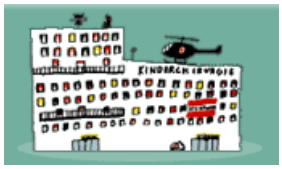

Physeal Reactions To Growth Plate Lesions

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Focusing On Specific Physeal Molecular Pathways (BMP-2)

eingereicht von

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unter der Anleitung von

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Graz, 02.12.2009 Marie-Therese Dolejschi

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Ich erkläre ehrenwörtlich, dass ich die vorliegende Arbeit selbstständig und ohne fremde Hilfe verfasst habe, andere als die angegebenen Quellen nicht verwende habe und die den benutzten Quellen wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

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ABSTRACT

Introduction: Post-traumatic bone growth disturbance is a very common complication and challenge in paediatric traumatology. Growth disturbances can be divided into a stimulating and inhibiting form. The inhibiting growth disturbance is associated with a partial or complete growth arrest due to the formation of physeal bone-bridges. To date, the underlying molecular mechanism has not been clarified, yet. The aim of this study is to elucidate bone morphogenetic protein 2 (BMP-2) expression in the process of physeal repair mechanisms in a physeal injury animal model. BMP-2 is a key signalling growth factor in the process of endochondral ossification at the growth plate. We hypothesise that deregulated BMP expression is involved in bone bridge formation after physeal injury.

Methods: Experimental Sprague-Dawley rats were subjected to a unilateral transepiphyseal drill-hole injury of the left proximal tibial growth plate. After euthanasia on days 1, 3, 7, 14, 28, and 82 post-injury, growth plate tissue of the left and right tibiae of the experimental and control animals were harvested and prepared for immunohistochemistry according to standardized protocols.

Results: The temporal-spatial expression of BMP-2 at the drill-hole injured growth plate suggests potential roles in the inflammatory response, as well as in the fibrogenic, osteogenic responses and in bone remodelling at the injured growth plate. On day 1 a weak positive BMP-2 staining among cells along the drill-hole injury was observed. The RZ (resting zone) and resorption zone were weakly positive. On day 3 BMP-2 positive immunostaining was observed in the RZ and PZ (proliferating zone). At day 7 mesenchymal cells, osteoblasts were highly stained positive, whereas the PZ showed highest staining. At day 14 a less positive immunostaining was observed compared to the growth plate and mesenchymal cells on day 1, 3 and 7. Cells of the growth plate next to the injury and mesenchymal cells along the drill-hole injury showed a denser positive staining compared to cells lateral of the defect. Day 28, 42 and 82 nearly the whole growth plate showed an abundant immunostaining, whereas cells of the PZ showed the highest. Further, chondrocytes of the articular cartilage were all consistently positive stained on day 1, 3, 7, 14, 28 and 82.

Discussion: These findings demonstrate a BMP-2 signalling gradient across the epiphyseal plate. This study provides evidence that the healing process after a transepiphyseal injury involves the whole growth plate by stimulating chondrocyte

proliferation and differentiation in growth plate cartilage by the expression of BMP-2. So one possible reason for the abundant staining of BMP-2 at the beginning of the osteogenic response on day 7 is that BMP-2 plays an important part in stimulating the differentiation of osteoblasts and further stimulating bone formation at the drill-hole injured growth plate. In addition, the results show that besides the early inflammatory phase, BMP-2 may also play an important role in bone formation/maturation and in bone bridge remodeling in the injured growth plate. The spatial differential expression of BMP-2 in the injured leg during various stages of bony repair suggests that BMP-2 has a critical and potential role in regulating the cellular events leading to the formation of bone bridges.

