 seems to be involved in osteoblast proliferation, chemotaxis, and also in collagen synthesis (Bouletreau *et al.*, 2000).

2.1 Expression and binding of TGF-β isoforms in bone

Bone cells synthesize all three TGF-β isoforms and express cell surface TGF-β receptors. Less is known about the molecular events that control TGF-β synthesis or differential isoform expression. One study on non-skeletal tissue

provided data for a positive autocrine feedback by TGF-βs on their own expression: in fetal murine fibroblasts, TGF-β_1 induces TGF-β_1 mRNA levels rapidly and solely. The TGF-β_2 has another stimulatory pattern in that it immediately induces TGF-β_2 mRNA, followed soon by TGF-β_3 mRNA, while the levels of TGF-β_1 mRNA increase only after longer treatment intervals (Bascom *et al.*, 1989). In both cases TGF-β_1 appeared to be regulated by transcriptional and posttranscriptional effects.

It has been noted that both TGF-β_1 and TGF-β_3 increase TGF-β_1 and TGF-β_3 transcripts in osteoblast-enriched cultures of fetal rat bone (Centrella *et al.*, 1994).

The TGF-β_1, -β_2 and -β_3 all produce analogous maximal stimulatory effects in primary cell cultures from fetal rat bone, and each isoform inhibits proliferation in rat osteosarcoma cultures. However, on a molar basis, TGF-β_3 was up to 10-fold more potent than TGF-β_1 or TGF-β_2 (Centrella *et al.*, 1994). The TGF-β_3 has approximately 3-fold higher binding affinity to fetal bone cells, but paradoxically binds with lower affinity to type I sites in rat osteosarcoma cultures (ten Dijke *et al.*, 1990), which suggests that TGF-β_3 couples binding site occupancy more effectively with signal transduction. Differences in biological potency seem to be determined by small differences in the midregion of TGF-β_1 and TGF-β_2, which may be related to their differential affinities to α2-macroglobulin (Burmester *et al.*, 1993).

3. Heterotopic ossification (HO)

Heterotopic ossification (HO) is defined as non-malignant abnormal formation of mature, lamellar bone (often containing bone marrow) in soft tissue structures where bone normally does not exist (Spry *et al.*, 1995; Balboni *et al.*, 2006). The HO was first described in 1692 by Patin in children with myositis ossificans progressiva (Geschickter and Maseritz, 1938). In 1883 and in 1918 a clearer description was provided by Riedel (Riedel, 1883) and by De´jerine and Ceillier (De´jerine and Ceillier, 1918), respectively.

HO has been given multiple names including myositis ossificans, paraosteo-arthropathy, periarticular ectopic ossification, periarticular new bone formation, neurogenic osteoma and neurogenic ossifying fibromyopathy (Sawyer *et al.*, 1991). A more common phenomenon is heterotopic calcification (calcium deposits in soft tissues), without formation of new bone (ossification), and thus heterotopic calcification is clearly differentiated from HO (Vanden Bossche and Vanderstraeten, 2005). In this thesis HO is therefore used when bone formation occurs while calcium deposits are called calcifications.

HO formation can occur at any site. However, it has been found most frequently in the hip following total hip arthroplasty. The hip is also the most common site of HO formation in patients with traumatic brain or spinal cord injury. The knee is less frequently affected (Vanden Bossche and Vander-straeten, 2005).

The most common causes of HO are direct muscle trauma, with accompanying bone fracture, spinal cord or cerebral injury (Sawyer *et al.*, 1991; Stołtny *et al.*, 2007). During World War I, HO was predominantly observed in soldiers who had become paraplegic from intramedullary gunshot wounds (De´jerine and Ceillier, 1918). The HO can be classified according to the inductive factors and extent of HO spreading (Puzas *et al.*, 1989; Thomas, 1992; Bosse, 1997):

1) generalized HO caused by systemic illnesses (e.g. fibrodysplasia ossificans progressiva) or by CNS injury (e.g. spinal cord injury)
2) local HO caused by local trauma (surgical trauma [e.g. after THA], fracture, muscle injuries, burns)
3) local HO caused by metabolic or organic tissue changes (e.g. intramuscular and subcutaneous injections, tumors) (Toom, 2007).

The reasons why soft tissues (muscle or connective tissue) are converted to bone remains poorly understood. It is possible that after hip replacement potentially bone-forming cells or fragments of bone tissue form a focus for further bone formation; however, in other cases, when bone formation occurs far from sites of neurological injury, there are no clues (Smith and Wordsworth, 2005). Although being a fairly common problem – approximately 37% of THA patients are affected (Toom *et al.*, 2001) – HO rarely causes serious clinical disturbances. However, if such disturbances do occur, they can be a serious burden causing pain and/or restriction of the movement of the joints. Sometimes surgical intervention is necessary.

3.1 HO formation and growth factor expression

Relatively little is known about the expression of growth factors during HO formation. The growth factors studied most in connection with bone are bone morphogenetic proteins (BMPs), among them BMP-2, in particular, that has a well known ability to induce bone formation (Yoshida *et al.*, 1998). However, only a few facts can be brought out regarding the roles of growth factors in HO formation in general. For example, it has been noted that basic fibroblast growth factor inhibits endochondral heterotopic ossification (Sakano *et al.*, 2002). Significant expression of BMP-2, TGF-β, and vascular endothelial growth factor (VEGF) has been noted in chondrocytes during thoracic ossification of the human ligamentum flavum (Yayama *et al.*, 2007) and impaired angiogenesis and endochondral bone formation have been noted in mice lacking certain isoforms of vascular endothelial growth factor (Maes *et al.*, 2002). Transforming growth factor beta and connective tissue growth factor are involved in ossification of the nevus of Nanta (Keida *et al.*, 2005). However, studies on the other growth factors, especially on TGF-β isoforms are scarce. Although TGF-β isoforms contribute to bone formation and functioning, they seem to be less studied compared to other effectors and little is known about

their specifics, especially in a HO setting. A few changes have been noted in the TGF-β isoform expression pattern. Changes in the expression pattern of TGF-β isoforms have been observed during bone formation in human atherosclerotic lesions (Jeziorska, 2001). Immunoreactivity against TGF-β_1 has been detected in pulmonary adenocarcinoma with HO (Kim *et al.*, 2009). The TGF-β_2 has been observed in young osteocytes during pathologic new bone formation around radicular cysts (Kusafuka *et al.*, 2006).

3.2 HO formation after total hip arthroplasty (THA)

As HO is a rather common side effect of total hip arthroplasty (THA), with approximately 37% of THA patients being affected (Toom *et al.*, 2001), the current thesis focuses on HO formation after THA, or in an animal model after the operation mimicking THA. The HO may cause pain and/or restriction of the movement of the joints depending on its location. The main risk factors for HO after THA are male gender, active ankylosing spondylitis, post-traumatic arthritis, hypertrophic arthrosis, previous HO, Paget's disease, extensive osteophytosis and nerve and/or spinal cord injury and the rare inherited disorder fibrodysplasia ossificans progressiva (Toom *et al.*, 2001; Iorio and Healy, 2002).

The etiology of HO is still not entirely known. The factors contributing to formation of HO include hypercalcemia, tissue hypoxia, changes in sympathetic nerve activity, prolonged immobilization, mobilization after prolonged immobilization and imbalance between parathyroid hormone and calcitonin (Shehab *et al.*, 2002). Regardless of what the initial effectors actually are, the initial stage of HO formation involves osteoblast differentiation and strong osteoblastic activity.

The most common HO develops in the soft tissues around the hip following total hip arthroplasty (THA). Although there is no direct evidence, it is hypothesized that after THA, HO may arise either from the osteoblastic cells migrating from the femoral canal (Bosse, 1997), or from pieces of the bone/ bone matrix that are left in the operation wound and induce bone formation, as has been shown by Urist (Urist, 1965), or from operation related connective tissue damage which can also induce mesenchymal cells to be differentiated into osteoblasts and to start forming bone (Nilsson and Persson, 1999). Morphological and biochemical analysis of HO has shown intense turnover and high content of growth factors, indicating that HO is a metabolically active tissue (Bosse, 1997). In the initial phases of HO formation cellular infiltration and inflammation can be seen. Inflammatory processes and released growth factors are thought to stimulate differentiation of mesenchymal cells into osteoblasts and osteoblast proliferation (Hughes *et al.*, 2006). Postoperative administration of NSAIDs is therefore generally considered a rather effective preventive treatment of HO (Gebuhr *et al.*, 1995; Dorn *et al.*, 1998; Persson *et al.*, 1998).

3.3 Maturation of HO

The HOs can be classified depending on their age and maturation. The HO formation is relatively rapid in the initial phases but slows down later during maturation of the ectopic bone, which usually takes 12–18 months (Park *et al.*, 2007). During the maturation period osteoblastic differentiation and proliferation take place and osteoblasts start forming the bone matrix, but calcification is limited and HO is then considered to be immature. At the later stage osteoblastic activity is lower but remodeling still continues.

Ackerman suggested that HO develops in centrifugal pattern (Ackerman, 1958) and consists of lamellar bone and proliferating osteoblasts, while stroma is mainly located centrally. Today it is generally accepted that although the histology is different, three zones can be distinguished: formed bone, zone of active bone formation and zone with fibrous connective and fibrocartilagineous tissue (Toom, 2007). This histological order does not always apply to larger ossificates which may contain multiple ossicles, with separately organized layers in each ossicle (Bosse *et al.*, 1994b). This is probably due to the multinucleate origin of larger ossifications.

Formed bone is mineralized bone matrix with osteocytes. On the surface of formed bone is the zone of active bone formation, which contains osteoblasts and osteoclasts. In the fibrous connective zone undifferentiated mesenchymal cells (star-shaped cells with large nuclei) can be noted. These cells differentiate into osteogenic cells and migrate to the zone of active bone formation (Urist *et al.*, 1978).

Mature HO shows cancellous bone and mature lamellar bone with blood vessels and bone marrow, with limited hematopoiesis (Vanden Bossche and Vanderstraeten, 2005). In our studies we used the age of the HO to differentiate between immature and mature HOs, as was originally suggested by Abrahamsson and co-workers (Abrahamsson *et al.*, 1984).

The HOs are usually removed during THA revision surgery, but occasionally they can be the reason for indicating surgery. The HOs have to be removed when they cause pain and/or restriction of the movement of the joints. The optimal timing of surgery is still controversial. Usually, it is suggested to wait until complete maturation of ectopic bone has occurred, which is believed to avoid recurrences. Another treatment strategy is to pay more attention to functional and neurological recovery rather than to maturity of the bone (Vanden Bossche and Vanderstraeten, 2005).

3.3.1 Grading of HO

Classifications of HO evaluate mostly the size of HO (Brookers', Della Valles') but also the size and localization of HO (Arcqs' or DeLees'). The aim of a classification of HO is to:
– allow to compare clinical HO cases or,
– facilitate the decision-making when setting up the treatment regimen.

Brooker's system is the most common and the most widely used classification system of HOs (Brooker *et al.*, 1973), but in some cases Della Valles', Arcqs' or DeLees' classifications are also used. In clinical practice quantitative and qualitative estimations of HO are usually carried out by comparison of frontal or plain x-rays of the proximal hip region, which are regularly made during the postoperative period and follow-up.

Brooker's classification. There are four classes of HOs according to Brooker's classification (Brooker *et al.*, 1973).
Class I: islets of bone in the soft tissue regardless of their size
Class II: bone originating from the pelvis or the femur with at least a 1 cm gap between opposite bone surfaces
Class III: bone originating from the pelvis or the femur but with less than a 1 cm gap between opposite bone surfaces
Class IV: ankylosis

Brooker's classification is the most widely used classification so far, despite its shortcomings that were pointed out later by Wright and co-workers (Wright *et al.*, 1994). Brooker's classification evaluates mostly the size of ossificates. Their localization is evaluated on the basis of whether the HO is connected to nearby bone or is located separately in soft tissues. Alternative classifications, which differ from Brooker's classification in some details, were suggested by Della Valle (Della Valle *et al.*, 2002), DeLee and coauthors (DeLee *et al.*, 1976) and Arcq (Arcq, 1973).

4. Preventive treatment of HO

Three types of treatments are proposed to prevent HO in patients at risk.
1) Postoperative treatment with non-steroidal inflammatory drugs (NSAIDs)
2) Pre- and postoperative irradiation of the hip region
3) Postoperative treatment with bisphosphonates

4.1 Treatment with NSAIDs

NSAID treatment is the most common and rather well proven method for prevention of HOs. The shortest effective treatment time is between 5 and 10 days, starting the treatment on the day of surgery (Gebuhr *et al.*, 1995; Wurnig *et al.*, 1997; Dorn *et al.*, 1998; Persson *et al.*, 1998). The mechanism by which the NSAIDs prevent formation of HOs is most likely through inhibition of prostaglandin (e.g. PGE$_2$) synthesis (Gebuhr *et al.*, 1995; Wurnig *et al.*, 1997; Persson *et al.*, 1998). Prostaglandins are mediators in inflammatory reactions and it is believed that inflammatory response may initiate formation of HO. NSAIDs inhibit the synthesis of prostaglandins by inhibiting enzymes cyclooxygenases (COX). Currently two COX isoenzymes are known: COX-1 and COX-2. It is suggested that there is also COX-3, which is thought to be a splice variant of COX-1 and is sometimes also called COX-1b or COX-1 variant (COX-1v) (Chandrasekharan *et al.*, 2002). Studies have shown equivalent efficacy for selective COX-2 inhibitors and non-selective NSAIDs in prevention of HO (Vastel *et al.*, 2005; van der Heide *et al.*, 2007).

The most common medications for prevention of HO after THA are indomethacin (standard dose 25 mg three times daily), ibuprofen (standard dose 400 mg three times daily), acetylsalicylic acid (standard dose 1 g three times daily) and naproxen (standard dose 0,5 g two times daily) (Schmidt *et al.*, 1988; Sodemann *et al.*, 1990; Elmstedt *et al.*, 1985). It has been shown that the minimum time of NSAID treatment should be 5 to 10 days (Elmstedt *et al.*, 1985) and treatment that exceeds three weeks does not reduce the incidence of HO any further (Persson *et al.*, 1998).

Major concern about NSAID treatment is that NSAIDs that inhibit heterotopic bone formation also inhibit bone remodeling and bone ingrowth in porous implants, which may increase the risk of loosening the prosthesis (Keller *et al.*, 1989). However there are no clinical studies to confirm this hypothesis.

4.2 Irradiation

In the initial stages of HO formation rapid osteoblastic differentiation and proliferation and DNA replication may be hindered by irradiation. It has been shown that radiation with a single dose of 5 to 8 Gy pre- or postoperatively effectively prevents HO formation (Fingeroth and Ahmed, 1995; Rumi *et al.*, 2005). However, the cost and risk of radiation-induced sarcoma as well as a decrease in both bone growth and fixation of implants limits the use of this method.

4.3 Usage of bisphosphonates

Bisphosphonates inhibit the action of osteoclasts and the resorption of bone by killing osteoclastic cells. Bisphosphonates are divided into non-nitrogenous and nitrogenous bisphosphonates. In the cell non-nitrogenous bisphosphonates (such as etidronate, clodronate and tiludronate) form a nonfunctional molecule that competes with adenosine triphosphate (ATP) in cellular energy metabolism and therefore the osteoclast initiates apoptosis and dies, leading to an overall decrease in the breakdown of bone (Frith *et al.*, 1997). Nitrogenous bisphosphonates (such as pamidronate, alendronate and risedronate) inhibit the enzymes of the mevalonate pathway. This pathway is among other actions responsible for attaching lipid moieties to small GTP-binding proteins present in the osteoclast, which are essential for cell survival and activity. Inhibition of this pathway inhibits resorptive function and triggers apoptosis (Selby *et al.*, 2002; van Beek *et al.*, 2003). Calcification of the formed osteoid can be postponed by postoperative treatment with bisphosphonates, but ossification develops when the medication is discontinued. Therefore bisphosphonates are generally not the first choice treatment in HO prevention (Urrutia and Bono, 2009).

5. Experimental HO

The first laboratory model for inducing bone formation in soft tissues was described by Marshall Urist in 1965 (Urist, 1965). Demineralized allogeneic bone-segments induced bone formation *in situ* when implanted into muscle pouches. Since then implantation of the demineralized allogeneic bone matrix (DABM) has been used to study bone metabolism.

However, it is known today that induction of bone (also in heterotopic locations) requires bone cells (i.e. active osteoblasts), a matrix/scaffold on which osteoblasts may start to form bone (e.g. demineralized allogeneic bone matrix (DABM)) and the specific growth factors (secreted by bone cells but also the surrounding cells). Growth factors help differentiate and activate cells so that they can start forming bone. In recent years matrixes with multipotent mesenchymal stem cells and different growth factors have been used to aid bone reparation but also to conduct bone research.

Although many growth factors tend to have osteoinductive properties, BMP-2 is considered to be the best for bone induction and it even has the potential to differentiate mesenchymal cells at the implantation site into bone cells. In our research we used rhBMP-2. Successful use of rhBMP-2 requires implantation with biomaterial which acts as a scaffold for cell invasion for osteoinduction and retains rhBMP-2 at the site of implantation (Uludag *et al.*, 1999). Nowadays collagenous sponges and also mineral based carriers (e.g. synthetic hydroxyapathite and tricalcium phosphate) are used besides the demineralized bone matrix (see also Materials and methods).

AIMS OF THE STUDY

General aim: to evaluate the cellular origin, morphological changes and differences in the localization and expression patterns and content of BMP-2 and TGF-β isoforms in HO in the human and in an experimental animal model.

1. To describe and compare the histology of immature and mature HOs and bone formation activity in HOs of different ages to that in orthotopic bone and its development in the human.

2. To determine differences in the gene expression of BMP-2 and TGF-β isoforms between the zones of HO and orthotopic bone as well as between immature and mature HOs and to estimate the content and localization of three TGF-β isoforms in immature and mature HOs in humans after THA.

3. To develop an animal model that mimicks the situation after THA for studying initial and early changes during HO formation.

4. To determine differences in the expression, localization and content of TGF-β isoforms at the onset of HO in order to elucidate their different roles in the initial and early ossification processes in a rat model.

5. To investigate how muscle damage and cells originating from the drilled femoral canal influence formation of heterotopic bone.

MATERIALS AND METHODS

1. Obtaining of patient samples

1.1 Experimental subjects

Patients undergoing endoprosthetic revision surgery due to aseptic loosening, quiescent endoprosthetic infection, or HO between the years 2001 and 2004 were invited to participate in this study. All samples were otherwise to be incinerated. Patients with apparent tissue changes related to active endoprosthetic infection as well as those having any rheumatic or systemic disease of the connective tissue were not enrolled. One patient was excluded after sample harvesting and histological investigation as secondary changes characteristic of osteoporosis were found in HO, as confirmed by histomorphometric analysis. In this case, there was an extensive period (34 years) between HO induction and sample harvesting.

Enrolment was voluntary, and all patients gave their informed consent. This study was approved by the Ethics Review Committee on Human Research of the University of Tartu.

1.2 Sample harvesting

1.2.1 For the mRNA study

Seven samples of HO were harvested during revision arthroplasty, and 12 control samples (fibrous part of the hip joint capsule and orthotopic bone from the femoral neck – from an age- and diagnosis-matched control group, which was enrolled according to the inclusion criteria for the study group) were harvested during primary arthroplasty. In the control group all patients except for one male, who had developed necrosis of the femoral head, were operated due to coxarthrosis of idiopathic causes.