Key words: BMP-2, growth plate injury and repair, bone bridge formation

ZUSAMMENFASSUNG

Einleitung: Knochenwachstumsstörungen im Kindesalter sind eine sehr häufige Komplikation, und in der Therapie eine Herausforderung in der Kindertraumatologie. Dieses Phänomen kann man sowohl in stimulierende, als auch in hemmende Wachstumsstörungen untergliedern. Ziel dieser Studie ist es, die Reaktion der Wachstumsfuge auf eine Fugenläsion zu untersuchen. Dabei liegt der Fokus auf der Evaluation der Expression eines wesentlichen Wachstumsfaktors der enchondralen Ossifikation, „Bone morphogenetic protein-2“ (BMP-2). Morphologisch reagiert die Fuge auf das Trauma mit der Bildung einer Knochenbrücke, die zu einem partiellen oder kompletten Wachstumsfugenverschluss führen kann. BMP-2 zählt zu den Haupt-Regulatoren der Knochenbildung. Die Hypothese dieser Studie ist, dass die BMP-2 Expression posttraumatisch in der Fuge erhöht ist und damit eine Knochenbrückenbildung auslöst.

Methodik: 70 männliche Sprague-Dawley Ratten (Alter: 5Wochen, Gewicht: 100-120g) wurden randomisiert in zwei Gruppen eingeteilt, in eine Experimental- und eine Kontrollgruppe. Den Tieren der Experimentalgruppe wurde unter Anästhesie ein Bohrloch in die Epiphysenfuge der linken proximalen Tibia zugeführt. Die rechte Tibia blieb unbehandelt. Je nach Zugehörigkeit zu den Gruppen wurden die Tiere an den postoperativen Tagen 1, 3, 7, 14, 28, und 82 getötet. Nach der Euthanasie wurden die linken und rechten Tibiae der Experimental- und Kontrolltiere gesammelt, für die Immunhistochemie in Methanol 100% fixiert und entkalkt. Die immunhistochemischen Färbungen wurden auf Kryoschnitten durchgeführt. Die Bestimmung der örtlichen Verteilung von BMP-2 wurde zum Vergleich im linken und rechten Bein durchgeführt.

Ergebnisse: Das zeitliche und räumliche Verteilungsmuster der BMP-2 Expression in der Wachstumsfugenläsion deutet auf eine bedeutende Funktion sowohl in der anfänglichen Entzündungsphase, als auch in der Granulations- und Remodelling-Phase hin. Am Tag 1 nach der Bohrlochläsion ist nur eine schwache Färbung der Zellen entlang des Defektes, in der Ruhezone und Resorptionszone vorhanden. Am Tag 3 konnte eine positive immunhistochemische BMP-2 Färbung in der Ruhezone und Proliferationzone beobachtet werden. Hingegen zeigten am Tag 7 Mesenchymzellen, Osteoblasten eine starke positive Färbung, wobei die Proliferationszone am stärksten angefärbt wurde. Die Epiphysenfuge und Mesenchymzellen zeigten am Tag 14 eine wesentlich schwächere positive Anfärbung

verglichen mit Tag 1,3 und 7. Auffallend war, dass Zellen angrenzend an den Fugendefekt und Mesenchymzellen entlang des Bohrlochdefektes eine stärkere BMP-2 Anfärbung hatten, als Zellen, die weiter lateral von der Verletzung waren. Beinahe die gesamte Wachstumsfuge war am Tag 28, 42 und 82 durchgehend stark angefärbt, wobei die Proliferationszone die stärkste Färbung zeigte. Darüber hinaus zeigten die Chondrozyten des Gelenksknorpels an allen Tagen eine positive Färbung für BMP-2.

Diskussion: Die Ergebnisse dieser Studie zeigen das Verteilungsmuster von BMP-2 entlang der Epiphysenfuge. Ferner wird gezeigt, dass eine transepiphyseale Verletzung die gesamte Wachstumsfuge im Heilungsprozess, durch Stimulation von der Proliferation und Differenzierung der Chondrozyten, umfasst. Diese Stimulation im Wachstumsfugenknorpel wird durch die Expression von BMP-2 gesteuert. BMP-2 spielt eine wichtige Rolle in der Stimulation der Differenzierung von Osteoblasten und weiter in der Stimulation von Knochenbildung in der mit einem Bohrlochdefekt versehenen Wachstumsfuge und könnte dadurch den Grund für die starke Anfärbung von BMP-2 zu Beginn der Frakturheilung am Tag 7 erklären. Darüber hinaus zeigen die Ergebnisse nicht nur eine essentielle Rolle von BMP-2 in der frühen Entzündungsphase, sondern auch in der Knochenbildung / Knochenreifung und in der Bildung von Knochenbrücken in der verletzten Wachstumsfuge. Das unterschiedliche Verteilungsmuster der BMP-2 Expression in der verletzten Epiphysenfuge gibt starke Hinweise darauf, dass BMP-2 eine wichtige und potentiell entscheidende Rolle in den Regulationsmechanismen der Zellinteraktionen spielt, die in weiterer Folge zur Bildung von Knochenbrücken führt.