1.2.2 For the histological and immunohistochemical study

Altogether material from 15 patients was harvested during revision arthroplasty and used in this study. Five of the patients had immature heterotopic ossifications (less than 17 months old) and 10 of them had mature ossifications (3–9 years old) (see Table 1).

In order to study the metabolic effects on HO formation, patients with septic loosening of the prosthesis were excluded from the study. However, no significant correlations were found between maturity of HOs and the characteristics of the patients or the prosthesis (see Table 1). Nor were significant differences noted in the content of TGF-β isoforms in HOs depending on age, type of prosthesis or bone cement.

Table 1. Table of patient characteristics.

| Patient no	Age	Gender	Pathology before prosthesis implantig	Reason for surgery	Type of hip prosthesis or preoperative local situation	Bone cements
Mature HOs						
1	77	male	Post-traumatic coxarthrosis	Aseptic loosening of endoprosthesis	Customized	Cemented prosthesis
2	53	male	Idiopathic coxarthrosis	Aseptic loosening of endoprosthesis + implant fracture	Link Lubinus IP	Cemented prosthesis
3	40	male	Post-traumatic coxarthrosis	Aseptic loosening of endoprosthesis	Link Lubinus IP	Cemented prosthesis
4	64	male	Post-traumatic coxarthrosis	Heterotopic ossifications	Link Lubinus IP	Cemented prosthesis
5	65	female	Idiopathic coxarthrosis	Aseptic loosening of endoprosthesis	Link Lubinus IP	Cemented prosthesis
6	75	male	Idiopathic coxarthrosis	Aseptic loosening of endoprosthesis	Müller prosthesis	Cemented prosthesis
7	51	male	Proximal femoral fracture	Girdlestone situation	Girdlestone situation	Girdlestone situation
8	76	male	Idiopathic coxarthrosis	Aseptic loosening of endoprosthesis	Link Lubinus IP	Cemented prosthesis
9	62	male	Idiopathic coxarthrosis	Aseptic loosening of endoprosthesis	Link Lubinus SP II	Cemented prosthesis
10	74	female	Idiopathic coxarthrosis	Aseptic loosening of endoprosthesis	Müller prosthesis	Cemented prosthesis
Immature HOs						
11	61	female	Idiopathic coxarthrosis + benign cyst of greater trochanter	Luxatio endoprosthesis	BICON PLUS/SL-PLUS	Uncemented prosthesis
12	80	female	Proximal femoral fracture	Pain after hemiarthroplasty	Austin-Moore	Uncemented prosthesis
13	38	female	Idiopathic coxarthrosis after dysplasia	Aseptic loosening of endoprosthesis	BICON PLUS/SL-PLUS	Uncemented prosthesis
14	39	female	Idiopathic coxarthrosis	Luxatio endoprosthesis (some large HOs)	BICON PLUS/SL-PLUS	Uncemented prosthesis
15	44	male	Proximal femoral fracture	Luxatio endoprosthesis	Link Lubinus SP II	Cemented prosthesis

1.3 Preparation of samples for the study

1.3.1 For the mRNA study

The HOs intended for gene expression analyses were repeatedly rinsed in normal saline and placed in the RNA preserving medium RNAlater (Ambion, Austin, TX, USA), at the time of macroscopic separation of HO from the adjacent tissue. Samples were flash-frozen in liquid nitrogen at about -196ºC until final dissection under a stereomicroscope.

The HOs were dissected according to three developmental zones as exemplified for a younger HO in Figure 2 and for older HOs in Figure 3. Separation procedures were performed in a dissection chamber at -68ºC (using solid carbon dioxide). Pieces from the border of the two different zones were discarded. Only pieces whose type could be identified by their hardness and visible color were collected for further investigation. Collected samples of different types of tissue were stored at –70ºC until RNA extraction.
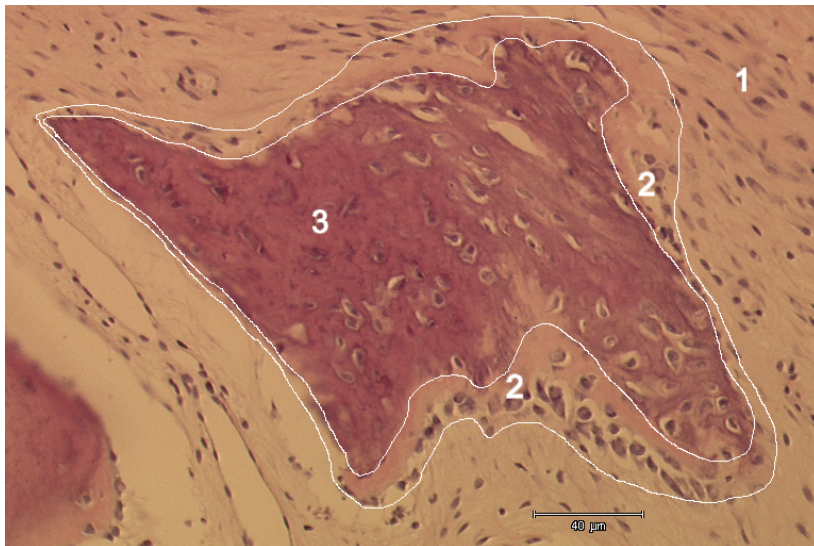
Figure 2. Three zones in immature HO: (1) zone of cellular proliferation, (2) zone of osteoid formation, and (3) zone of formed bone. White lines indicate approximate cutting lines during separation of the immature HO into three zones. Hematoxylin and eosin staining.
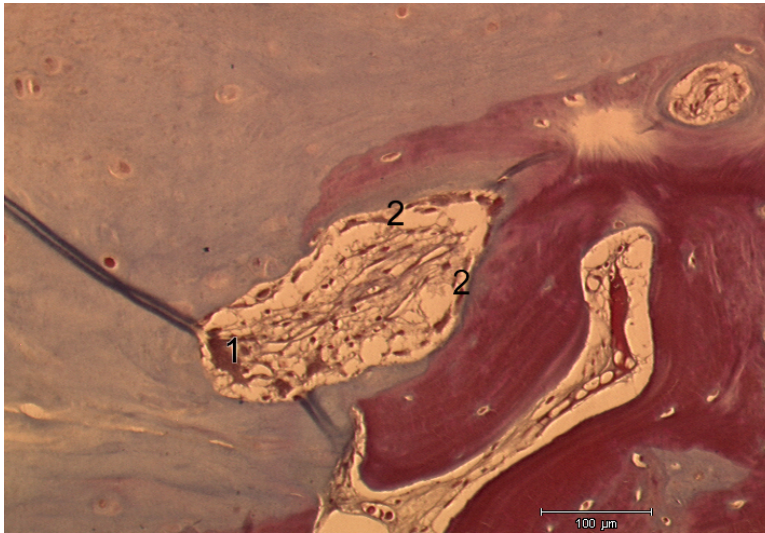
Figure 3. Bone formation in the middle zone of a mature ossificate. (1) Chondroclasts resorbing calcified fibrocartilage; (2) osteoblasts replacing it with osteoid. Abundance of this kind of remodeling activity is reflected also by the higher amount of osteoid surface in mature HOs compared to control bone. Azan staining.

1.3.2. For the histological and immunohistochemical study

Harvested samples were first fixed in neutral buffered 4% formaldehyde solution, then decalcified in EDTA-solution, dehydrated by alcohol-chloroform solutions and embedded into paraffin according to standard methods.

2. Obtaining of experimental samples from the rat model

2.1 Experimental animals

Twenty 9-month old Bkl Wistar strain adult male rats weighing between 500–600 g were purchased from Scanbur BK AB (Sweden). The rats were housed in polycarbonate cages (Tecniplast, Italy) (Eurostandard type III) and were maintained under specific pathogen free (SPF) conditions – water, cages, lids and bedding were autoclaved. Room temperature was 20±2 ℃ and relative humidity was 50±5 %. Food (Labfor R70, Lactamin, Sweden) and autoclaved water were available *ad libitum*. The rats were exposed to a 12 h: 12 h light/dark cycle. Lights were switched on from 08:00 to 20:00. Autoclaved aspen chips (chip size 4×4×1 mm, Estap, Estonia) were used as bedding. The cages were changed each Monday.

This project received the approval of the Animal Ethics Committee at the University of Tartu.

2.2 Experimental HO

We used rhBMP-2 to induce bone growth. In order to use rhBMP-2 successfully, one needs to implant it with a biomaterial which acts as scaffold for cell invasion for osteoinduction and retains rhBMP-2 at the site of implantation (Uludag *et al.*, 1999). Collagenous sponges, mineral based carriers (e.g. synthetic hydroxyapathite and tricalcium phosphate) and demineralized bone matrix can be used for this purpose. It has been shown that, unlike collagen-based carriers, in which rhBMP-2 retention was continuously decreased, mineral-based carriers (except for synthetic hydroxyapatite) seemed to retain a fraction of rhBMP-2 in the implant (Uludag *et al.*, 1999). Therefore we decided that use of a mineral-based carrier (β-tricalcium phosphate) would be the most appropriate for our research. And since we decided to apply stereological analysis for evaluation of the relative content of the growth factor in induced bone, the cube-shaped β-tricalcium phosphate implant served best.

2.3 Operative procedure and implantation technique

The animals were anesthetized with isoflurane inhalation (Isoflurane Baxter®, Baxter Medical AB, Sweden). Antibacterial prophylaxis was performed using a single intramuscular dose of ampicillin prior to the operation. Analgesia was provided before the operation and for 72 hours postoperatively using morphine sulphate. No anti-inflammatory drugs were applied.

The surgical procedure was the following: The hip joint was exposed, the gluteus maximus was retracted, and 3 mm of the gluteus medius was pinched for 2 minutes with a standard vascular clamp giving the same clamp force to produce muscular damage. Further, the compressed muscle was immediately visually inspected for the extent of damages. Bilateral femoral capsulotomies were then performed. On the right side, a 1.6 mm diameter canal with a depth of 8 mm was created in the femur with the opening at the tip of the greater trochanter, slightly medial to the highest point. A conic drill-bit was used for cortical opening, after that the opening of the canal was continued using a standard-shaped 1.6 mm drill-bit. Tissue remnants were not removed after the opening of the femoral canal. No canal was created on the left side and care was taken to avoid any periostal injury during the capsulotomy and implantation procedures.

A 36 mm^3 cube-shaped implant of beta-tricalcium phosphate (ChronOS™ Block, Mathys Medical Ltd, Bettlach, Switzerland, with 70% porosity and with the capacity to contain approx. 25 mm^3 liquid) was used. For half of the animals, the implant was immersed in a solution of rhBMP-2 (supplied by Prof.

Walter Sebald, Biozentrum der Universität Würzburg, Am Hubland, Germany). The estimated amount was 12.5 µg/20 µl per implant. Control implants were immersed in sterile phosphate-buffered isotonic saline. The implants were placed into the capsulotomy wounds. This surgical procedure has been described earlier by Toom and co-workers (Toom *et al.*, 2006).

2.4 Euthanasia

The rats were decapitated under sedation with isoflurane 3 or 21 days after the operation.

2.5 Study groups

Differences in the expression and localization of BMP-2 and three TGF-β isoforms were studied in 4 groups of rats with 5 rats in each group. Two implants were inserted into each rat so that each group consisted of 10 implants.

Group I – implants were immersed in saline and the rats were killed 3 days after the operation and the implants were removed.

Group II – as in group I but the implants were immersed in rhBMP-2 solution.

Group III – as in group I but the rats were killed after 21 days.

Group IV – as in group III but the implants were immersed in rhBMP-2 solution.

The beta-tricalcium phosphate implants were removed after 3 or 21 days and systematic cryosections were made. A three-day endpoint was used for studying the initial stages of bone formation and the expression of the growth factors at the site where ossification was expected. A 21-day endpoint was used for studying the early stage of bone formation, where bone and cartilage formation had already occurred, i.e. for characterizing the expression and localization of the above mentioned growth factors in developing/remodeling bone.

3. Preparation of samples for the study

3.1 For the histological study

Samples from animal experiments were fixed in neutral buffered formalin, then decalcified in EDTA-solution, dehydrated in alcohol-chloroform solutions and embedded into paraffin.

3.2 For the mRNA study and for the immunohistochemical study

Beta-tricalcium phosphate implants were removed after 3 or 21 days and systematic cryosections were made according to the principle of systematic uniform and random selection. Sections with a thickness of 10 μm for surface analysis and cell counting according to Cavalieri's principle (Gundersen *et al.*, 1988) were collected systematically after every 80 μm.

4. Processing of samples

4.1 For the histological study

Samples were systematically sectioned and azan staining was performed (the method combines staining with azocarmine, aniline blue and orange G) (Kiernan, 1999). All stains were obtained from Sigma-Aldrich (St. Louis, USA).

4.2 Total RNA extraction

For RNA extraction, bone tissue was crushed using a custom metal bone-crushing device that maintains low temperature of the tissue by using liquid nitrogen around the crushing tube. After sample crushing, the pieces were warmed up to 4ºC, rinsed thoroughly with buffered saline in order to eliminate the free bone marrow cells, and thereafter dried. The sample was then homogenized with a polytrone (Ultra-Turrax IKAT25; Labassco, Partille, Sweden) in Trizol reagent (Invitrogen Life Technologies, Stockholm, Sweden). The RNA extraction procedure was then conducted according to the manu-facturers' instructions. Total RNA concentrations were measured spectrophoto-metrically, and RNA integrity was controlled by agarose gel electrophoresis. Absence of DNA contamination was assessed by polymerase chain reaction (PCR).

4.2.1 Analysis of mRNA expression (on human samples)

Total RNA (1 μg) was used for semiquantitative reverse-transcription PCR (RT-PCR) with gene-specific primers. The RT-PCR was performed using the commercial kit Access RT-PCR System (Promega, Falkenberg, Sweden) according to the manufacturers' instructions. Primers for RT-PCR were designed using the Oligo Primer Analysis Software, version 6 (MedProbe, Oslo, Norway). The primers were ordered from CyberGene (Huddinge, Sweden). Primer sequences were the following: human β-actin (product length 427 bp), forward primer 5´-GGCACCACACCTTCTACAAT-3´ and reverse primer 5´-

CCATCTCTTGCTCGAAGT-3´; human BMP-2 (product length 330 bp), forward primer 5´-GCAAAGAAAAGGAACGGACA-3´ and reverse primer 5´-GTCTCTGTTTCAGGCCGAAC-3´; human TGF-β_2 (product length 407 bp), forward primer 5´-TGCCTG AACAACGGAT-3´ and reverse primer 5´-GGTCTGTTGTGACTCAAGTCT-3´; human TGF-β_3 (product length 409 bp), forward primer 5´-TGCTGAACTTTGCCACGGT-3´ and reverse primer 5´-CTGCTCGGAATAGGTTGGTTCT-3´.

The β-actin primers were used as internal control because β-actin has been shown to be constantly expressed in mesenchymal tissues as well as unaffected by the activity of TGF-βs (Pisano *et al.*, 2003). The PCRs were optimized according to standard procedures (Saiki *et al.*, 1988) in order to achieve linearity for all primer pairs under the same general conditions. The PCR products were labeled with 32P-ATP (Amersham Biosciences, Aylesbury, UK) and separated on a 1.5% agarose gel. The bands and some blank gel pieces of equal size were cut from the gel and dissolved in 4 mL scintillation fluid (UltimaGold; Packard Bioscience, Groningen, the Netherlands) overnight. The blank gel from the lanes was used as control to set the zero values. Radioactivity was measured with a Wallac (Turku, Finland) 1409 Liquid Scintillation Counter, and the expression levels of mRNA relative to β-actin mRNA were calculated.

4.3 *In situ* hybridization

The β-tricalcium phosphate implants were removed after 3 or 21 days and systematic cryosections were made. The sections were bleached with 0.6% H_2O_2. The sections were then washed in PBT (phosphate buffered saline containing 0.1% Tween 20) and treated with proteinase K solution for 5 minutes. Proteinase K reaction was stopped in glycine solution. Slides were refixed in 4% PFA containing 0.2% of glutaraldehyde. Commercial DIG-labeled probes (from GeneDetect, Auckland, New Zealand) were used to hybridize mRNAs of BMP-2; TGF-β_1; TGF-β_2 and TGF-β_3. For BMP-2 the hybridization probe was complementary to nucleotides 237–284 (TGTTTGGCCTGAAGCAGAGACCC AGGACGTCGTGGTGC), for TGF-β_1, the standard GeneDetect oligonucleotide probe pack for TGF-β_1 was used (code: GD1254-OP), for TGF-β_2 the hybridization probe was complementary to nucleotides 342–389 (TGCATCTGGTC CCGGTGGCGCTCAGTCTGTCTACCTGCAGCACCCTCG) and for TGF-β_3 the hybridization probe was complementary to nucleotides 720–767 (TTC GACATGATCCAGGGGCTGGCGGAGCACAATGAACTGGCAGTCTGC). Hybridization was carried out in RNA hybridization buffer (50% formamide, 5xSSC, 1% sodium dodecyl sulphate [SDS], at 70°C overnight, and post-hybridization washes were performed at a final stringency of 50% formamide/ 2xSSC at 70°C for 2 x 30 min. The anti-DIG antibody (Roche 11 093 274 910, Bromma Sweden) was used at a dilution of 1:5000, in blocking solution (1x 10x TBST (0.25 M Tris-HCl pH 7.5, 1.4 M NaCl, 27 mM KCl, 1% Tween 20) + 9 X MQ water) at 4°C overnight. Detection of the antibody was done using

NBT/BCIP stock solution (Roche (1 681 451)), which was diluted in (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, 0.1% Tween 20, 2 mM levamisole). The washing steps were performed in PBT.

4.4 Immunohistochemistry

Samples were sectioned and deparaffinized or cryosections were treated with 0.6% H_2O_2 to inactivate endogenous peroxidase and then with 1% BSA to block non-specific binding. After blocking, the sections were incubated with the mouse monoclonal antibody to TGF-β_1 (ab27969) or with the rabbit polyclonal antibodies to TGF-β_2 (ab15539), TGF-β_3 (ab15537) or BMP-2 (ab14933) overnight at 4ºC (all antibodies produced by Abcam Ltd., United Kingdom). In the study on the effects of femoral canal cells, the mouse monoclonal antibody to osteonectin (Acris Antibodies GmbH, Germany), the mouse monoclonal antibody to osteocalcin (Abcam Ltd., United Kingdom), or the rabbit polyclonal anti-Collagen type I antibody (Research Diagnostics Inc., NJ, USA) were used. Visualization of the primary antibodies was performed using the commercial kit "Strept ABComplex/HRP Duet Mouse/Rabbit system" (Dako Cytomation Denmark A/S, Denmark) and DAB+ Chromogen (Dako Cytomation, USA) as the substrate. The washing steps in-between were carried out in phosphate buffered saline (PBS) which contained 0.07% of Tween 20 as the detergent. Thionine blue (Sigma-Aldrich, St. Louis, USA) was used for background staining. No immunohistochemical staining was noted in negative controls where the primary antibody was omitted.

5. Evaluation of samples

5.1 Cell counting (on rat samples)

Cell counting and evaluation of growth factor distribution in different tissues was performed with the light microscope "Olympus BX51" (Olympus, Olympus Company Ltd., Japan) and analysis was made with the software "Cast 2" (Olympus, Olympus Company Ltd., Japan). Cavalieri's principle (which states that the volumes of two objects are equal if the areas of their corresponding cross-sections are in all cases equal) was applied and the relative as well as the absolute volumes of different types of tissues in the implants were calculated. Quantified data was analyzed by comparing the groups with the paired t-test.

5.2 Semiquantitative evaluation of *in situ* hybridization (on rat samples)

A subjective score system was adopted for visual interpretation of *in situ* hybridization slides for semiquantitative analysis of the data. The scale ranged from 0 to +++ (0 - absence/faint staining; + - weak staining; ++ - moderate staining; +++ - strong staining) as described by Matsuzaki and co-authors (Matsuzaki *et al.*, 1999). Differences between the groups were tested by the Mann-Whitney U test.

5.3 Semi-quantitative estimation of the content of proteins in heterotopic bone (on human samples)

Estimation was performed on randomly selected representative digital images of HO sections. The pictures of comparative slides were taken from the areas where only bone cells and the bone matrix were seen. At least 5 randomly selected pictures were evaluated for each HO. All pictures were taken at standard conditions using a 40x objective (Olympus BX-50 microscope equipped with a ColorView IIIu camera). Analysis of staining intensity in the bone was performed using the software Adobe Photoshop CS2 (Adobe Systems) as described by Lehr and co-workers (Lehr *et al.*, 1999). The procedure of determination of immunostaining intensity included the following steps. Using the Magic Wand tool in the Select menu, a typical stained area was selected with tolerance level set at 15 units. Applying the Similar command in the Select menu, all stained areas were automatically selected. An optical density plot of the selected area was generated using the Histogram tool in the Image menu.

The results are presented as the proportion of the stained areas in the whole analyzed HO area. The Mann-Whitney test was used to analyze the data.

6. Statistical analysis

Analysis of variance (ANOVA) with post-hoc testing was applied to reveal statistical significance in mRNA expression between different zones of human HO. The results obtained by systematic cell counting (according to Cavalieri's principle) were analysed with the t-test. Non-parametric statistics like the semiquantitative data of *in situ* hybridization and analysis of staining intensity in bone according to method by Lehr and co-workers (Lehr *et al.*, 1999) were analysed with the Mann-Whitney test.

RESULTS

1. Morphology of HO – description and comparison of the histological structure of immature and mature HOs in humans (Paper I)

### 1.1 Histology of immature and mature HOs

In our study HOs were divided into immature HOs (less than 17 months old) and mature HOs (3–9 years old). Immature ossifications consisted of ossicles formed almost completely of woven bone (see Figure 4). There were plenty of osteoblasts on the outer surface of immature HOs and active bone formation was noted. Only a small amount of cartilage was found. The HOs were surrounded by the fibrous connective tissue which contained many star-shaped cells with multiple processes and large nuclei, thus morphologically resembling undifferentiated mesenchymal cells. Also, proliferation of low-differentiated cells with the morphological appearance, resembling preosteoblastic and prechondroblastic cells, were seen.

Mature ossifications consisted of either one or a few ossicles where cross-sectional morphology was typical of trabecular bones: spongy area with bone marrow spaces surrounded by cortical bone (see Figure 5). The HOs were mostly surrounded by the fibrocartilaginous tissue. Remodeling of bone, including osteoblastic activity, was often seen on the surface of the formed HO.
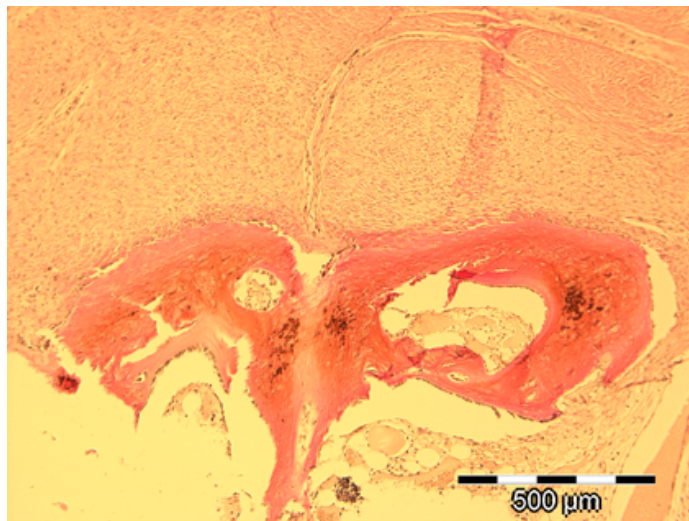
Figure 4. Immature HO (7–19 months old).
Azan staining. Red indicates formed bone.
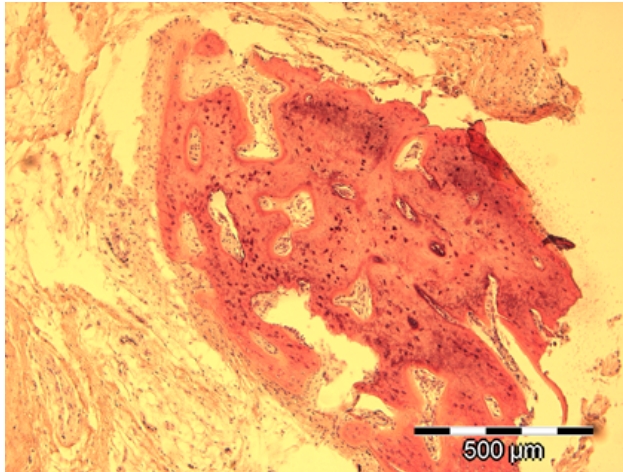
Figure 5. Mature HO (3–9 years). Azan staining. Red indicates formed bone. Bone marrow is visible in bone cavitations.

1.2 HO – an actively remodeling tissue

The HO is an actively remodeling bone tissue compared to regular bone tissue. The histological structure of the HO is similar to callus formation of a healing fracture and the onset of the ossification process seems to involve fibroblastic metaplasia (Vanden Bossche and Vanderstraeten, 2005).

Histological studies have demonstrated a zone of fibroblastic proliferation in the HO, followed by chondroblasts, which eventually are transformed into osteoblasts. However, HO formation could also be intramembranous. In this case the undifferentiated mesenchymal cells start producing bone straight from the connective tissue. Regardless of the origin of the bone, HO formation is rapid at first and slows down in the later stages, thus resembling the fracture healing process. Although remodeling and active tissue formation slow down with maturation of HO, remodeling activity remains higher than it is in normal bone, without turning into a malignant process.

2. Detection of differences in the expression and production of the BMP-2 and TGF-β subtypes between the zones of HO and orthotopic bone and in HO of different ages (Papers I and II)

2.1 mRNA expression in HOs compared to normal bone and the fibrous tissue

Specific changes in the expression of different growth factors may help understand the etiology of HO and therefore the expression and production of TGF-β isoforms were evaluated. As no clear differences were noted in the amount of TGF-β_1 in HO during the immunohistochemical study and the TGF-β_1 protein seemed to be universally present in many tissues, we only quantified the expressions of TGF-β_2 and TGF-β_3.

The expression of TGF-β_2 in the bone formation zone of ossifications (0.79 ± 0.35) was significantly higher than in control bone (0.32 ± 0.04, P = 0.050), while the difference was not significant for the capsular tissue. Relative expression levels of TGF-β_3 were 0.61 ± 0.12 and 0.59 ± 0.09 in the bone formation zone and in the surrounding tissue, respectively, which were significantly higher than in control bone (0.30 ± 0.03, P = 0.003 and 0.001, respectively) but not in the capsular tissue (0.43 ± 0.06). Expression levels of BMP-2 were similar in all groups. However, the highest expression (0.56 ± 0.16) was detected in the zone of bone formation, which was significantly higher than the mean value in the zone of formed bone (0.24 ± 0.02, P = 0.040) but, at the same time did not differ significantly from the values in the control sample.

### 2.2 mRNA expression in different zones of HOs

Expression levels of growth factors were normalized to the zone of formed bone (Figure 6). All middle zones (zones of bone formation) had higher levels of BMP-2, TGF-β_2, and TGF-β_3. In the outer zone, TGF-β_2 and TGF-β_3 were higher than in the central zone (zone of formed bone) (Figure 6).

All ossifications

Immature ossifications
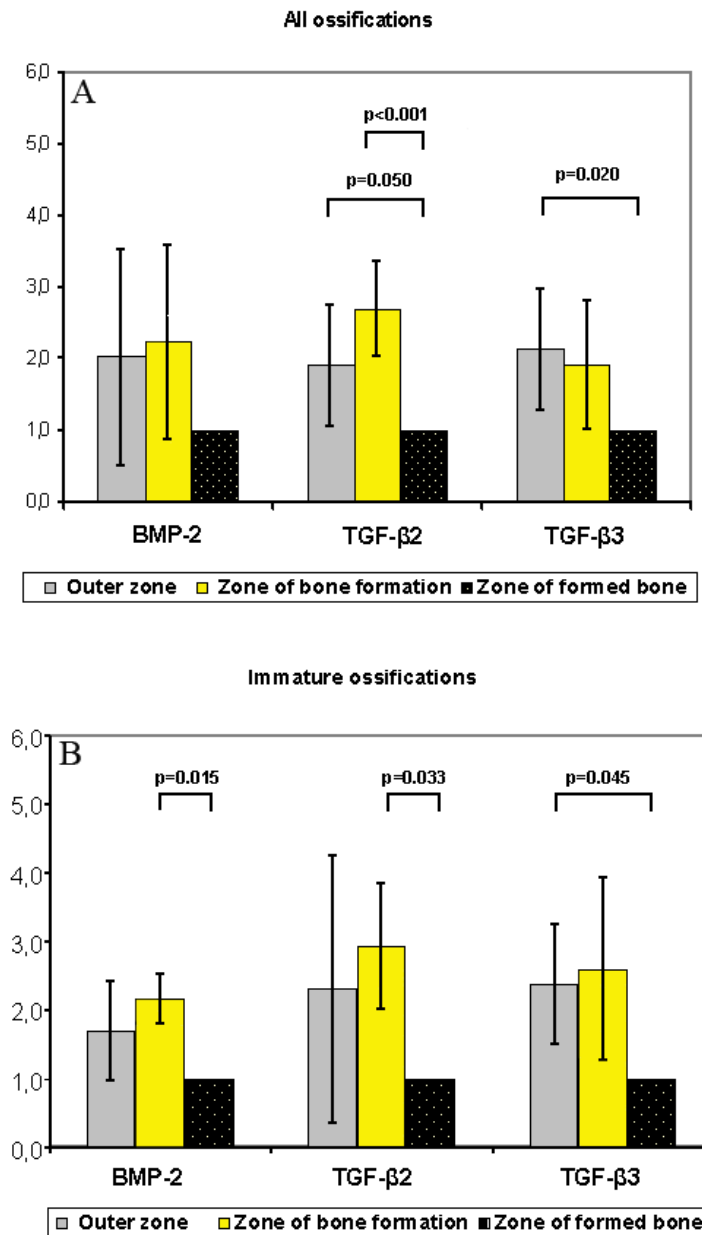

Figure 6. Expression of RNA for BMP-2, TGF-β_2, and TGF-β_3 genes in different zones of all (n = 7) (A) and immature (n = 3) (B) ossifications normalized to the zone of formed bone from the same patient.

2.3 The amount of growth factors in heterotopic bone

Immunohistochemistry showed the presence of all TGF-βs in the bone-forming zones of HOs. The three TGF-β isoforms showed different localization patterns in the heterotopic bone. The TGF-β_1 was mostly found in the connective tissue surrounding the HO and in the areas of osteoblastic bone formation (see Figure 7). A clear signal of TGF-β_2 was detected exclusively in the bone formation zones in undifferentiated mesenchymal cells and preosteoblasts (small, round cells lining bone surface), while during remodeling it was clearly detectable also in differentiated osteoblasts. Staining for TGF-β_2 was noted in the osteocytes of the immature HO (see Figure 8). The TGF-β_3 was located in the cells lining the formed HO (in all stages of differentiation of osteoblastic cells) in the bone formation zone but also in osteocytes in the deeper parts of the HO away from the bone formation area, as well as in osteoclasts. Staining for TGF-β_3 was also detected in the walls of the newly formed blood vessels as well as in the connective tissue surrounding the HO (see Figure 9). The protein expression of BMP-2 was detected mainly in the matrix of the woven bone, in osteoblasts, and in areas surrounding the osteoblasts in all stages of differentiation in immature ossificates (Figure 10), whereas in mature ossifications the expression was hardly detectable.
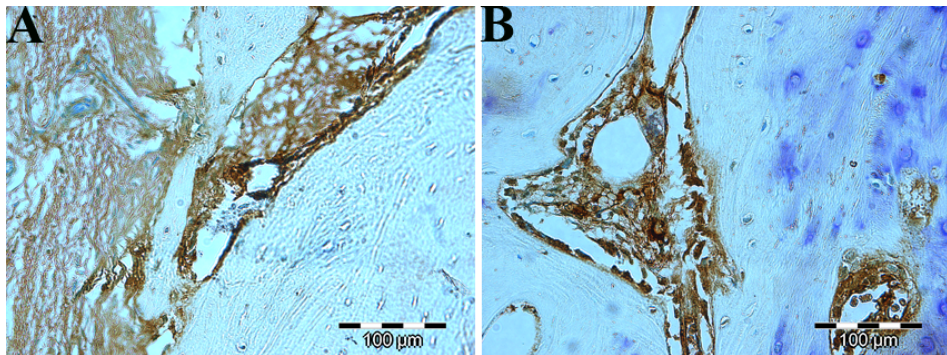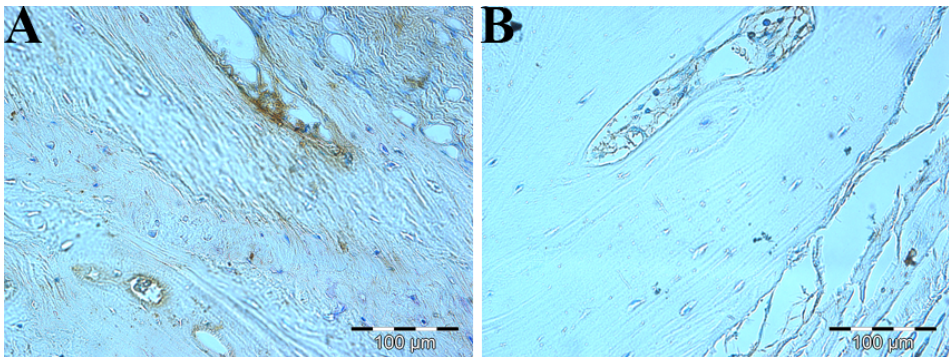

Figure 7. A – TGF-β_1 in an immature HO (7–19 months); B - TGF-β_1 in a mature HO (3–9 years). Staining can mostly be seen in osteoblasts and osteocytes but also in the connective tissue. Positive staining for the growth factor TGF-β_1 is brown.
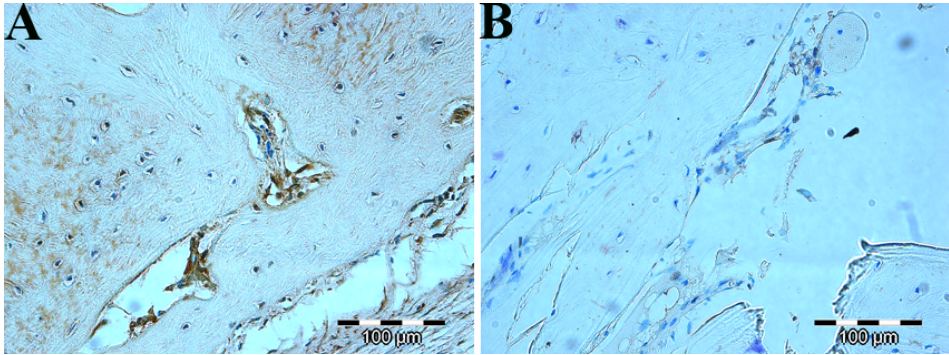
Figure 8. A – TGF-β_2 in an immature HO (7–19 months); B - TGF-β_2 in a mature HO (3–9 years). Staining can be seen in osteoblasts and osteocytes of the immature HO. Positive staining for the growth factor TGF-β_2 is brown.
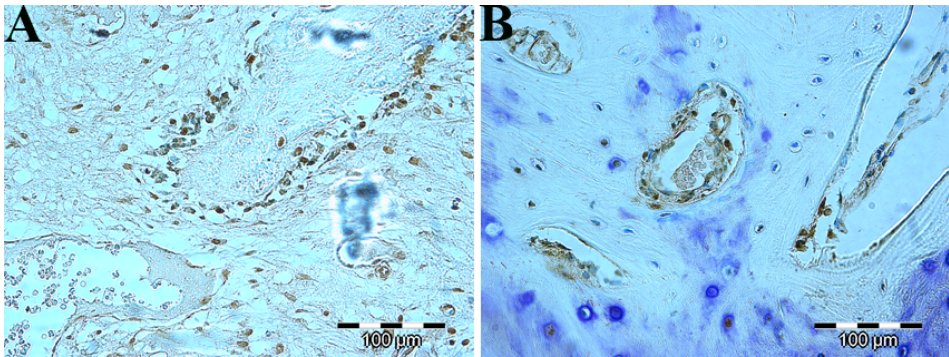
Figure 9. A - TGF-β_3 in an immature HO (7–19 months); B - TGF-β_3 in a mature HO (3–9 years). Staining can be seen in both osteoblasts and osteocytes. Positive staining for the growth factor TGF-β_3 is brown.

Figure 10. A – BMP-2 in an immature HO (7–19 months); B – BMP-2 in a mature HO (3–9 years). Staining can be seen in osteoblasts on bone surface. Positive staining for the growth factor BMP-2 is brown.

2.4 Changes in the content of growth factors
in heterotopic bone over time

Analysis of TGF-β isoforms showed that TGF-β_1 was the most abundant growth factor in the HO followed by TGF-β_3 and TGF-β_2. The only significant difference found between the immature and mature HO groups was that the content of TGF-β_2 was almost three fold higher in the immature heterotopic bone (P=0.0064; see Table 2). This difference was clear even despite the relatively small amount of immature HO samples.

Table 2. The percentages of TGF-β_1, TGF-β_2 and TGF-β_3 in the heterotopic bone, i.e. the proportion of immunostaining in the whole analyzed area $\pm$ SEM (standard error of the mean). Each analyzed area was a microscopic field within the HO. Adobe Photoshop was used for estimation of IHC staining. For further details see Materials and Methods.
* – P=0.0064.

TGF-β isoform	in immature HO	in mature HO
TGF-β_1	2.97 ($\pm$ 0.72)	2.22 ($\pm$ 0,87)
TGF-β_2	0.33 ($\pm$ 0.10)*	0.11 ($\pm$ 0.02)*
TGF-β_3	0.93 ($\pm$ 0.26)	0.90 ($\pm$ 0.18)

3. Differences in the expression, localization and content of the TGF-β isoforms at onset of HO in a rat model (Paper III)

In rats of group I (implants immersed in saline) and group II (implants immersed in rhBMP-2 solution) initial changes were studied at the site of expected HO formation on the third day after the inserting of beta-tricalcium phosphate implants into the capsulotomy wounds of the hip joint. In both groups the inner pores of the implants were mostly empty and the outward pores were filled with the connective tissue together with inflammatory and undifferentiated mesenchymal-like cells. A higher expression of TGF-β_2 and TGF-β_3 in group II, where rhBMP-2 was applied, was found by semi-quantitative evaluation of *in situ* hybridization (see Table 3 and Figure 11). Immunohistochemical staining of the proteins of all TGF-β isoforms was discovered in the connective tissue and mesenchymal-like cells. In group II the undifferentiated mesenchymal-like cells stained 49.6% more for TGF-β_3 compared to group I (p=0.024; see Table 3 and Figure 11). No other significant differences were found in the expression or production of growth factors at day three. Although immunohistochemical BMP-2 staining was increased in group II, it probably was due to the remaining exogenous protein.