Schlagwörter: BMP-2, Epiphysenfugenverletzungen und Heilungsmechanismen, Knochenbrückenbildung

ABBREVIATIONS

ABC	Avidin – Biotin - Complex
AC	Articular cartilage
BESTT	BMP-2 Evaluation in Surgery for Tibial Trauma
BGP	Bone Gla Protein or osteocalcin
BMP	Bone Morphogenetic Protein
BMPR	Bone Morphogenetic Protein Receptor
cAMP	cyclic AMP = cyclice Adenosine Monophosphate
DHI	Drill Hole Injury
ECB	Endochondral Bone
EPI	Epiphysis
FGF	Fibroblast Growth Factor
FGFRs	Fibroblast Growth Factor Receptors
GDF	Growth and Differentiation Factor
GH	Growth Hormone
HCl	Hydrochloride
HZ	Hypertrophic Zone
IBF	Institute for Biomedical Research
IGF	Insulin-like Growth Factor
IgG	Immunoglobulin G
IHC	Immunohistochemistry
Ihh	Indian Hedgehog
IL (-1, -1α, 1β -6)	Interleukin (-1, -1α, 1β -6)
IMB	Intramembranous Bone
INF-γ	Interferon γ
kDa	Kilodalton
M-CSF	Macrophage/Monocyte-Colony Stimulating Factor
META	Metaphysis
MMPs	Matrix Metalloproteinases
mRNA	Messenger Ribonucleic Acid
MSC	Mesenchymal Stem Cell
MSCs	Mesenchymal Stem Cells

MUG	Medical University of Graz
NF-κB	Nuclear Factor – kappa B
OP	Osteogenic Proteins
OPG	Osteoprotegerin
PBS	Phosphate Buffered Saline
Ptc-1	Patched-1
PTH	Parathyroid Hormone
PTHlh	Parathyroid Hormone-like-Hormone
PTHrP	Parathyroid Hormone-related Protein
PZ	Proliferating Zone
RANKL	Receptor Activator of NF-B Ligand
rDNA	Recombinant Deoxyribonucleic Acid
rhBMP-2	recombinant human BMP-2
R-Smads	Receptor Smads
RZ	Resting (reserve) Zone
Smad	Sma and Mad
Smo	Smoothened
T3	Triiodothyronine
T4	Thyroxine
TGF-β	Transforming Growth Factor beta
TNF-α	Tumor Necrosis Factor-α
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular endothelial growth factor receptor
ZMF	Center of Medical Research of Medical University of Graz

TABLE OF CONTENTS

1. INTRODUCTION

Bones are rigid organs that have mechanical, synthetic, and further metabolic functions and play an extraordinary role in the human bodies. The growing skeleton presents particular advantages in the healing of bone fracture. For instance, bone has the capacity to regenerate nearly 100 percent of the original structure of bones after fracture. Furthermore, short time to union, the rare occurrence of non-union, or delayed union as well as the potency for spontaneous remodelling of post-traumatic bone deformities are advantages of the paediatric skeleton. Post-traumatic bone deformities include sideway shift, angulation, and rotational deformities. As paediatric fractures and the healing potency are different from the adult's, therapeutic strategies have to be adapted.[1] Due to the advantages of the paediatric skeleton, unnecessary reductions, anaesthesia, and post-traumatic deformities can be avoided in certain cases as the growing bone has a high remodelling ability. The common treatment of paediatric fractures is the conservative therapy.