In the implants removed from the rats 21 days after the operation (group III (implants immersed in saline) and group IV (implants immersed in rhBMP-2 solution)), early events of HO formation were studied. In group III, the surface of outer pores contained a limited amount of osteoid and cartilage. In group IV, where rhBMP-2 was applied, significant bone formation was found and cells penetrated the implants from all sides and widespread osteoid formation was observed. Evaluation of *in situ* hybridization suggested that the mRNA expression of TGF-β_3 was stronger in group IV compared to group III, but this difference was not obvious for immunohistochemical staining (see Table 4). Immunohistochemistry showed, that in groups III and IV all growth factors were present in all types of tissues, however in the connective tissue surrounding the heterotopic bone, all TGF-β isoforms were generally more prevalent than BMP-2 (see Table 4). In group IV osteoblasts stained 27.2% more for TGF-β_3 compared to TGF-β_1 (p=0.045) and seemed to stain more for TGF-β_2 compared to TGF-β_1 (p=0.09) (see Table 4). As seen in Table 4, in group IV, the bone tissue contained 32.1% and 47.8% more of TGF-β_2 and TGF-β_3 respectively, compared to TGF-β_1 (p=0.007 and p=0.006, respectively, for representative images, see Figure 12). In group IV the implants were largely reabsorbed and replaced by bone, indicating good osteoconductive properties of beta-tricalcium phosphate.

Table 3. Percentage of immunohistochemically (IHC) stained mesenchymal-like cells (± standard deviation; n=10 in each group). [a] – p=0.024 in Group II vs Group I for TGF-β_3. Total mRNA staining (*in situ* hybridization, ISH) in the implant graded as: 0 – absence/faint staining; + – weak staining; ++ – moderate staining; +++ – strong staining. [b] – p=0.0005 TGF-β_2 in Group II vs TGF-β_2 in Group I; [c] – p=0.001 TGF-β_3 in Group II vs TGF-β_3 in Group I.

	TGF-β_1		TGF-β_2		TGF-β_3	
	Group I	Group II	Group I	Group II	Group I	Group II
IHC	55.67±16.96	63.81±22.28	41.61±28.12	52.31±19.84	47.11±21.68[a]	70.46±22.55[a]
ISH	+	+	0[b]	+[b]	0[c]	+[c]

Table 4. Percentage of immunohistochemically (IHC) stained cells/tissue (±standard deviation; n=10 in each group). NP – not present

		BMP-2		TGF-β1		TGF-β2		TGF-β3	
		Gr III	Gr IV	Gr III	Gr IV	Gr III	Gr IV	Gr III	Gr IV
IHC	Osteoblasts	NP	71.73±15.88	NP	63.84±26.53^a	NP	77.87±14.03	NP	81.20±11.39^a
	Connective tissue	33.12±18.62^{b,c,d}	36.46±28.64	51.99±15.38^b	55.58±26.10	50.78±10.16^c	49.13±11.67	50.62±18.98^d	55.03±19.16
	Bone tissue except osteoblasts	NP	52.92±7.68	NP	46.76±3.49^{e,f}	NP	61.79±6.92^e	NP	69.12±11.82^f
ISH	Total mRNA staining in the implant	+	+	+	+	+	++	+^{g}	++^{g}

IHC of TGF-β3 in group I
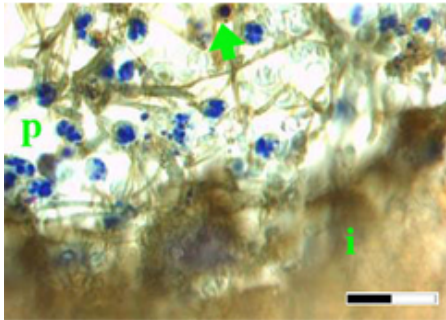
ISH of TGF-β3 in group I
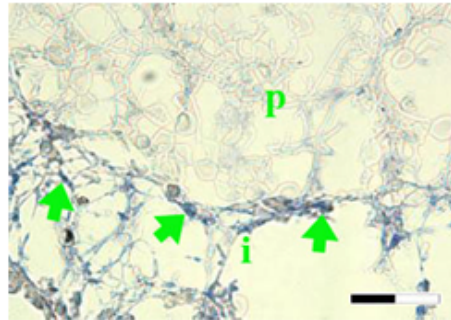
IHC of TGF-β3 in group II
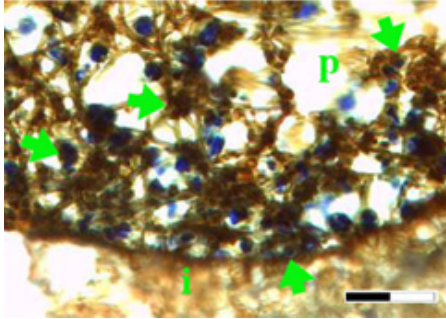
ISH of TGF-β3 in group II
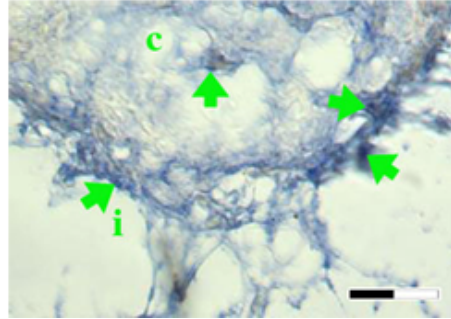
Figure 11. In group II (where rhBMP-2 was applied) the undifferentiated mesenchymal-like cells produced more TGF-β_3 compared to group I (brown staining marks the protein). Arrows show examples of positive staining. Some differences were also seen in mRNA expression (blue staining on the right). Legend: IHC – immunohistochemistry; ISH – *in situ* hybridization; i – remnants of the implant; p – pore of the implant; c – connective tissue. The scale bar represents 20 μm.
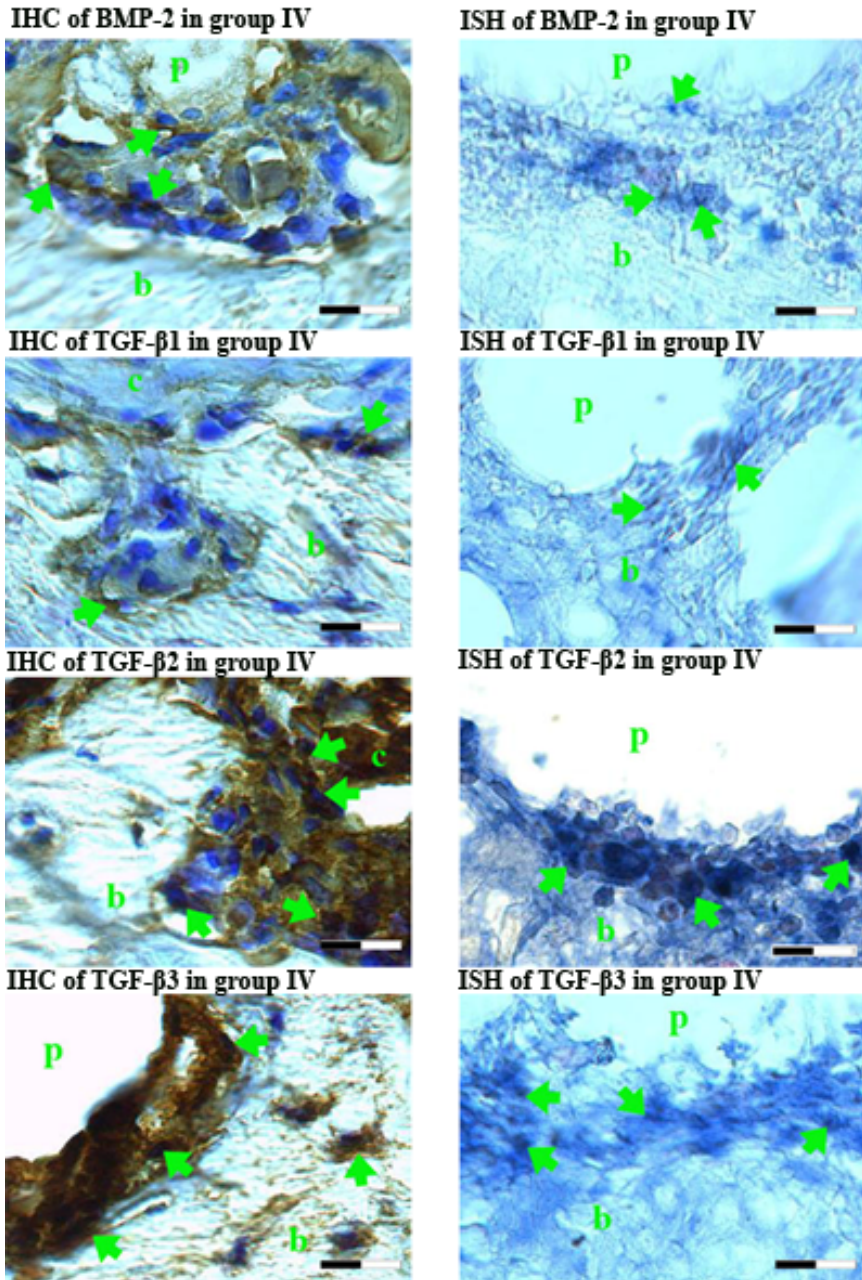
Figure 12. In group IV the cells lining the newly formed bone stained more for TGF-β_3 compared to TGF-β_1 (see Table 4). After the application of rhBMP-2, the newly formed bone tissue produced more TGF-β_2 and TGF-β_3 compared to TGF-β_1 (see Table 4) (brown staining marks the protein). Some differences were also seen in mRNA expression (blue staining on the right). Legend: IHC – immunohistochemistry; ISH – *in situ* hybridization; p – pore of the implant; b – bone tissue; c – connective tissue. The scale bar represents 20 μm.

4. How cells originating from the femoral canal influence heterotopic bone (Paper IV)

In order to study how cells originating from drilled femoral canals and damaged muscles influence formation of heterotopic bone, a model of heterotopic ossification, which allows or omits drilling of femoral canals, was designed to evaluate the effect of access of bone marrow cells to the joint area and periarticular tissues. To follow cellular dynamics in time, two timepoints were set (3 days and 21 days).

At 3 days the pores of the cubes were mostly filled with inflammatory cells and there was no significant difference between the implants in the degree of cellular penetration. Poorly differentiated connective tissue cells were present in the outer pores of all implants. The proportion of connective tissue-filled implant volume in total implant volume was significantly different for the rhBMP-2 groups and for the saline treated groups, 5.22±1.67% and 1.31± 0.35%, respectively (p=0.004). There was no difference between the implants where femoral canal cells were present and the implants where they were absent with regard to the presence of connective tissue cells. Considering the endothelial lining the reference of the vascular lumen, this histological finding suggests activation of perivascular cells.

At 21 days no bone tissue had been formed in the saline treated groups, except for one implant from the group with the open femoral canal, where an osteoid region with a volume of 0.075 mm^3 was detected (Figure 13). In the other samples, osteoblast-like cells were only occasionally found on the surface of the implant, without any recognizable osteoid or bone formation. Negligible fibrous cartilage formation was detected with metachromatic staining in the outer pores of some samples from the saline treated groups. Nor was there any difference between the saline treated groups in the number of fibrous tissue cells, capillary sprouts, or inflammatory cells. The saline impregnated implants were mostly surrounded by cell-rich fibrous connective tissue with fibers positioned in arbitrary directions, which were observed similarly in both groups. There was no qualitatively distinguishable difference between the osteocalcin and osteonectin expression patterns as revealed by immunohistochemical staining, either.
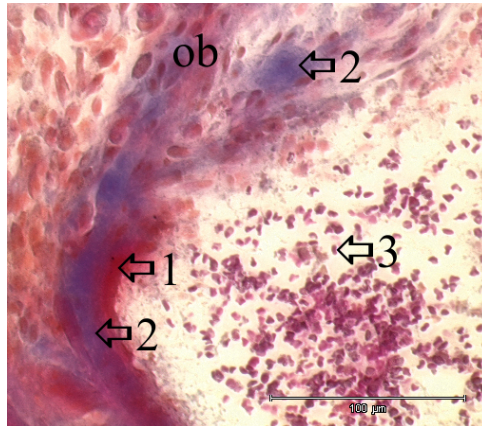
Figure 13. Picture of an implant where no rhBMP-2 was added but access of the femoral canal cells was granted. There was only one limited area in one sample from group 1A where osteoid formation was recognizable. (AZAN stain): 1 – osteoid mineralization, 2 – osteoid seal, 3 – implant, note the presence of some inflammatory cells inside the implant material, ob – osteoblastic cells.

Heterotopic bone was induced in all implants in the rhBMP-2 treated groups. Ossification occurred around the implants, in the pores of the implants, and in the implant itself, partly replacing the cube with newly formed bone (Figure 14A and 14B). Complete replacement of the implant occurred in one of the five in the group with the open femoral canal and in two of the five in the group with the closed femoral canal. A rigid bridging between the implant and the greater trochanter was seen in 60% of the group with the open femoral canal and in 80% of the group with the closed femoral canal. There was no case of completely ossified bridging; the connections were predominantly of the calcified fibrocartilage or hyaline cartilage.

The mean ratio of bone volume to total sample volume (BV/TV) was similar for the groups with the open and closed femoral canal, 33.1% (SD 7.4%) vs 30.0% (SD 7.6%) (p=0.234). However, comparison between these groups revealed that more bone had been mineralized in the group with the closed femoral canal, where mineralized volume (Md.V/TV) was higher than in the group with the open femoral canal, 18.2% ($\pm$ 4.5%) vs 12.7% ($\pm$ 2.9%) (p=0.019). The ratio of osteoid volume to bone volume (OV/BV) was higher in the group with the open femoral canal than in the group with the closed femoral canal, 57.3% ($\pm$ 5.0%) vs 45.3% ($\pm$ 3.0%) (p=0.010). The ratio of osteoid surface to total bone surface (OS/BS), 61.3% ($\pm$ 5.8%) in the group with the open femoral canal and 56.8% ($\pm$ 6.8%) in the group with the closed femoral canal, did not differ significantly between the groups (p=0.184). There was no significant difference in the ratio of osteoblast surface to bone surface (Ob.S/BS) or in the ratio of eroded surface to bone surface (ES/BS) between these two groups, either.
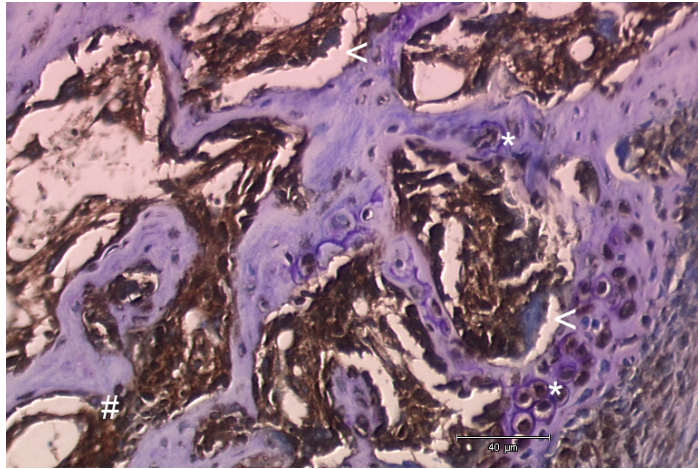
Figure 14A. Representative microanatomical photo of an implant. Immunohistochemical staining for osteocalcin. Note very fast bone formation expressed as intense resorption of the implant material by osteoclasts (<), newly formed bone trabeculae (#) and marked osteocalcin expression (brown stain) in actively synthesizing osteoblasts but also in osteocytes and in some cartilage cells present in the cartilage remnants (*) (Osteocalcin, counterstain with toluidin).
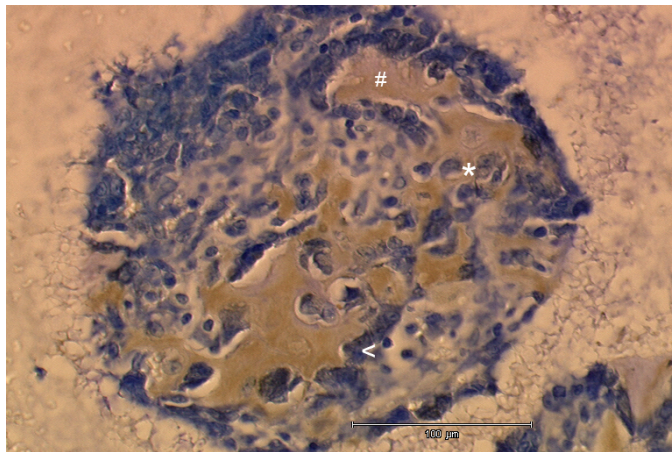
Figure 14B. Representative microanatomical photo of an implant. Note the remodeling of implant structure expressed as osteoclastic resorptive activity (<), as well as the formation of cartilagineous (*) and bony structures inside the pores (#); (toluidin stain).

DISCUSSION

Transforming growth factor beta (TGF-β) isoforms have shown to have versatile effects on bone metabolism. Different levels of expression of TGF-β isoforms and their receptors have been shown in human bone formation (Horner *et al.*, 1998). TGF-βs differentially affect osteoblast differentiation by promoting the early stages and suppressing final differentiation, while their role in osteoclast functioning and bone resorption seem to be very complex as TGF-β have been found to either stimulate or repress osteoclast differentiation depending on TGF-β concentration and on the model system used (Janssens *et al.*, 2005). This research focused on TGF-β isoforms in development of human heterotopic ossification (HO) and on the initial and early stages of HO formation in an animal model mimicking the situation after THA, as well as and also on local morphological changes in both cases.

1. TGF-β_1, TGF-β_2 and TGF-β_3 in human HO

Although it is generally agreed that TGF-β isoforms play an important role in HO formation, little is known about the functions of TGF-β isoforms in this process. In our study we evaluated the dynamics of expression and production of TGF-β isoforms in time after THA. In 1994 Bosse and coworkers described higher expression of TGF-β_2 in the zones of bone formation and higher expression of TGF-β_3 in the zone of cellular differentiation (Bosse *et al.*, 1994a). They demonstrated the expression of TGF-β_1 in the areas of endochondral ossification by using digoxigenin-labeled cDNA probes. Our results indicate that TGF-β_2 and TGF-β_3 isoforms are related to intramembranous ossification, since it seems to be the main mode of HO formation after THA (Paper II). We also showed increased expression of TGF-β_2 and TGF-β_3 in immature HOs as compared to mature HOs, using a method with β-actin serving as control for expression (Paper II). We confirmed that the HO content of TGF-β_2 is clearly higher in immature HOs compared to mature HOs and that increased expression thus leads to increased formation of TGF-β_2 (Paper I). It should, however, be noted that the overall bone content of TGF-β_2 was lower compared to the other TGF-β isoforms (see Table 2). We also demonstrated that TGF-β_3 expression was increased (Paper II) while no difference was seen in protein level. However, it should be emphasized that in tissues protein level does not always correspond to the mRNA profile. This difference in mRNA expression and the protein content of TGF-β_3 can be explained by the fact that TGF-β genes also appear to be regulated posttranscriptionally, which may account for lack of correlation between mRNA expression and level of protein secreted from some cells (Bonewald, 2002).

Our results suggest that after THA, HO originates mostly from connective tissue damage (Paper II), in which several effectors like BMP-2, TGF-β iso-

forms, PGE_2, IL-6, etc can be involved (Roelen and Dijke, 2003; Bartlett *et al.*, 2006; De Benedetti *et al.*, 2006).

Since TGF-βs have the ability to accelerate bone formation (Joyce *et al.*, 1990a) and since growing HO could be problematic after THA if not treated, it was in our interest to find out specific changes in TGF-β isoforms that can be related to bone formation.

Immunohistochemically, there are distinctive differences in the location of the three isoforms in HOs: TGF-$β_2$ and TGF-$β_3$ are both produced more in the osteoblastic and preosteoblastic cells compared to TGF-$β_1$ which seemed to be more prominent in the surrounding connective tissue.

We also found strong TGF-$β_3$ protein staining in the walls of the vessels located densely in the region surrounding the bone formation zone. It may be suggested that TGF-$β_3$ is involved in the neovascularization of the HO since TGF-$β_3$ is known to promote mesenchymal cell proliferation and angiogenesis (Muraoka *et al.*, 2005). Detection of TGF-$β_3$ in blood vessels and osteocytes, but also in osteoblasts suggests that TGF-$β_3$ is involved in the normal function and remodeling of the HO. The TGF-$β_2$, which is detected in osteoblasts, seems to participate in their functioning and is produced more in immature HOs. Since osteoblasts and preosteoblasts have a high osteogenic potential (Puzas *et al.*, 1989), inhibition of TGF-$β_2$ may also specifically inhibit HO formation.

2. Initial and early changes of TGF-β in HO in the rat model

The experimental rat model involving application of implants immersed in saline or in rhBMP-2 solution was used to investigate changes in the localization, expression and content of TGF-β isoforms during the initial and early stages of HO formation. The implants were removed 3 days (groups I and II) and 21 days (groups III and IV) after implantation.

In this experimental model undifferentiated mesenchymal-like cells and connective tissue cells invaded the outer parts of the implant after three days. Although no overall changes in histological appearance were noted, a proportion of TGF-$β_3$ was increased in the mesenchymal-like cells when rhBMP-2 was added. Therefore, we can conclude that addition of rhBMP upregulates the production of TGF-$β_3$ and that more TGF-$β_3$ is produced in the area where HO formation is expected. This was also supported by the *in situ* hybridization findings of mRNA of TGF-$β_3$, which showed stronger expression in group II compared to group I (see Table 3). The total expression of mRNA of TGF-$β_3$ in the implant was stronger in group IV compared to group III (see Table 4). This difference was not obvious in the estimations of immunohistochemical staining of TGF-$β_3$ in the connective tissue in the implant, while it was not possible to compare staining of the bone tissue as there was almost no bone formation in group III. However, compared to TGF-$β_1$, TGF-$β_3$ was produced more in the osteoblasts lining the newly formed heterotopic bone in group IV, which was

not noted in group III (see Table 4). These results indicate the probable regulating role of this growth factor for the process of HO formation, since it has been described to participate in the differentiation of mesenchymal stem cells (Barry *et al.*, 2001). There were more immunohistochemically detectable TGF-β_2 and TGF-β_3 in newly formed heterotopic bone compared to TGF-β_1 (see Table 4). Although this difference was not evident at the mRNA level, it suggests that these isoforms are produced more during the remodelation of a newly formed HO. Since the bone content of these two isoforms increased in the early stages of the HO formation, it may be that these isoforms take part in both formation and remodeling of the HO.

In induced HOs the expression of TGF-β_1 has been noted to increase by day 30 (Ripamonti, 2005). Our results indicate that during induced HO formation changes in the production of the other isoforms (TGF-β_2 and TGF-β_3) take place earlier. Since isoforms TGF-β_2 and TGF-β_3 rather than TGF-β_1 have been shown to be expressed during the course of fracture healing in murine bone (Cho and Gerstenfeld, 2002), these results show that the pattern of changes in the tissue content of TGF-β_2 and TGF-β_3 during HO formation is similar to the pattern noted during fracture healing. Specific changes in mRNA levels of TGF-β_2 and TGF-β_3 rather than TGF-β_1 have also been shown during experimental HO formation after Achilles tenotomy (Lin *et al.*, 2009), which indicates their role in the early phases of HO formation. Although TGF-β_1 is considered the most abundant isoform of the three, with 200 µg/kg of TGF-β_1 present in bone (Seyedin *et al.*, 1985), it seems that this accumulation does not take place within 21 days from induction and may occur later during bone remodeling.

3. Factors related to the open femoral canal

Our method was developed to study heterotopic bone formation under standardized conditions regarding bone induction and osteoconduction by using homogenous materials with an exact chemical composition (beta-tricalcium phosphate, rhBMP-2) and mimicking the situation after total hip replacement surgery.

Many reports address occurrence of the HO in the region of the abductor musculature, especially in the gluteus medius and gluteus minimus muscles (Nollen and Slooff, 1973; Bisla *et al.*, 1976; Søballe *et al.*, 1988; Puzas *et al.*, 1989; Kjaersgaard-Andersen *et al.*, 1990; Ahrengart, 1991), which correspond to the structures often subjected to tensile forces and damaged during total hip replacement surgery. The same location, i.e. the greater trochanter underlying the abductor musculature, involving the capsulotomy wound, was used in our model by determining the exact amount of osteoconductive and/or osteoinductive factors, our method allows to reduce variability of bone induction and the number of subjects needed for studying local factors.

Osteoid volume (OV/BV) was very high in the rhBMP-2 treated groups, which indicates a high rate of formation of new bone. Thus, HO formation was

evident in both hind limbs in all animals, regardless of the presence or absence of femoral canal cells. It is difficult to explain the significantly lower mineralized volume of bone (Md.V/TV) on the side with the open femoral canal, while the volume of formed bone (BV/TV) itself was similar on both sides. This may be due to an increased washout of rhBMP-2 caused by bleeding from the femoral canal; presence of inhibitory factors originating from the femoral canal cannot be ruled out.

This study suggests that presence or absence of femoral canal cells has no significant effect on development of HO. Secondly, tissue injury and implant alone are insufficient to cause heterotopic ossification and osteoinductive signal, as e.g. rhBMP-2 plays a crucial role in heterotopic ossification. Thirdly, we believe that this method can be used in *in vivo* studies of HO.

4. General discussion

Formation of HO is a complex process. Involvement of four factors is necessary in the pathogenesis of heterotopic bone (Kaplan *et al.*, 2004). The first factor is usually an episode of trauma which may include a hematoma. In true myositis ossificans trauma is often minimal consisting in only a few torn muscle or collagen fibers (Jackson *et al.*, 2009). The second factor is a signal from the site of injury. This signal consists of proteins secreted from cells of the injured tissue and from inflammatory cells migrating to the site in response to the tissue injury (Ahn *et al.*, 2003; Kaplan *et al.*, 2004; Paper I). Also a change in the chemical environment may serve as the signal (e.g. in the case of pulmonary HO formation (Chan *et al.*, 2002)) or dystrophic calcification (ossification of the cardiac valves (Mohler *et al.*, 2001)). The third factor is availability of cells that can produce bone (e.g. undifferentiated mesenchymal cells, preosteoblasts). With the help of the appropriate signal at the site of injury, the genes which synthesize osteoid and chondroid and direct differentiation of mesenchymal cells into osteoblasts or chondroblasts are activated (McCarthy and Sundaram, 2005; Jackson *et al.*, 2009). Heterotopic bone formation can occur anywhere where these uncommitted mesenchymal cells are present, like in the skeletal muscle, the perivascular tissue and the fibrous tissue. The fourth factor is an appropriate environment conductive to the continued production of heterotopic bone (e.g. formation of a calcified area or scaffold) (Ekelund *et al.*, 1991). The relationship between these four factors is debatable. According to McCarthy and Sundaram (2005), signaling agents appear to play the most important role and progress has been made recently in the understanding of these agents.

One finding that may change the perspective of factors important for HO formation is presented in this thesis: we found that TGF-β isoforms have different expression patterns during HO formation and their relative content changes differently during HO maturation. These changes were notable for the less studied isoforms TGF-β_2 and TGF-β_3, and especially in undifferentiated mesenchymal cells in the area of HO formation (Paper I; Paper III).

The HOs removed from patients after total hip arthroplasty reveal morphological (rapid bone formation, presence of bone forming zones) and biochemical changes (growth factors, like BMP-2 and isoforms of TGF-β, but also IL-6, PGE_2, type-I collagen, etc) occurring over the time (Paper I; Paper II; Wilkinson *et al.*, 2003). In order to investigate initial and early changes at the site of HO formation, an animal model was created which mimicks the situation after THA. The advantage of our model compared to other methods is that osteo-induction occurs in the joint area and its variability is low, which was achieved by determining the exact amount of osteoconductive and/or osteoinductive factors. The method of HO formation introduced by Schneider and co-workers (Schneider *et al.*, 1998) mimicked the situation after THA but in that case osteoinduction varied considerably. According to the method of Kantorowitz and co-authors (Kantorowitz *et al.*, 1990), HO formation was studied in a subcutaneous location and in a situation not occurring after THA. We standardized the osteoinductive signal by using exogenous rhBMP-2 (whose osteoinductive properties have been well described (Urist, 1965; Wozney *et al.*, 1988)) and chose beta-tricalcium phosphate as the carrier of rhBMP-2 since it has the ability to form a depot of recombinant BMP-2 (Uludag *et al.*, 1999; Seeherman and Wozney, 2005). It can be noted that the concentration of rhBMP-2 in the implant remains relatively constant and beta-tricalcium phosphate possesses good osteoconductive properties (Seeherman and Wozney, 2005). The usage of an external inductor (BMP-2) in this model can be considered a disadvantage. However, this was absolutely necessary to ensure stable bone growth. Although the animal model raises the question about the comparability and extrapolation of these results to humans, we have shown that the histological appearance of HOs and the dynamics of TGF-β isoforms are similar in the rat and in the human during the growth of HO, which confirms that the introduced model is suitable for experiments exploring the dynamics of TGF-β isoforms during HO formation (Paper III; Paper IV).

The TGF-β isoforms have similar but not overlapping effects and their expression patterns are different during normal bone formation (Cho and Gerstenfeld, 2002). Changes in the expression and production of HOs in time show that their relative functions in HOs change in time. Differences in the expression and HO content of TGF-β isoforms at different time points suggest that these isoforms may have different roles in formation of HO and further demonstrate the complexity of the HO formation.

Numerous factors affect bone as well as HO formation and it is clear that TGF-β isoforms play an important role in this process. So far the literature data about the functions of TGF-β isoforms have been controversial. In this thesis we showed that TGF-β isoforms are present in HO and their dynamics can be followed in time. Our studies suggest that there are distinctive differences in the expression and content of TGF-β isoforms in HO depending on the age or type of HO formation.

Although $TGF-\beta_1$ is considered to be the most abundant isoform of the three, we showed that the expression and production of the isoforms $TGF-\beta_2$ and

TGF-β_3 are more prevalent in the early stages of HO formation. In healing fractures, the isoform expressions of the TGF-βs have also been observed, with more prominent expressions of the TGF-β_2 and TGF-β_3 isoforms (Rosier *et al.*, 1998). Since the bone content of these two isoforms was also elevated in the early stages of HO formation, it may be that these isoforms take part in both formation and remodeling of HO, particularly during the initial and early stages of heterotopic bone formation (Paper III). Moreover, it has been noted that the histological structure of the HO is similar to the histology noted in callus formation of a healing fracture and the onset of the ossification process seems to involve fibroblastic metaplasia (Vanden Bossche and Vanderstraeten, 2005). Considering the above findings, it can be suggested that the process of HO formation resembles the process of fracture healing and is (like fracture healing) initiated by tissue damage and an inflammatory process involving several inflammatory markers (e.g. PGE$_2$), inflammatory cells, growth factors (vascular growth factors, BMP-2, TGF-β isoforms) (Tatsuyama *et al.*, 2000; Rapuano *et al.*, 2008; Paper I; Paper III). This is also confirmed by the fact that therapy with NSAIDs has been proven to be successful in prevention of HO after THA (Gebuhr *et al.*, 1995; Dorn *et al.*, 1998; Persson *et al.*, 1998). Therefore, suppression of inflammatory response immediately after THA is an important tool in HO prevention.

Also recent results support the notion that HO is initiated by tissue damage and not by multipotent cells which migrate from the femoral canal to the surrounding tissues (Paper IV).

Further research should establish whether usage of TGF-β isoform specific inhibitors might have some pharmacological potential and whether they could be used for HO suppression.

An overall review of the literature shows tens of biomolecules that are active at some point of bone formation and growth. Although the problem has been studied by different authors it is still not completely solved; further testing and introduction of new animal models would better elucidate this issue. The picture emerging from the study of the TGF-β isoforms indicates that they are somehow involved in almost every aspect of HO formation. Their way of action is intricate with overlapping features, which makes evaluation of their importance ambiguous. This complication is further confused by our finding in this thesis that importance of the TGF-β isoforms changes over time. Further research, probably largely restricted to *in vitro* action studies, are needed to clarify the role of the TGF-β isoforms.

CONCLUSIONS

1. Immature HOs had multiple ossicles with clearly distinguishable active bone formation on their outer surfaces, with bone marrow but also undifferentiated mesenchymal-like cells remaining inside. Mature HOs were histologically very similar, consisting of one or a few ossicles whose cross-sectional morphology was typical of trabecular bones: spongy area with bone marrow spaces surrounded by cortical bone. The OS/Ps ratios were higher for mature HOs compared to control bone, which indicates that remodeling activity in mature HOs is still high. The HO has high bone forming activity which slows down with time. Based on morphological and morphometric findings, the HO can be considered as mature after three years.

2. The expressions of TGF-β_2 and TGF-β_3 were increased during HO formation and remodeling (in the zone of bone formation and in the zone of cellular differentiation of HO) and the bone tissue content of TGF-β_2 was clearly reduced in mature HOs suggesting that the isoform TGF-β_2 is definitely involved in the growth of immature HO.

3. Stable and reliable bone formation, similar to the situation after THA, was achieved in the joint area in the presented animal model, which confirms that this model can be used to evaluate initial and early changes during HO formation.

4. A specific increase in the expression and tissue content of TGF-β_2 and TGF-β_3 was shown in the initial and early stages of heterotopic bone formation; it is likely that these TGF-β isoforms had a particular effect on HO formation at these developmental stages in the experimental rat model.

5. Presence of femoral canal cells did not exert an additional osteoinductive effect, or an effect on the amount of bone to be formed and osteoinductive signal from exogenous BMP-2 was prevalent in our experimental HO rat model, suggesting that local signals and cells of local origin have a more significant role in HO formation compared to the effect of the multipotent cells of the femoral canal.

General conclusion: From the point of view of the histology and dynamics of the TGF-β isoforms, the process of HO formation resembles the fracture healing process rather than normal bone development.

REFERENCES

Abrahamsson SO, Ahlgren SA, Dahlström JA, Ohlin P, Stigsson L (1984) Ectopic bone after hip replacement. Excision and free fat transplants in four cases. Acta Orthop Scand 55: 589–592.

Ackerman LV (1958) Extra-osseous localized non-neoplastic bone and cartilage formation (so-called myositis ossificans). J Bone Joint Surg Am 40: 279–298.

Ahn J, Serrano de la Pena L, Shore EM, Kaplan FS (2003) Paresis of a bone morphogenetic protein-antagonist response in a genetic disorder of heterotopic skeletogenesis. J Bone Joint Surg Am 85-A(4): 667–674.

Ahrengart L (1991) Periarticular heterotopic ossification after total hip arthroplasty. Risk factors and consequences. Clin Orthop 263: 49–58.

Arcq M (1973) [Ectopic ossification: a complication after total hip replacement (author's transl)] Arch Orthop Unfallchir 77(2): 108–131.

Arsura M, Panta GR, Bilyeu JD, Cavin LG, Sovak MA, Oliver AA (2003) Transient activation of NF-kappaB through a TAK1/IKK kinase pathway by TGF-beta1 inhibits AP-1/SMAD signaling and apoptosis: implications in liver tumor formation. Oncogene 22: 412–425.

Assoian R, Komoriya A, Meyers C, Miller D, Sporn M (1983) Transforming growth factor-beta in human platelets. Identification of a major storage site, purification, and characterization. J Biol Chem 258 (11): 7155–7160.

Balboni TA, Gobezie R, Mamon HJ (2006) Heterotopic ossification: Pathophysiology, clinical features, and the role of radiotherapy for prophylaxis. Int J Radiat Oncol Biol Phys 65(5): 1289–1299.

Barry F, Boynton RE, Liu B, Murphy JM (2001) Chondrogenic differentiation of mesenchymal stem cells from bone marrow: differentiation-dependent gene expression of matrix components. Exp Cell Res 268(2): 189–200.

Bartlett CS, Rapuano BE, Lorich DG, Wu T, Anderson RC, Tomin E, Hsu JF, Lane JM, Helfet DL (2006) Early changes in prostaglandins precede bone formation in a rabbit model of heterotopic ossification. Bone 38: 322–332.

Bascom CC, Wolfshohl JR, Coffey RJ Jr, Madisen L, Webb NR, Purchio AR, Derynck R, Moses HL (1989) Complex regulation of transforming growth factor beta 1, beta 2, and beta 3 mRNA expression in mouse fibroblasts and keratinocytes by transforming growth factors beta 1 and beta 2. Mol Cell Biol 9(12): 5508–5515.

Bisla RS, Ranawat CS, Inglis AE (1976) Total hip replacement in patients with ankylosing spondylitis with involvement of the hip, J Bone Joint Surg (Am) 58: 233.

Blobe GC, Schiemann WP, Lodish HF (2000) Role of transforming growth factor beta in human disease. N Engl J Med 342(18): 1350–1358.

Bonewald LF (2002) Transforming growth factor-β. In: Bilezikian JP, Raisz LG, Rodan GA (Eds.) Principles of bone biology (second edition) volume 1. Academic press, p 903–918.

Bonniaud P, Margetts PJ, Kolb M, Haberberger T, Kelly M, Robertson J, Gauldie J (2003) Adenoviral gene transfer of connective tissue growth factor in the lung induces transient fibrosis. Am J Respir Crit Care Med 168(7): 770–778.

Bosse A (1997) Clinical aspects, differential diagnosis and histogenesis of heterotopic ossification. Veroff Pathol 146: 1–168.

Bosse A, Wulf M, Wiethege T, Voss B, Müller KM (1994)a Collagens and growth factors in heterotopic ossification. Pathologe 15: 216–225.

Bosse A, Wanner KF, Weber A, Müller KM (1994)b Morphological and clinical aspects of heterotopic ossification in sports. A case study. Int J Sports Med 15(6): 325–329.