Besides the high remodelling capacity of growing bones, bone trauma can also lead to growth disturbances. Due to open growth plates, paediatric fractures are unique in developing growth disturbances that can be divided into a stimulating and inhibiting form. The stimulating one describes the overgrowth of a bone after fracture **(see Figure 1)**, while the inhibiting one describes partial or full growth arrest due to the formation of bone bridges crossing the growth plate.[2] The occurrence of growth disturbances and remodelling has been related to the age of the children, the location of the fracture, the type of displacement, as well as to the distance of the fracture to the epiphyseal plate.[1]

Growth disturbances can only occur as long as the corresponding growth plate of the fractured bone has not closed. Growth disturbances can lead to both limb length discrepancy, and angular or joint deformities. They eventually represents an extraordinary risk factor for the development of early arthrosis.[3] In the overgrowth phenomenon, the physeal growth process is stimulated by the fracture. Either the whole size or just a partial part of the epiphyseal plate can be affected. The partial stimulation of occurs very rare, mostly at the distal humerus and the proximal tibia after a metaphyseal fracture.[2, 3]

In contrast, to the upper extremities, the length differences in legs are critical complications. Length discrepancies in the upper extremities do not have any clinical consequence, concerning the functionality. However, length discrepancies of the lower limbs can have the consequence of gait abnormalities, pelvic obliquity with following

lumbar scoliosis, disturbances in function of the hip, the knee, and the ankle joint, and further may cause psychological problems in children.[4] As already mentioned above, growth disturbances depend on different factors. The stimulating growth disorder depends heavily on the age of the children and less on the location of the fracture. It is known that patients under the age of ten rather have problems with growth stimulation, while children older than ten have to deal with bone shortening. An injury, like a femoral fracture in premature age, initially leads to an increase of growth which results in a premature epiphyseal closure. The premature closure of the physis results in bone shortening. In contrast, in the early growth years a high level of post-traumatic overgrowth is observed.[1-3]

It is postulated that, on the one side, the influence of hormones and an increased release of growth factors and, on the other side, an increased blood flow to the growth plate are responsible for the stimulation of the physis.[1-3] For instance, Laer et al. showed that post-traumatic leg length difference occurs in about 70 percent after femoral shaft fractures and in about 40 percent after fractures of the tibia and the fibula. Moreover, he described an identical difference of the bone length in 25 percent. In addition, he determined an average overgrowth: which is after a femoral fracture about 10 mm and after a fracture of the lower leg about 7 mm.[5]

The growth plate itself is the most fragile structure in growing long bones, thus, trauma injury to the epiphyseal plate is a common occurrence. When the mechanical loading localized on the long bones exceeds the mechanical strength of the physis it could lead to an epiphyseal fracture. Regarding injuries of the epiphyseal plate it has been clinically documented, that injured growth plate cartilage is replaced by bony tissue which may lead to impaired bone growth.[6]

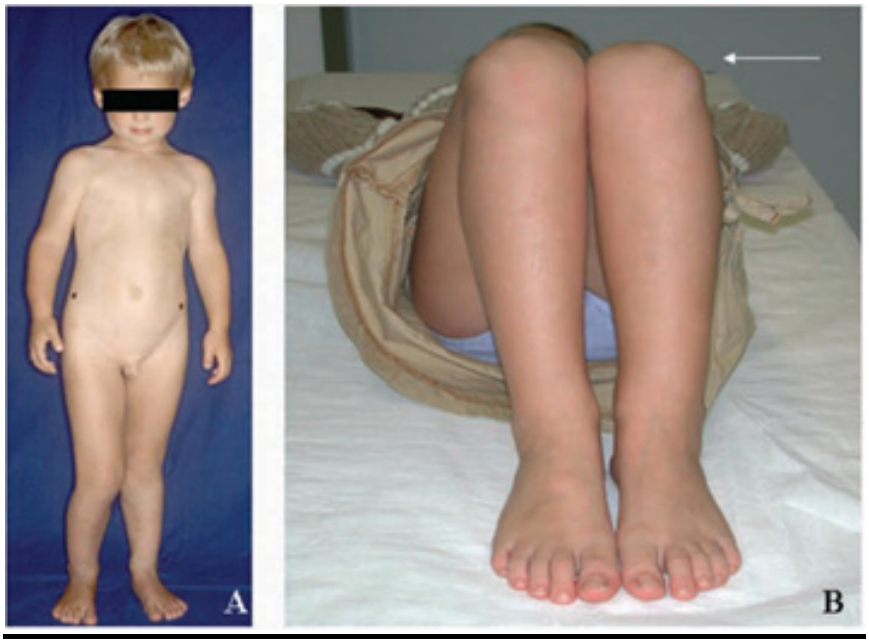
Growth plate fractures are classified as Salter Harris fractures type I-V. Type I and II describe extra-articular epiphyseal and type III and IV intra-articular fractures.[7]