Bouletreau PJ, Steinbrech D, Spector JA, Warren SM, Greenwald JA, Mehrara BJ, Detch RC, Longaker MT (2000) Gene expression of transforming growth factor-beta 3 and tissue inhibitor of metalloproteinase type 1 during membranous bone healing in rats. J Craniofac Surg 11(6): 521–526.

Brooker AF, Bowerman JW, Robinson RA, Riley LH Jr (1973) Ectopic ossification following total hip replacement. Incidence and a method of classification. J Bone Joint Surg Am 55(8): 1629–1632.

Burmester JK, Qian SW, Roberts AB, Huang A, Amatayakul-Chantler S, Suardet L, Odartchenko N, Madri JA, Sporn MB (1993) Characterization of distinct functional domains of transforming growth factor beta. Proc Natl Acad Sci USA 90(18): 8628–8632.

Centrella M, Horowitz MC, Wozney JM, McCarthy TL (1994) Transforming growth factor-beta gene family members and bone. Endocr Rev 15(1): 27–39.

Chandrasekharan NV, Dai H, Roos KL, Evanson NK, Tomsik J, Elton TS, Simmons DL (2002) COX-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other analgesic/antipyretic drugs: Cloning, structure, and expression. Proc Natl Acad Sci USA 99(21): 13926–13931.

Chan ED, Morales DV, Welsh CH, McDermott MT, Schwarz MI (2002) Calcium deposition with or without bone formation in the lung. Am J Respir Crit Care Med 165(12): 1654–1669.

Chang H, Brown CW, Matzuk MM (2002) Genetic analysis of the mammalian transforming growth factor-beta superfamily. Endocr Rev 23(6): 787–823.

Cho TJ, Gerstenfeld LC (2002) Differential temporal expression of members of the transforming growth factor beta superfamily during murine fracture healing. J Bone Miner Res 17: 513–520.

Copland IB, Adamson SL, Post M, Lye SJ, Caniggia I (2002) TGF-beta 3 expression during umbilical cord development and its alteration in pre-eclampsia. Placenta 23(4): 311–321.

De Benedetti F, Rucci N, Del Fattore A, Peruzzi B, Paro R, Longo M, Vivarelli M, Muratori F, Berni S, Ballanti P, Ferrari S, Teti A (2006) Impaired skeletal development in interleukin-6-transgenic mice: a model for the impact of chronic inflammation on the growing skeletal system. Arthritis Rheum 54: 3551–3563.

de Caestecker M (2004) The transforming growth factor-beta superfamily of receptors. Cytokine Growth Factor Rev 15(1): 1–11.

de Caestecker MP, Piek E, Roberts AB (2000) Role of transforming growth factor-beta signaling in cancer. J Natl Cancer Inst 92(17): 1388–1402.

Dejerine A, Ceillier A (1918) Para-osteo-arthropathies des paraplegigues par lesion medullarie; etude clinique et radiographique. Ann Med 5: 497.

DeLee J, Ferrari A, Charnley J (1976) Ectopic bone formation following low friction arthroplasty of the hip. Clin Orthop Relat Res 121: 53–59.

Della Valle AG, Ruzo PS, Pavone V, Tolo E, Mintz DN, Salvati EA (2002) Heterotopic ossification after total hip arthroplasty: a critical analysis of the Brooker classification and proposal of a simplified rating system. J Arthroplasty 17(7): 870–875.

Dorn U, Grethen C, Effenberger H, Berka H, Ramsauer T, Drekonja T (1998) Indomethacin for prevention of heterotopic ossification after hip arthroplasty. A randomized comparison between 4 and 8 days of treatment. Acta Orthop Scand 69(2): 107–110.

Ekelund A, Brosjö O, Nilsson OS (1991) Experimental induction of heterotopic bone. Clin Orthop Relat Res 263: 102–112.

Elmstedt E, Lindholm TS, Nilsson OS, Törnkvist H (1985) Effect of ibuprofen on heterotopic ossification after hip replacement. Acta Orthop Scand 56(1): 25–27.

Erlebacher A, Derynck R (1996) Increased expression of TGF-beta 2 in osteoblasts results in an osteoporosis-like phenotype. J Cell Biol 132(1–2): 195–210.

Fingeroth RJ, Ahmed AQ (1995) Single dose 6 Gy prophylaxis for heterotopic ossification after total hip arthroplasty. Clin Orthop Relat Res 317: 131–140.

Faler BJ, Macsata RA, Plummer D, Mishra L, Sidawy AN (2006) Transforming growth factor-beta and wound healing. Perspect Vasc Surg Endovasc Ther 18(1): 55–62.

Frith J, Mönkkönen J, Blackburn G, Russell R, Rogers M (1997) Clodronate and liposome-encapsulated clodronate are metabolized to a toxic ATP analog, adenosine 5'-(beta, gamma-dichloromethylene) triphosphate, by mammalian cells in vitro. J Bone Miner Res 12(9): 1358–1367.

Gatherer D, Ten Dijke P, Baird DT, Akhurst RJ (1990) Expression of TGF-beta isoforms during first trimester human embryogenesis. Development 110(2): 445–460.

Gebuhr P, Wilbek H, Soelberg M (1995) Naproxen for 8 days can prevent heterotopic ossification after hip arthroplasty. Clin Orthop Relat Res 314: 166–169.

Geschickter CF, Maseritz I (1938) Myositis ossificans. J Bone Joint Surg Am 20: 661–674.

Gundersen HJ, Bagger P, Bendtsen TF, Evans SM, Korbo L, Marcussen N, Møller A, Nielsen K, Nyengaard JR, Pakkenberg B (1988) The new stereological tools: disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis. APMIS 96(10): 857–881.

Hardie LJ, Laing KJ, Daniels GD, Grabowski PS, Cunningham C, Secombes CJ (1998) Isolation of the first piscine transforming growth factor beta gene: analysis reveals tissue specific expression and a potential regulatory sequence in rainbow trout (Oncorhynchus mykiss). Cytokine 10(8): 555–563.

Herpin A, Lelong C, Favrel P (2004) Transforming growth factor-beta-related proteins: an ancestral and widespread superfamily of cytokines in metazoans. Dev Comp Immunol 28(5): 461–485.

Horner A, Kemp P, Summers C, Bord S, Bishop NJ, Kelsall AW, Coleman N, Compston JE (1998) Expression and distribution of transforming growth factor-beta isoforms and their signaling receptors in growing human bone. Bone 23(2): 95–102.

Hughes FJ, Turner W, Belibasakis G, Martuscelli G (2006) Effects of growth factors and cytokines on osteoblast differentiation. Periodontol 2000 41: 48–72.

Iorio R, Healy WL (2002) Heterotopic ossification after hip and knee arthroplasty: risk factors, prevention, and treatment. J Am Acad Orthop Surg 10(6): 409–416.

Jackson WM, Aragon AB, Bulken-Hoover JD, Nesti LJ, Tuan RS (2009) Putative heterotopic ossification progenitor cells derived from traumatized muscle. J Orthop Res 27(12): 1645–1651.

Janssens K, ten Dijke P, Janssens S, Van Hul W (2005) Transforming growth factor-beta1 to the bone. Endocr Rev 26(6): 743–774.

Jeziorska M (2001) Transforming growth factor-betas and CD105 expression in calcification and bone formation in human atherosclerotic lesions. Z Kardiol 90 Suppl 3: 23–26.

Joyce ME, Jinguishi S, Bolander ME (1990)a Transforming growth factor-beta in the regulation of fracture repair. Orthop Clin North Am 21(1): 199–209.

Joyce ME, Roberts AB, Sporn MB, Bolander ME (1990)b Transforming growth factor-beta and the initiation of chondrogenesis and osteogenesis in the rat femur. J Cell Biol 110(6): 2195–2207.

Kantorowitz DA, Miller GJ, Ferrara JA, Ibbott GS, Fisher R, Ahrens CR (1990) Preoperative versus postoperative irradiation in the prophylaxis of heterotopic bone formation in rats. Int J Radiat Oncol Biol Phys 19: 1431–1438.

Kaplan FS, Glaser DL, Hebela N, Shore EM (2004) Heterotopic ossification. J Am Acad Orthop Surg 12: 116–125.

Keida T, Hayashi N, Kawakami M, Kawashima M (2005) Transforming growth factor beta and connective tissue growth factor are involved in the evolution of nevus of Nanta. J Dermatol 32(6): 442–445.

Keller JC, Trancik TM, Young FA, St Mary E (1989). Effects of indomethacin on bone ingrowth. J Orthop Res 7(1): 28–34.

Kiernan JA (1999) Heidenhain's AZAN. In: Kiernan JA (Ed.). Histological & Histochemical Methods - Theory & Practice (Third edition). Oxford: Butterworth-Heinemann p 156–158.

Kim GY, Kim J, Kim TS, Han J (2009) Pulmonary adenocarcinoma with heterotopic ossification. J Korean Med 24(3): 504–510.

Kjaersgaard-Andersen P, Sletgard J, Gjerloff C, Lund F (1990) Heterotopic bone formation after noncemented total hip arthroplasty. Location of ectopic bone and the influence of postoperative antiinflammatory treatment. Clin Orthop 252: 156–162.

Kusafuka K, Sasaguri K, Sato S, Takemura T, Kameya T (2006) Runx2 expression is associated with pathologic new bone formation around radicular cysts: an immuno-histochemical demonstration. J Oral Pathol Med 35(8): 492–499.

Lebrin F, Deckers M, Bertolino P, Ten Dijke P (2005) TGF-beta receptor function in the endothelium. Cardiovasc Res 65(3): 599–608.

Lehr HA, van der Loos CM, Teeling P, Gown AM (1999) Complete chromogen separation and analysis in double immunohistochemical stains using Photoshop-based image analysis. J Histochem Cytochem 47: 119–126.

Letterio J, Roberts A (1998) Regulation of immune responses by TGF-beta. Annu Rev Immunol 16: 137–161.

Lin L, Shen Q, Xue T, Yu C (2009) Heterotopic ossification induced by Achilles tenotomy via endochondral bone formation: Expression of bone and cartilage related genes. Bone doi:10.1016/j.bone.2009.08.057.

Maes C, Carmeliet P, Moermans K, Stockmans I, Smets N, Collen D, Bouillon R, Carmeliet G (2002) Impaired angiogenesis and endochondral bone formation in mice lacking the vascular endothelial growth factor isoforms VEGF164 and VEGF188. Mech Dev 111(1–2): 61–73.

Matsaba T, Ramoshebi LN, Crooks J, Ripamonti U (2001) Transforming growth factor-beta1 supports the rapid morphogenesis of heterotopic endochondral bone initiated by human osteogenic protein-1 via the synergistic upregulation of molecular markers. Growth Factors 19(2): 73–86.

Matsuzaki S, Fukaya T, Suzuki T, Murakami T, Sasano H, Yajima A (1999) Oestrogen receptor alpha and beta mRNA expression in human endometrium throughout the menstrual cycle. Mol Hum Reprod 5(6): 559–564.

McCarthy EF, Sundaram M (2005) Heterotopic ossification: a review. Skeletal Radiol 34(10): 609–619.

McCartney-Francis NL, Wahl SM (1994) Transforming growth factor beta: a matter of life and death J Leukoc Biol 55(3): 401–409.

Mercado-Pimentel ME, Runyan RB (2007) Multiple transforming growth factor-beta isoforms and receptors function during epithelial-mesenchymal cell transformation in the embryonic heart. Cells Tissues Organs 185(1–3): 146–156.

Millan FA, Denhez F, Kondaiah P, Akhurst RJ (1991) Embryonic gene expression patterns of TGF beta 1, beta 2 and beta 3 suggest different developmental functions in vivo. Development 111(1): 131–143.

Miller DA, Lee A, Matsui Y, Chen EY, Moses HL, Derynck R (1989) Complementary DNA cloning of the murine transforming growth factor-beta 3 (TGF beta 3) precursor and the comparative expression of TGF beta 3 and TGF beta 1 messenger RNA in murine embryos and adult tissues. Mol Endocrinol 3(12): 1926–1934.

Mittl PR, Priestle JP, Cox DA, McMaster G, Cerletti N, Grutter MG (1996) The crystal structure of TGF-beta 3 and comparison to TGF-beta 2: implications for receptor binding. Protein Sci 5(7): 1261–1271.

Mohler ER 3rd, Gannon F, Reynolds C, Zimmerman R, Keane MG, Kaplan FS (2001) Bone formation and inflammation in cardiac valves. Circulation 103(11): 1522–1528.

Moioli EK, Hong L, Mao JJ (2007) Inhibition of osteogenic differentiation of human mesenchymal stem cells. Wound Repair Regen 15(3): 413–421.

Mundy GR (1991) The effects of TGF-beta on bone. Ciba Found Symp 157: 137–143; discussion 143–151.

Muraoka N, Shum L, Fukumoto S, Nomura T, Ohishi M, Nonaka K (2005) Transforming growth factor-beta3 promotes mesenchymal cell proliferation and angiogenesis mediated by the enhancement of cyclin D1, Flk-1, and CD31 gene expression during CL/Fr mouse lip fusion. Birth Defects Res A Clin Mol Teratol 73: 956–965.

Nilsson OS, Persson PE (1999) Heterotopic bone formation after joint replacement. Curr Opin Rheumatol 11: 127–131.

Nollen AJ, Slooff TJ (1973) Para-articular ossifications after total hip replacement. Acta Orthop Scand 44: 230–241.

Opperman LA, Adab K, Gakunga PT (2000) Transforming growth factor-beta 2 and TGF-beta 3 regulate fetal rat cranial suture morphogenesis by regulating rates of cell proliferation and apoptosis. Dev Dyn 219(2): 237–247.

Orlic I, Borovecki F, Simic P, Vukicevic S (2007) Gene expression profiling in bone tissue of osteoporotic mice. Arh Hig Rada Toksikol 58(1): 3–11.

Park JB, Watthanaaphisit T, Riew KD (2007) Timing of development of adjacent-level ossification after anterior cervical arthrodesis with plates. Spine J 7(6): 633–636.

Pelton RW, Nomura S, Moses HL, Hogan BL (1989) Expression of transforming growth factor beta 2 RNA during murine embryogenesis. Development 106(4): 759–767.

Persson PE, Sodemann B, Nilsson OS (1998) Preventive effects of ibuprofen on periarticular heterotopic ossification after total hip arthroplasty. A randomized double-blind prospective study of treatment time. Acta Orthop Scand 69(2): 111–115.

Pfeilschifter J, Seyedin SM, Mundy GR (1988) Transforming growth factor beta inhibits bone resorption in fetal rat long bone cultures. J Clin Invest 82(2): 680–685.

Pisano MM, Mukhopadhyay P, Greene RM (2003) Molecular fingerprinting of TGFbeta-treated embryonic maxillary mesenchymal cells. Orthod Craniofac Res 6: 194–209.

Poncelet AC, Schnaper HW (2001) Sp1 and Smad proteins cooperate to mediate transforming growth factor-beta 1-induced alpha 2 (I) collagen expression in human glomerular mesangial cells. J Biol Chem 276: 6983–6992.

Proetzel G, Pawlowski SA, Wiles MV, Yin M, Boivin GP, Howles PN, Ding J, Ferguson MW, Doetschman T (1995) Transforming growth factor-beta 3 is required for secondary palate fusion. Nat Genet 11(4): 409–414.

Puzas JE, Miller MD, Rosier RN (1989) Pathologic bone formation. Clin Orthop Relat Res 245: 269–281.

Rapuano BE, Boursiquot R, Tomin E, Macdonald DE, Maddula S, Raghavan D, Lane JM, Helfet DL (2008) The effects of COX-1 and COX-2 inhibitors on prostaglandin synthesis and the formation of heterotopic bone in a rat model. Arch Orthop Trauma Surg 128(3): 333–344.

Riedel B (1883) Demonstration line durch ach Hagiges Umhergehen total destruirten kniegelenkes von einem patienten mit stichverletzing des ruckans. Verh Dtsch Gesellschaft Chirurg 12: 93.

Ripamonti U (2005) Bone induction by recombinant human osteogenic protein-1 (hOP-1, BMP-7) in the primate Papio ursinus with expression of mRNA of gene products of the TGF-beta superfamily. J Cell Mol Med 9(4): 911–928.

Ripamonti U, Ferretti C, Teare J, Blann L (2009) Transforming growth factor-beta isoforms and the induction of bone formation: implications for reconstructive craniofacial surgery. J Craniofac Surg 20(5): 1544–1555.

Roelen BA, Dijke P (2003) Controlling mesenchymal stem cell differentiation by TGFBeta family members. J Orthop Sci 8: 740–748.

Rosier RN, O'Keefe RJ, Hicks DG (1998) The potential role of transforming growth factor beta in fracture healing. Clin Orthop (355 Suppl): 294–300.

Rumi MN, Deol GS, Bergandi JA, Singapuri KP, Pellegrini VD Jr (2005) Optimal timing of preoperative radiation for prophylaxis against heterotopic ossification. A rabbit hip model. J Bone Joint Surg Am 87(2): 366–373.

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239: 487–491.

Sakano S, Hasegawa Y, Murata Y, Ito T, Genda E, Iwata H, Ishiguro N, Seo H (2002) Inhibitory effect of bFGF on endochondral heterotopic ossification Biochem Biophys Res Commun 293(2): 680–685.

Sanford LP, Ormsby I, Gittenberger-de Groot AC, Sariola H, Friedman R, Boivin GP, Cardell EL, Doetschman T (1997) TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. Development 124(13): 2659–2670.

Sawyer JR, Myers MA, Rosier RN, Puzas JE (1991) Heterotopic ossification: Clinical and cellular aspects. Calcif Tissue Int 49: 208–215.

Schabort EJ, van der Merwe M, Loos B, Moore FP, Niesler (2009) CU TGF-beta's delay skeletal muscle progenitor cell differentiation in an isoform-independent manner. Exp Cell Res 315(3): 373–384.

Schmidt SA, Kjaersgaard-Andersen P, Pedersen NW, Kristensen SS, Pedersen P, Nielsen JB (1988) The use of indomethacin to prevent the formation of heterotopic bone after total hip replacement. A randomized, double-blind clinical trial. J Bone Joint Surg Am 70(6): 834–838.

Schneider DJ, Moulton MJ, Singapuri K, Chinchilli V, Deol GS, Krenitsky G, Pellegrini VD Jr (1998) Inhibition of heterotopic ossification with radiation therapy in an animal model. Clin Orthop 355: 35–46.

Seeherman H, Wozney JM (2005) Delivery of bone morphogenetic proteins for orthopedic tissue regeneration. Cytokine Growth Factor Rev 16: 329–345.

Selby PL, Davie MW, Ralston SH, Stone MD (2002) Guidelines on the management of Paget's disease of bone. Bone 31(3): 366–373.

Seyedin SM, Thomas TC, Thompson AY, Rosen DM, Piez KA (1985) Purification and characterization of two cartilage-inducing factors from bovine demineralized bone. Proc Natl Acad Sci 82(8): 2267–2271.

Shehab D, Elgazzar AH, Collier BD (2002) Heterotopic ossification. J Nucl Med 43(3): 346–353.

Shi Y, Massagué J (2003) Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell 113(6): 685–700.

Smith R, Wordsworth P (2005) Ectopic ossification. In: Smith R, Wordsworth P (Eds.) Clinical and biochemical disorders of the skeleton. New York, Oxford University Press, p 430–444.

Sodemann B, Persson PE, Nilsson OS (1990) Nonsteroid anti-inflammatory drugs prevent the recurrence of heterotopic ossification after excision. Arch Orthop Trauma Surg 109(2): 53–56.