Particularly, growth plate injuries of Salter's types III and type IV can cause severe problems as these fractures represent a total vertical destruction of the physis. These fractures lead to structural disorganization of the epiphyseal plate and interruption of endochondral longitudinal bone formation, resulting in formation of bone bridges and limb length discrepancy and/or angulation deformity.[8]

The underlying cellular and molecular mechanism of bone bridge formation of the injured epiphyseal plate has not yet been fully elucidated.

Figure 1: Child with limb length discrepancy as a result of overgrowth after femoral fracture

(photograph A is friendly provided by the Department of Pediatric and Adolescent Surgery – Medical University of Graz, Austria; Photograph B is friendly provided by Weinberg, Tscherne – Unfallchirurgie im Kindesalter Band II, page 777, Abb. 22-33-g)**[9, 10]**



1.1. Aim of the study

Bone growth disturbances in children are a very common clinical posttraumatic phenomenon representing a complication during fracture repair in paediatric traumatology. Up to now, the underlying molecular mechanisms that cause stimulating or inhibiting growth disturbances have not yet been elucidated.

The aim of this study is to investigate the phenomenon of physeal repair mechanisms due to growth plate lesions, focusing on the growth factor BMP-2, a known key factor in the process of endochondral ossification. This study addressed to determine the spatial distribution of BMP-2 expression in the growth plate.

Animals in the control group sustained a transepiphyseal drill-hole injury to the left tibia, simulating a transepiphyseal fracture. After the left tibiae were compared to the right tibiae. Spatial distribution of BMP-2 expression in the epiphyseal plate was determined by the use of immunohistochemistry on frozen sections.

Following objects are addressed in this study:

- The general morphological physeal reactions to a transepiphyseal drill-hole fracture.
- Illustration of the spatial distribution of BMP-2 expression in the growth plate, in order to gain knowledge about its function in bone bridge formation.
- To what extent does the spatial distribution of BMP-2 expression in the epiphyseal plate of the fractured left tibia differ from the intact contra-lateral right tibia within the experimental group and compared to the control group.

2. BACKGROUND

2.1. Bone Types

In general, bones are divided into four main groups:

1. short bones (ossa brevia)
2. long bones (ossa longa)
3. flat bones (ossa plana)
4. irregular bones (ossa irregularia)

Furthermore, there are pneumatic, sesamoid, and accessory bones.[11] The *long bones* have a central shaft called diaphysis and at each end, they have an epiphysis. You can find the so-called metaphysis between the epiphysis and the diaphysis. The metaphysis can be defined as the wider part at both ends of the diaphysis. The tubular or pipe bones of the limbs, like the tibia or femur are examples of long bones.[12] **(see Figure 2)** During growth, the growth plate (epiphyseal plate or physis) separates the diaphysis and each corresponding epiphysis. The epiphyseal plate consists of hyaline cartilage and is responsible for the longitudinal growth of bones. When growth has finished, the physis becomes completely calcified and remains as the epiphyseal line.[13] In contrast to long bones, short bones are cubed-shaped and chiefly composed of cancellous bone enclosed in a thin layer of compact bone. The carpal and tarsal bones are good examples of short bones. In the group of flat bones, for instance the scapula, ilium, sternum, ribs and bones of calvaria can be found. Flat bones consists of two parallel layers of compact bone sandwiching one layer of spongy bone and a have a thin shape.[11, 14]

Figure 2: Components of a humerus as an example of a typical long bone[7]
(Weinberg, Tscherne, 2006, Unfallchirurgie im Kindesalter Band I, page.4, Abb. 1.2.)

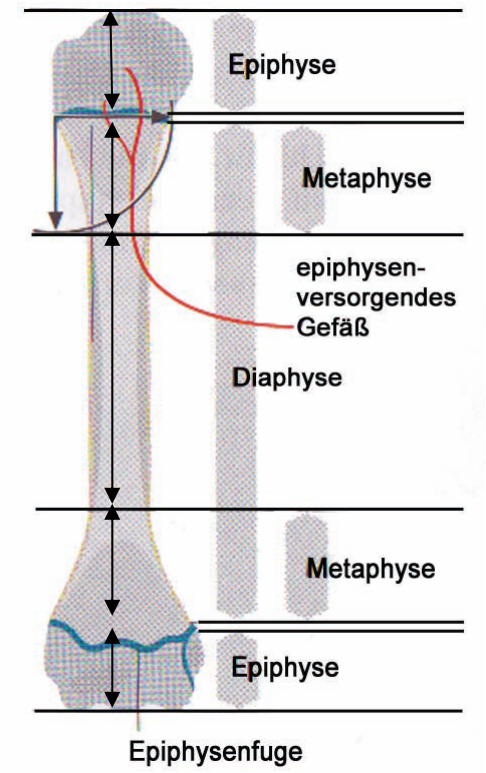
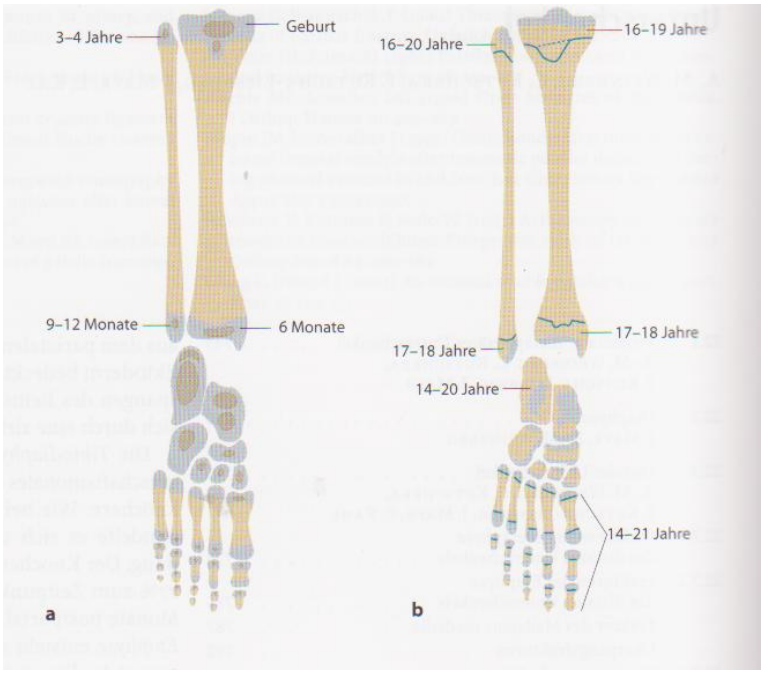


Figure 3: View of a lower leg of a young child. a) Time of occurrence of ossification center and b) Time of occurrence of growth plate closure



2.2. Macroscopic aspects of bones

Besides, macroscopically, there are two different types of bone substances:

1. *substantia compacta* (synonymous cortical bone or compact bone)
2. *substantia spongiosa* (synonymous trabecular, cancellous or spongy bone)

The epiphysis mainly consists of cancellous bone enveloped by a thin layer of cortical bone, whereas the diaphysis consists of a thick cylinder of compact bone with only a few trabeculae. Furthermore, articular hyaline cartilage covers the epiphysis on its ends to shape the synovial joint. Macroscopically, *cortical bone* has a solid texture containing only few spaces and has more density then the spongy bone. As its name implies cortical bone forms the cortex of most bones and is of great importance for providing the rigidity and strength and therefore for reducing the stress brought along by weight bearing and gravidity. Compared to the cortical bone the *trabecular bone* is less dense, softer and weaker but has a higher surface area. Its structure resembles honeycomb pattern, consisting of an irregular lattice of osseous trabeculae with many large cavities between them. For that reason, cancellous bone is of great importance in metabolic functions. The large cavities are filled with either red haematopoietic or yellow fat marrow depending on the age and type of bones. The strong cortical bone of the diaphysis encases a large medullary cavity where you can find thin trabeculae. Usually, in adults this large medullary cavity contains mainly fatty yellow marrow that is not involved in haematopoiesis.[13, 14]