Spry NA, Dally MJ, Benjamin B, Chapman P, Morum P, Christie DR (1995) Heterotopic bone formation affecting the hip joint is preventable in high risk patients by post-operative radiation. Australas Radiol 39(4): 379–383.

Søballe K, Christensen F, Kristensen SS (1988) Ectopic bone formation after total hip arthroplasty, Clin Orthop 228: 57–62.

Stołtny T, Koczy B, Wawrzynek W, Miszczyk L (2007) Heterotopic ossification in patients after total hip replacement. Ortop Traumatol Rehabil 9(3): 264–272.

Tatsuyama K, Maezawa Y, Baba H, Imamura Y, Fukuda M (2000) Expression of various growth factors for cell proliferation and cytodifferentiation during fracture repair of bone. Eur J Histochem 44(3): 269–278.

ten Dijke P, Iwata KK, Goddard C, Pieler C, Canalis E, McCarthy TL, Centrella M (1990) Recombinant transforming growth factor type beta 3: biological activities and receptor-binding properties in isolated bone cells. Mol Cell Biol 10(9): 4473–4479.

Thomas BJ (1992) Heterotopic bone formation after total hip arthroplasty. Orthop Clin North Am 23(2): 347–358.

Toom A (2007) Heterotopic ossification after total hip arthroplasty: clinical and pathogenic investigation. Dissertationes Medicinae Universitas Tartuensis 140.

Toom A, Haviko T, Rips L (2001) Heterotopic ossification after total hip arthroplasty. Int Orthop 24(6): 323–326.

Toom A, Suutre S, Arend A, Märtson A (2006) Cellular dynamics in bone formation. A biomaterial based model. 5th World Congress of Biomechanics; Munich, Germany July Liepsch D, Monduzzi Editore 261–265.

Uludag H, D'Augusta D, Palmer R, Timony G, Wozney J (1999) Characterization of rhBMP-2 pharmacokinetics implanted with biomaterial carriers in the rat ectopic model. J Biomed Mater Res 46(2): 193–202.

Urist MR (1965) Bone: formation by autoinduction. Science 150(698): 893–899.

Urist MR, Nakagawa M, Nakata N, Nogami H (1978) Experimental myositis ossificans: cartilage and bone formation in muscle in response to diffusible bone matrix-derived morphogen. Arch Pathol Lab Med 102: 312–316.

Urrutia J, Bono CM (2009) Long-term results of surgical treatment of dysphagia secondary to cervical diffuse idiopathic skeletal hyperostosis. Spine J 9(9):13–17.

van Beek E, Cohen L, Leroy I, Ebetino F, Löwik C, Papapoulos S (2003) Differentiating the mechanisms of antiresorptive action of nitrogen containing bisphosphonates Bone 33(5): 805–811.

van der Heide HJ, Koorevaar RC, Lemmens JA, van Kampen A, Schreurs BW (2007) Rofecoxib inhibits heterotopic ossification after total hip arthroplasty. Arch Orthop Trauma Surg 127(7): 557–561.

Vanden Bossche L, Vanderstraeten G (2005) Heterotopic ossification: a review. J Rehabil Med 37(3): 129–136.

Vastel L, Rosencher N, Siney H, Courpied JP (2005) Prevention of heterotopic ossifications in hip arthroplasty: effectiveness of selective Cox-2 inhibitors (celecoxib) versus ketoprofen. Rev Chir Orthop Reparatrice Appar Mot 91(1): 64–69.

Wahl SM, Wen J, Moutsopoulos N (2006) TGF-beta: a mobile purveyor of immune privilege. Immunol Rev 213: 213–227.

Wilkinson JM, Stockley I, Hamer AJ, Barrington NA, Eastell R (2003) Biochemical markers of bone turnover and development of heterotopic ossification after total hip arthroplasty. J Orthop Res 21(3): 529–534.

Williams S, Souchelnytskyi S, Danik M (2002) TGFbeta2 mediates rapid inhibition of calcium influx in identified cholinergic basal forebrain neurons. Biochem Biophys Res Commun 290(4): 1321–1327.

Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Whitters MJ, Kriz RW, Hewick RM, Wang EA (1988) Novel regulators of bone formation: molecular clones and activities. Science 242: 1528–1534.

Wright JG, Moran E, Bogoch E (1994) Reliability and validity of the grading of heterotopic ossification. J Arthroplasty 9(5): 549–553.

Wurnig C, Auersperg V, Boehler N, Steindl M, Kiss H, Zweymuller K, Kotz R (1997) Short term prophylaxis against heterotopic bone after cementless hip replacement. Clin Orthop Relat Res 334: 175–183.

Yayama T, Uchida K, Kobayashi S, Kokubo Y, Sato R, Nakajima H, Takamura T, Bangirana A, Itoh H, Baba H (2007) Thoracic ossification of the human ligamentum flavum: histopathological and immunohistochemical findings around the ossified lesion. J Neurosurg Spine 7(2): 184–193.

Yoshida K, Bessho K, Fujimura K, Kusumoto K, Ogawa Y, Tani Y, Iizuka T (1998) Osteoinduction capability of recombinant human bone morphogenetic protein-2 in intramuscular and subcutaneous sites: an experimental study. J Craniomaxillofac Surg 26(2): 112–115.

Zhang R, Supowit SC, Hou X, Simmons DJ (1999) Transforming growth factor-beta2 mRNA level in unloaded bone analyzed by quantitative *in situ* hybridization. Calcif Tissue Int 64(6): 522–526.

Zúñiga JE, Groppe JC, Cui Y, Hinck CS, Contreras-Shannon V, Pakhomova ON, Yang J, Tang Y, Mendoza V, López-Casillas F, Sun L, Hinck AP (2005) Assembly of TbetaRI:TbetaRII:TGFbeta ternary complex in vitro with receptor extracellular domains is cooperative and isoform-dependent. J Mol Biol 354(5): 1052–1068.

SUMMARY IN ESTONIAN

TGF-β isovormide ja luukoe eelrakkude roll heterotoopse ossifikatsiooni patogeneesis. Eksperimentaalne ja kliiniline uuring puusaliigese endoproteesimisest

TGF-β perekond ja heterotoopne ossifikatsioon

Transformeerivate kasvufaktorite superperekonna liikmetest mõjutavad heterotoopse luu teket kindlasti kõige enam luu morfogeneetilised proteiinid (*bone morphogenetic proteins* – BMP). Samas on suhteliselt vähe teada organismis olulisi funktsioone täitvate TGF-β isovormide (imetajatel täheldatud isovormid: TGF-β_1, TGF-β_2 ja TGF-β_3) rolli kohta selles protsessis – ehkki on teada, et TGF-β isovormidel puuduvad osteoinduktiivsed omadused, on näidatud, et need stimuleerivad luuteket olulisel määral (Joyce *et al.*, 1990a). Isovormide funktsionaalset erinevust iseloomustab näiteks see, et kuigi luukoes on kõige levinumaks isovormiks TGF-β_1, mille funktsioonide hulka kuuluvad osteoblastide diferentseerumise ja prolifereerumise mõjutamine (Seyedin *et al.*, 1985), mängivad olulist rolli ka teised isovormid. Näiteks osaleb TGF-β_2 endokondraalses ossifikatsioonis ja kondrogeneesis (Joyce *et al.*, 1990b) ning TGF-β_3 reguleerib kollageeni sünteesi, mitogeneesi ja alkaalse fosfataasi aktiivsust (Opperman *et al.*, 2000).

Heterotoopne ossifikatsioon (HO) on teisisõnu skeletiväline luustumine ja seda tuleb eristada heterotoopsest kaltsifitseerumisest, mille puhul luukudet ei teki (Vanden Bossche and Vanderstraeten, 2005). HO võib tekkida erinevates kohtades, kuid kõige sagedamini esineb seda pärast puusaliigese endoproteesimist (Toom *et al.*, 2001). Kuna pehmete kudede luustumise täpsed põhjused ei ole veel siiski teada, on selles dissertatsioonis keskendutud mesenhümaalsete tüvirakkude ja kasvufaktorite rollile HO tekkes.

Uurimuse eesmärgid

Uurimuse üldeesmärk oli hinnata muutusi BMP-2 ja TGF-β isovormide ekspressioonimustris ja sisalduses ning morfoloogilisi muutusi HO-s nii inimesel kui loommudelis. Täpsemalt:

1. Kirjeldada HO morfoloogiat ja luutekke aktiivsust ajalises dünaamikas ja võrrelda seda inimesel normaalse luu arenguga.
2. Hinnata ajalises dünaamikas erinevusi BMP-2 ja TGF-β isovormide ekspressioonis, sisalduses ja lokalisatsioonis inimese HO erinevates tsoonides ning küpsetes ja mitte-küpsetes HO-des pärast puusaliigese endoproteesimist.

3. Töötada välja loommudel, mille abil saab jäljendada puusaliigese endoproteesimisel tekkivat olukorda ja uurida HO tekke esialgseid ja varajasi etappe.
4. Hinnata erinevusi TGF-β isovormide ekspressioonis, lokalisatsioonis ja sisalduses HO tekkimise esialgsetes ning varajastes etappides (loommudel).
5. Uurida luuüdikanalist pärinevate tüvirakkude ja lihaskoe kahjustuse mõju HO moodustumisele (loommudel).

Materjalid ja meetodid

I ja II artiklis kasutasime patsientidelt kogutud proove, et uurida muutusi luukoe morfoloogias ja kasvufaktorite ekspressioonis ning sisalduses ossifikaadi küpsemise käigus. Selleks rakendasime vastavalt histomorfomeetrilist analüüsi ja semikvantitatiivset pöördtranskriptaasi reaktsiooni ning immuunohistokeemilist uuringut.

III ja IV artiklis modelleerisime katseloomadel totaalendoproteesimise järgset situatsiooni, et uurida luukoe kasvufaktorite sisalduse muutusi HO-s ja luuüdikanali tüvirakkude ja lokaalse trauma mõju heterotoopse ossifikatsiooni tekkimisele HO moodustumise esialgsetes ja varajastes etappides, kasutades selleks immuunhistokeemilist uuringut ja stereoloogilist analüüsi.

Tulemustest lähtuvad järeldused

1. Inimmaterjali histomorfomeetriline analüüs näitas, et küpses HO-s on võrreldes normaalse luuga suurem osteoidi pinna/periostaalse pinna suhe ja luukoe remodelleerumine toimus aktiivselt ka küpsetes HO-des. Küpsete HO-de histoloogia sarnanes trabekulaarsete luude puhul täheldatuga: selles esinesid spongioosse luu piirkonnad, milles leidus ka luuüdi ja mida ümbritses kortikaalne luu. Samas oli luuteke veelgi aktiivsem nooremates ossifikaatides, mida iseloomustasid muuhulgas ka muutused kasvufaktorite ekspressioonis ja produktsioonis. Nooremate ossifikaatide välispinnal oli selgelt eristatav luutekke piirkond ning luukoe sees võis leida nii luuüdi kui diferentseerumata mesenhümaalseid rakke. Küpseks võib lugeda üle 3-aastast heterotoopset ossifikaati.
2. Pärast puusaliigese endoproteesimist tekkinud HO-de hindamisel täheldasime, et transformeeriva kasvufaktor beeta (TGF-β) isovormide ekspressioon ja produktsioon muutub ossifikaadi küpsedes ja erinevusi võib näha ka TGF-β isovormide lokalisatsioonis. TGF-$β_2$ ja TGF-$β_3$ mRNA ekspressiooni suurenemine HO tekkimise ja remodelleerumise ajal ja TGF-$β_2$ hulga oluline langus küpsemates ossifikaatides viitab TGF-$β_2$ võimalikule rollile HO varajases arengus.
3. Töötasime välja loommudeli, mille puhul tagatakse rotil stabiilne HO teke situatsioonis, mis sarnaneb puusaliigese endproteesimise järgsele olukorrale inimesel ja lähtudes morfoloogilistest muutustest ja muutusest kasvufaktorite

sisalduses võime öelda, et loommudelit võib kasutada hindamaks muutusi HO tekke esialgsetes ja varajastes etappides.

4. Näitasime loommudelil, et muutused kasvufaktorite (eelkõige TGF-β_2 and TGF-β_3) tasemetes on täheldatavad juba HO tekke esialgsetes ja varajastes staadiumites, mis viitab sellele, et need kasvufaktorid võivad antud loom-mudelis mõjutada skeletivälise luutekke algstaadiume.

5. Reieluu kanalist pärinevad rakud ei avaldanud täiendavat mõju HO tekkele ega selle kasvamisele ning konkreetses loommudelis on tunduvalt suurem osakaal lokaalsel osteoinduktiivsel signaalil (BMP-2).

Histoloogilisest vaatepunktist ja TGF-β isovormide dünaamikast lähtudes tundub, et HO teke sarnaneb rohkem luumurru paranemise protsessi kui tavapärase luu arenguga.

ACKNOWLEDGEMENTS

PUBLICATIONS

CURRICULUM VITAE

Siim Suutre

Date and Place of Birth: October 14th 1980, Tartu, Estonia
Country of Citizenship: Estonia
Marital Status: single
Address: Department of Anatomy, University of Tartu,
                       Ravila 19, 51014, Tartu, Estonia
Phone: +372 737 4255
Fax: +372 737 4252
e-mail: siim.suutre@ut.ee

Education

2004–…   PhD student, University of Tartu, Faculty of Medicine,
            Department of Anatomy
1999–2004   University of Tartu, Faculty of Medicine,
            Department of Pharmacy
1996–1999   Hugo Treffner Gymnasium

Special courses

2006   Immunohistochemistry course; Southampton, UK
2005   Laboratory Animal Science C-category Competence Course;
            Tartu, Estonia
2004   Stereology course; Tartu, Estonia

Professional employment

2006–…   University of Tartu, Department of Anatomy, specialist

Scientific work

Main field of research:
– heterotopic ossification after total hip arthroplasty;
– growth factors in heterotopic bone formation;
– effects of biomaterials and growth factors on heterotopic bone formation
  4 publications in peer-reviewed journals and 8 conference abstracts.

CURRICULUM VITAE

Siim Suutre

Sünniaeg- ja koht:	14.10.1980, Tartu, Eesti
Kodakondsus:	Eesti
Perekonnaseis:	vallaline
Aadress:	Anatoomia Instituut, Tartu Ülikool, Ravila 19, 51014, Tartu, Eesti
Telefon:	+372 737 4255
Faks:	+372 737 4252
e-post:	siim.suutre@ut.ee

Haridus

2004–…   Tartu Ülikool, Arstiteaduskond, Anatoomia Instituut, doktorant
1999–2004   Tartu Ülikool, Arstiteaduskond, proviisoriõpe
1996–1999   Hugo Treffneri Gümnaasium

Erialane enesetäiendus

2006   Immunohistochemistry course; Southampton, UK
2005   Katseloomateaduse C kategooria kursus; Tartu, Eesti
2004   Stereoloogia kursus; Tartu, Eesti

Erialane teenistuskäik

2006–…   Tartu Ülikool, Arstiteaduskond, Anatoomia Instituut, spetsialist

Teadustegevus

Peamine teadustöö valdkond:
– heterotoopne ossifikatsioon puusaliigese endoproteesimise järgselt;
– kasvufaktorite roll heterotoopse luu moodustumises;
– biomaterjalide ja kasvufaktorite mõju heterotoopse luu moodustumisele
   4 artiklit eelretsenseeritavates ajakirjades ja 8 konverentsi teesi.

DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS

1. **Heidi-Ingrid Maaroos.** The natural course of gastric ulcer in connection with chronic gastritis and *Helicobacter pylori*. Tartu, 1991.
2. **Mihkel Zilmer.** Na-pump in normal and tumorous brain tissues: Structural, functional and tumorigenesis aspects. Tartu, 1991.
3. **Eero Vasar.** Role of cholecystokinin receptors in the regulation of behaviour and in the action of haloperidol and diazepam. Tartu, 1992.
4. **Tiina Talvik.** Hypoxic-ischaemic brain damage in neonates (clinical, biochemical and brain computed tomographical investigation). Tartu, 1992.
5. **Ants Peetsalu.** Vagotomy in duodenal ulcer disease: A study of gastric acidity, serum pepsinogen I, gastric mucosal histology and *Helicobacter pylori*. Tartu, 1992.
6. **Marika Mikelsaar.** Evaluation of the gastrointestinal microbial ecosystem in health and disease. Tartu, 1992.
7. **Hele Everaus.** Immuno-hormonal interactions in chronic lymphocytic leukaemia and multiple myeloma. Tartu, 1993.
8. **Ruth Mikelsaar.** Etiological factors of diseases in genetically consulted children and newborn screening: dissertation for the commencement of the degree of doctor of medical sciences. Tartu, 1993.
9. **Agu Tamm.** On metabolic action of intestinal microflora: clinical aspects. Tartu, 1993.
10. **Katrin Gross.** Multiple sclerosis in South-Estonia (epidemiological and computed tomographical investigations). Tartu, 1993.
11. **Oivi Uibo.** Childhood coeliac disease in Estonia: occurrence, screening, diagnosis and clinical characterization. Tartu, 1994.
12. **Viiu Tuulik.** The functional disorders of central nervous system of chemistry workers. Tartu, 1994.
13. **Margus Viigimaa.** Primary haemostasis, antiaggregative and anticoagulant treatment of acute myocardial infarction. Tartu, 1994.
14. **Rein Kolk.** Atrial versus ventricular pacing in patients with sick sinus syndrome. Tartu, 1994.
15. **Toomas Podar.** Incidence of childhood onset type 1 diabetes mellitus in Estonia. Tartu, 1994.
16. **Kiira Subi.** The laboratory surveillance of the acute respiratory viral infections in Estonia. Tartu, 1995.
17. **Irja Lutsar.** Infections of the central nervous system in children (epidemiologic, diagnostic and therapeutic aspects, long term outcome). Tartu, 1995.
18. **Aavo Lang.** The role of dopamine, 5-hydroxytryptamine, sigma and NMDA receptors in the action of antipsychotic drugs. Tartu, 1995.
19. **Andrus Arak.** Factors influencing the survival of patients after radical surgery for gastric cancer. Tartu, 1996.
20. **Tõnis Karki.** Quantitative composition of the human lactoflora and method for its examination. Tartu, 1996.

21. **Reet Mändar.** Vaginal microflora during pregnancy and its transmission to newborn. Tartu, 1996.
22. **Triin Remmel.** Primary biliary cirrhosis in Estonia: epidemiology, clinical characterization and prognostication of the course of the disease. Tartu, 1996.
23. **Toomas Kivastik.** Mechanisms of drug addiction: focus on positive reinforcing properties of morphine. Tartu, 1996.
24. **Paavo Pokk.** Stress due to sleep deprivation: focus on $GABA_A$ receptor-chloride ionophore complex. Tartu, 1996.
25. **Kristina Allikmets.** Renin system activity in essential hypertension. Associations with atherothrombogenic cardiovascular risk factors and with the efficacy of calcium antagonist treatment. Tartu, 1996.
26. **Triin Parik.** Oxidative stress in essential hypertension: Associations with metabolic disturbances and the effects of calcium antagonist treatment. Tartu, 1996.
27. **Svetlana Päi.** Factors promoting heterogeneity of the course of rheumatoid arthritis. Tartu, 1997.
28. **Maarike Sallo.** Studies on habitual physical activity and aerobic fitness in 4 to 10 years old children. Tartu, 1997.
29. **Paul Naaber.** *Clostridium difficile* infection and intestinal microbial ecology. Tartu, 1997.
30. **Rein Pähkla.** Studies in pinoline pharmacology. Tartu, 1997.
31. **Andrus Juhan Voitk.** Outpatient laparoscopic cholecystectomy. Tartu, 1997.
32. **Joel Starkopf.** Oxidative stress and ischaemia-reperfusion of the heart. Tartu, 1997.
33. **Janika Kõrv.** Incidence, case-fatality and outcome of stroke. Tartu, 1998.
34. **Ülla Linnamägi.** Changes in local cerebral blood flow and lipid peroxidation following lead exposure in experiment. Tartu, 1998.
35. **Ave Minajeva.** Sarcoplasmic reticulum function: comparison of atrial and ventricular myocardium. Tartu, 1998.
36. **Oleg Milenin.** Reconstruction of cervical part of esophagus by revascularised ileal autografts in dogs. A new complex multistage method. Tartu, 1998.
37. **Sergei Pakriev.** Prevalence of depression, harmful use of alcohol and alcohol dependence among rural population in Udmurtia. Tartu, 1998.
38. **Allen Kaasik.** Thyroid hormone control over β-adrenergic signalling system in rat atria. Tartu, 1998.
39. **Vallo Matto.** Pharmacological studies on anxiogenic and antiaggressive properties of antidepressants. Tartu, 1998.
40. **Maire Vasar.** Allergic diseases and bronchial hyperreactivity in Estonian children in relation to environmental influences. Tartu, 1998.
41. **Kaja Julge.** Humoral immune responses to allergens in early childhood. Tartu, 1998.
42. **Heli Grünberg.** The cardiovascular risk of Estonian schoolchildren. A cross-sectional study of 9-, 12- and 15-year-old children. Tartu, 1998.

43. **Epp Sepp.** Formation of intestinal microbial ecosystem in children. Tartu, 1998.
44. **Mai Ots.** Characteristics of the progression of human and experimental glomerulopathies. Tartu, 1998.
45. **Tiina Ristimäe.** Heart rate variability in patients with coronary artery disease. Tartu, 1998.
46. **Leho Kõiv.** Reaction of the sympatho-adrenal and hypothalamo-pituitary-adrenocortical system in the acute stage of head injury. Tartu, 1998.
47. **Bela Adojaan.** Immune and genetic factors of childhood onset IDDM in Estonia. An epidemiological study. Tartu, 1999.
48. **Jakov Shlik.** Psychophysiological effects of cholecystokinin in humans. Tartu, 1999.
49. **Kai Kisand.** Autoantibodies against dehydrogenases of α-ketoacids. Tartu, 1999.
50. **Toomas Marandi.** Drug treatment of depression in Estonia. Tartu, 1999.
51. **Ants Kask.** Behavioural studies on neuropeptide Y. Tartu, 1999.
52. **Ello-Rahel Karelson.** Modulation of adenylate cyclase activity in the rat hippocampus by neuropeptide galanin and its chimeric analogs. Tartu, 1999.
53. **Tanel Laisaar.** Treatment of pleural empyema — special reference to intrapleural therapy with streptokinase and surgical treatment modalities. Tartu, 1999.
54. **Eve Pihl.** Cardiovascular risk factors in middle-aged former athletes. Tartu, 1999.
55. **Katrin Õunap.** Phenylketonuria in Estonia: incidence, newborn screening, diagnosis, clinical characterization and genotype/phenotype correlation. Tartu, 1999.
56. **Siiri Kõljalg.** *Acinetobacter* – an important nosocomial pathogen. Tartu, 1999.
57. **Helle Karro.** Reproductive health and pregnancy outcome in Estonia: association with different factors. Tartu, 1999.
58. **Heili Varendi.** Behavioral effects observed in human newborns during exposure to naturally occurring odors. Tartu, 1999.
59. **Anneli Beilmann.** Epidemiology of epilepsy in children and adolescents in Estonia. Prevalence, incidence, and clinical characteristics. Tartu, 1999.
60. **Vallo Volke.** Pharmacological and biochemical studies on nitric oxide in the regulation of behaviour. Tartu, 1999.
61. **Pilvi Ilves.** Hypoxic-ischaemic encephalopathy in asphyxiated term infants. A prospective clinical, biochemical, ultrasonographical study. Tartu, 1999.
62. **Anti Kalda.** Oxygen-glucose deprivation-induced neuronal death and its pharmacological prevention in cerebellar granule cells. Tartu, 1999.
63. **Eve-Irene Lepist.** Oral peptide prodrugs – studies on stability and absorption. Tartu, 2000.
64. **Jana Kivastik.** Lung function in Estonian schoolchildren: relationship with anthropometric indices and respiratory symptomas, reference values for dynamic spirometry. Tartu, 2000.

65. **Karin Kull.** Inflammatory bowel disease: an immunogenetic study. Tartu, 2000.

66. **Kaire Innos.** Epidemiological resources in Estonia: data sources, their quality and feasibility of cohort studies. Tartu, 2000.

67. **Tamara Vorobjova.** Immune response to *Helicobacter pylori* and its association with dynamics of chronic gastritis and epithelial cell turnover in antrum and corpus. Tartu, 2001.

68. **Ruth Kalda.** Structure and outcome of family practice quality in the changing health care system of Estonia. Tartu, 2001.

69. **Annika Krüüner.** *Mycobacterium tuberculosis* – spread and drug resistance in Estonia. Tartu, 2001.

70. **Marlit Veldi.** Obstructive Sleep Apnoea: Computerized Endopharyngeal Myotonometry of the Soft Palate and Lingual Musculature. Tartu, 2001.

71. **Anneli Uusküla.** Epidemiology of sexually transmitted diseases in Estonia in 1990–2000. Tartu, 2001.

72. **Ade Kallas.** Characterization of antibodies to coagulation factor VIII. Tartu, 2002.

73. **Heidi Annuk.** Selection of medicinal plants and intestinal lactobacilli as antimicrobil components for functional foods. Tartu, 2002.

74. **Aet Lukmann**. Early rehabilitation of patients with ischaemic heart disease after surgical revascularization of the myocardium: assessment of health-related quality of life, cardiopulmonary reserve and oxidative stress. A clinical study. Tartu, 2002.

75. **Maigi Eisen.** Pathogenesis of Contact Dermatitis: participation of Oxidative Stress. A clinical – biochemical study. Tartu, 2002.

76. **Piret Hussar.** Histology of the post-traumatic bone repair in rats. Elaboration and use of a new standardized experimental model – bicortical perforation of tibia compared to internal fracture and resection osteotomy. Tartu, 2002.

77. **Tõnu Rätsep.** Aneurysmal subarachnoid haemorrhage: Noninvasive monitoring of cerebral haemodynamics. Tartu, 2002.

78. **Marju Herodes.** Quality of life of people with epilepsy in Estonia. Tartu, 2003.

79. **Katre Maasalu.** Changes in bone quality due to age and genetic disorders and their clinical expressions in Estonia. Tartu, 2003.

80. **Toomas Sillakivi.** Perforated peptic ulcer in Estonia: epidemiology, risk factors and relations with *Helicobacter pylori*. Tartu, 2003.

81. **Leena Puksa.** Late responses in motor nerve conduction studies. F and A waves in normal subjects and patients with neuropathies. Tartu, 2003.

82. **Krista Lõivukene**. *Helicobacter pylori* in gastric microbial ecology and its antimicrobial susceptibility pattern. Tartu, 2003.

83. **Helgi Kolk.** Dyspepsia and *Helicobacter pylori* infection: the diagnostic value of symptoms, treatment and follow-up of patients referred for upper gastrointestinal endoscopy by family physicians. Tartu, 2003.

84. **Helena Soomer.** Validation of identification and age estimation methods in forensic odontology. Tartu, 2003.

85. **Kersti Oselin.** Studies on the human MDR1, MRP1, and MRP2 ABC transporters: functional relevance of the genetic polymorphisms in the *MDR1* and *MRP1* gene. Tartu, 2003.

86. **Jaan Soplepmann.** Peptic ulcer haemorrhage in Estonia: epidemiology, prognostic factors, treatment and outcome. Tartu, 2003.

87. **Margot Peetsalu.** Long-term follow-up after vagotomy in duodenal ulcer disease: recurrent ulcer, changes in the function, morphology and *Helicobacter pylori* colonisation of the gastric mucosa. Tartu, 2003.

88. **Kersti Klaamas.** Humoral immune response to *Helicobacter pylori* a study of host-dependent and microbial factors. Tartu, 2003.

89. **Pille Taba.** Epidemiology of Parkinson's disease in Tartu, Estonia. Prevalence, incidence, clinical characteristics, and pharmacoepidemiology. Tartu, 2003.

90. **Alar Veraksitš**. Characterization of behavioural and biochemical phenotype of cholecystokinin-2 receptor deficient mice: changes in the function of the dopamine and endopioidergic system. Tartu, 2003.

91. **Ingrid Kalev.** CC-chemokine receptor 5 (CCR5) gene polymorphism in Estonians and in patients with Type I and Type II diabetes mellitus. Tartu, 2003.

92. **Lumme Kadaja.** Molecular approach to the regulation of mitochondrial function in oxidative muscle cells. Tartu, 2003.

93. **Aive Liigant**. Epidemiology of primary central nervous system tumours in Estonia from 1986 to 1996. Clinical characteristics, incidence, survival and prognostic factors. Tartu, 2004.

94. **Andres, Kulla.** Molecular characteristics of mesenchymal stroma in human astrocytic gliomas. Tartu, 2004.

95. **Mari Järvelaid.** Health damaging risk behaviours in adolescence. Tartu, 2004.

96. **Ülle Pechter.** Progression prevention strategies in chronic renal failure and hypertension. An experimental and clinical study. Tartu, 2004.

97. **Gunnar Tasa.** Polymorphic glutathione S-transferases – biology and role in modifying genetic susceptibility to senile cataract and primary open angle glaucoma. Tartu, 2004.

98. **Tuuli Käämbre.** Intracellular energetic unit: structural and functional aspects. Tartu, 2004.

99. **Vitali Vassiljev.** Influence of nitric oxide syntase inhibitors on the effects of ethanol after acute and chronic ethanol administration and withdrawal. Tartu, 2004.

100. **Aune Rehema.** Assessment of nonhaem ferrous iron and glutathione redox ratio as markers of pathogeneticity of oxidative stress in different clinical groups. Tartu, 2004.

101. **Evelin Seppet.** Interaction of mitochondria and ATPases in oxidative muscle cells in normal and pathological conditions. Tartu, 2004.

102. **Eduard Maron.** Serotonin function in panic disorder: from clinical experiments to brain imaging and genetics. Tartu, 2004.
103. **Marje Oona.** *Helicobacter pylori* infection in children: epidemiological and therapeutic aspects. Tartu, 2004.
104. **Kersti Kokk.** Regulation of active and passive molecular transport in the testis. Tartu, 2005.
105. **Vladimir Järv.** Cross-sectional imaging for pretreatment evaluation and follow-up of pelvic malignant tumours. Tartu, 2005.
106. **Andre Õun.** Epidemiology of adult epilepsy in Tartu, Estonia. Incidence, prevalence and medical treatment. Tartu, 2005.
107. **Piibe Muda.** Homocysteine and hypertension: associations between homocysteine and essential hypertension in treated and untreated hypertensive patients with and without coronary artery disease. Tartu, 2005.
108. **Külli Kingo.** The interleukin-10 family cytokines gene polymorphisms in plaque psoriasis. Tartu, 2005.
109. **Mati Merila.** Anatomy and clinical relevance of the glenohumeral joint capsule and ligaments. Tartu, 2005.
110. **Epp Songisepp**. Evaluation of technological and functional properties of the new probiotic *Lactobacillus fermentum* ME-3. Tartu, 2005.
111. **Tiia Ainla.** Acute myocardial infarction in Estonia: clinical characteristics, management and outcome. Tartu, 2005.
112. **Andres Sell.** Determining the minimum local anaesthetic requirements for hip replacement surgery under spinal anaesthesia – a study employing a spinal catheter. Tartu, 2005.
113. **Tiia Tamme.** Epidemiology of odontogenic tumours in Estonia. Pathogenesis and clinical behaviour of ameloblastoma. Tartu, 2005.
114. **Triine Annus**. Allergy in Estonian schoolchildren: time trends and characteristics. Tartu, 2005.
115. **Tiia Voor.** Microorganisms in infancy and development of allergy: comparison of Estonian and Swedish children. Tartu, 2005.
116. **Priit Kasenõmm.** Indicators for tonsillectomy in adults with recurrent tonsillitis – clinical, microbiological and pathomorphological investigations. Tartu, 2005.
117. **Eva Zusinaite.** Hepatitis C virus: genotype identification and interactions between viral proteases. Tartu, 2005.
118. **Piret Kõll.** Oral lactoflora in chronic periodontitis and periodontal health. Tartu, 2006.
119. **Tiina Stelmach.** Epidemiology of cerebral palsy and unfavourable neurodevelopmental outcome in child population of Tartu city and county, Estonia Prevalence, clinical features and risk factors. Tartu, 2006.
120. **Katrin Pudersell.** Tropane alkaloid production and riboflavine excretion in the field and tissue cultures of henbane (*Hyoscyamus niger* L.). Tartu, 2006.
121. **Külli Jaako.** Studies on the role of neurogenesis in brain plasticity. Tartu, 2006.

122. **Aare Märtson.** Lower limb lengthening: experimental studies of bone regeneration and long-term clinical results. Tartu, 2006.
123. **Heli Tähepõld.** Patient consultation in family medicine. Tartu, 2006.
124. **Stanislav Liskmann.** Peri-implant disease: pathogenesis, diagnosis and treatment in view of both inflammation and oxidative stress profiling. Tartu, 2006.
125. **Ruth Rudissaar.** Neuropharmacology of atypical antipsychotics and an animal model of psychosis. Tartu, 2006.
126. **Helena Andreson.** Diversity of *Helicobacter pylori* genotypes in Estonian patients with chronic inflammatory gastric diseases. Tartu, 2006.
127. **Katrin Pruus.** Mechanism of action of antidepressants: aspects of serotoninergic system and its interaction with glutamate. Tartu, 2006.
128. **Priit Põder.** Clinical and experimental investigation: relationship of ischaemia/reperfusion injury with oxidative stress in abdominal aortic aneurysm repair and in extracranial brain artery endarterectomy and possibilities of protection against ischaemia using a glutathione analogue in a rat model of global brain ischaemia. Tartu, 2006.
129. **Marika Tammaru.** Patient-reported outcome measurement in rheumatoid arthritis. Tartu, 2006.
130. **Tiia Reimand.** Down syndrome in Estonia. Tartu, 2006.
131. **Diva Eensoo.** Risk-taking in traffic and Markers of Risk-Taking Behaviour in Schoolchildren and Car Drivers. Tartu, 2007.
132. **Riina Vibo.** The third stroke registry in Tartu, Estonia from 2001 to 2003: incidence, case-fatality, risk factors and long-term outcome. Tartu, 2007.
133. **Chris Pruunsild.** Juvenile idiopathic arthritis in children in Estonia. Tartu, 2007.
134. **Eve Õiglane-Šlik.** Angelman and Prader-Willi syndromes in Estonia. Tartu, 2007.
135. **Kadri Haller.** Antibodies to follicle stimulating hormone. Significance in female infertility. Tartu, 2007.
136. **Pille Ööpik.** Management of depression in family medicine. Tartu, 2007.
137. **Jaak Kals.** Endothelial function and arterial stiffness in patients with atherosclerosis and in healthy subjects. Tartu, 2007.
138. **Priit Kampus.** Impact of inflammation, oxidative stress and age on arterial stiffness and carotid artery intima-media thickness. Tartu, 2007.
139. **Margus Punab.** Male fertility and its risk factors in Estonia. Tartu, 2007.
140. **Alar Toom**. Heterotopic ossification after total hip arthroplasty: clinical and pathogenetic investigation. Tartu, 2007.
141. **Lea Pehme.** Epidemiology of tuberculosis in Estonia 1991–2003 with special regard to extrapulmonary tuberculosis and delay in diagnosis of pulmonary tuberculosis. Tartu, 2007.
142. **Juri Karjagin.** The pharmacokinetics of metronidazole and meropenem in septic shock. Tartu, 2007.
143. **Inga Talvik.** Inflicted traumatic brain injury shaken baby syndrome in Estonia – epidemiology and outcome. Tartu, 2007.

144. **Tarvo Rajasalu.** Autoimmune diabetes: an immunological study of type 1 diabetes in humans and in a model of experimental diabetes (in RIP-B7.1 mice). Tartu, 2007.

145. **Inga Karu.** Ischaemia-reperfusion injury of the heart during coronary surgery: a clinical study investigating the effect of hyperoxia. Tartu, 2007.

146. **Peeter Padrik.** Renal cell carcinoma: Changes in natural history and treatment of metastatic disease. Tartu, 2007.

147. **Neve Vendt.** Iron deficiency and iron deficiency anaemia in infants aged 9 to 12 months in Estonia. Tartu, 2008.

148. **Lenne-Triin Heidmets.** The effects of neurotoxins on brain plasticity: focus on neural Cell Adhesion Molecule. Tartu, 2008.

149. **Paul Korrovits.** Asymptomatic inflammatory prostatitis: prevalence, etiological factors, diagnostic tools. Tartu, 2008.

150. **Annika Reintam.** Gastrointestinal failure in intensive care patients. Tartu, 2008.

151. **Kristiina Roots.** Cationic regulation of Na-pump in the normal, Alzheimer's and CCK_2 receptor-deficient brain. Tartu, 2008.

152. **Helen Puusepp.** The genetic causes of mental retardation in Estonia: fragile X syndrome and creatine transporter defect. Tartu, 2009.

153. **Kristiina Rull.** Human chorionic gonadotropin beta genes and recurrent miscarriage: expression and variation study. Tartu, 2009.

154. **Margus Eimre.** Organization of energy transfer and feedback regulation in oxidative muscle cells. Tartu, 2009.

155. **Maire Link.** Transcription factors FoxP3 and AIRE: autoantibody associations. Tartu, 2009.

156. **Kai Haldre.** Sexual health and behaviour of young women in Estonia. Tartu, 2009.

157. **Kaur Liivak.** Classical form of congenital adrenal hyperplasia due to 21-hydroxylase deficiency in Estonia: incidence, genotype and phenotype with special attention to short-term growth and 24-hour blood pressure. Tartu, 2009.

158. **Kersti Ehrlich.** Antioxidative glutathione analogues (UPF peptides) – molecular design, structure-activity relationships and testing the protective properties. Tartu, 2009.

159. **Anneli Rätsep.** Type 2 diabetes care in family medicine. Tartu, 2009.

160. **Silver Türk.** Etiopathogenetic aspects of chronic prostatitis: role of mycoplasmas, coryneform bacteria and oxidative stress. Tartu, 2009.

161. **Kaire Heilman.** Risk markers for cardiovascular disease and low bone mineral density in children with type 1 diabetes. Tartu, 2009.

162. **Kristi Rüütel.** HIV-epidemic in Estonia: injecting drug use and quality of life of people living with HIV. Tartu, 2009.

163. **Triin Eller.** Immune markers in major depression and in antidepressive treatment. Tartu, 2009.