2.3. Microscopic aspects of bones

Compact bones have function units called the Haversian systems or osteons, which have the capability to reach a length of 1 cm and a thickness of 250 to 350 μm.[14] The Haversian canal is in the center of such a function unit located and runs longitudinally containing nerves and blood and lymphatic vessels. The central canal, in the centre of an osteon, is surrounded by 5 to 20 concentric lamellar systems. These concentric lamellar systems, special lamellae, are composed of osteocytes and mineralized extracellular matrix collagen fibres are in circular branching bundles. The so-called osteocytes have their origin

from osteoblasts, which have cemented themselves into the extracellular matrix they have produced. The mature bone cells constitute the main cellular component of cortical bone.[15] Osteocytes lay down in spaces (lacunae) within the mineralized matrix and enlarge by forming filo-podial processes to surrounding osteocytes and keep in contact with gap junctions. The contact via gap junctions constitutes a network of osteocytes and plays an essential role in cell communication and a fundamental role in functional bone adaptation to external stimuli.[15] Starting from the osteocytes lacunae many branched channels, called canaliculi extend. They are free of extracellular matrix but filled with extracellular fluid. These canaliculi contain the cytoplasmatic projections of osteocytes, and supply a route for the exchange of nutrients, gases, and waste products between mature bone cells and the blood vessels.[16]

The Haversian canals in the centre of the Haversian systems are connected to each other as well as to blood and lymphatic vessels of the medullary cavity and periosteum by oblique and transverse canals, called Volkmann canals. Between each osteon there is an interstitial lamella, constituted by remnants of circumferential lamellae of older, partially degraded bone tubules. The so-called cement line surrounds and circumscribes each lamellae system. This line is located where the ground substance consists of a few collagen fibrils[17] but includes many glycoproteins and proteoglycans instead[16]. All Haversian systems or osteons with their special lamellae and the interstitial lamellae systems in between are circumscribed by the outer and inner general lamellae.[17]

In contrast to compact bone, spongy bone does not consist of true Haversian systems or osteons, but of an irregular lattice of thin bony plates or spicules with a large surface-to-volume ratio. These trabeculae show an enormous variety in widths and lengths and show an inclusion of many spaces filled up with bone marrow between them.[13] Trabecular bones receive their nutrients through diffusion from the adjacent medullary space, instead of an actual vascular supply. Consequently single trabeculae cannot reach a thickness over about 200 to 300 μm, because that is the limit distance for functional diffusion.[11]

2.4. The growth plate (epiphysis or physis)

The epiphyseal plate is a cartilaginous tissue located between the epiphysis and metaphysis bone tissue toward the ends of children's long bones as described above and can be divided in several horizontal zones, characterized by chondrocytes at different stages of differentiation. Postnatal, the growth plate is of great importance for bone growth in length by endochondral ossification.[18-20]

Depending on the author the classification of the growth plate zones are different, but one possible classification says that according to defined stages of differentiation there are three distinct zones: **(see Figure 4)**

- reserve zone (or germinal zone or resting zone) RZ
- proliferating zone (or proliferative zone) PZ
- hypertrophic zone HZ

The process of endochondral ossification includes chondrocyte proliferation at the PZ, maturation, hypertrophy, apoptosis, and further mineralization at the HZ. Through this process the epiphyseal plate is of greatest significance for making a calcified cartilaginous template for bone deposition for the longitudinal growth of young long bones.[6] Once blood vessels from metaphysis begin to invade the calcified cartilage the conversion of calcified mature cartilage to new bony tissue at metaphysis bone tissue is initiated. Blood vessels bring in bone-formation cells, called osteoblasts and further bone-resorbing cells, called osteoclasts. Osteoclasts partially remodel calcified mature cartilage, and activation of osteoblasts leads to new bone on the surface of partially remodelled calcified cartilage.[21]

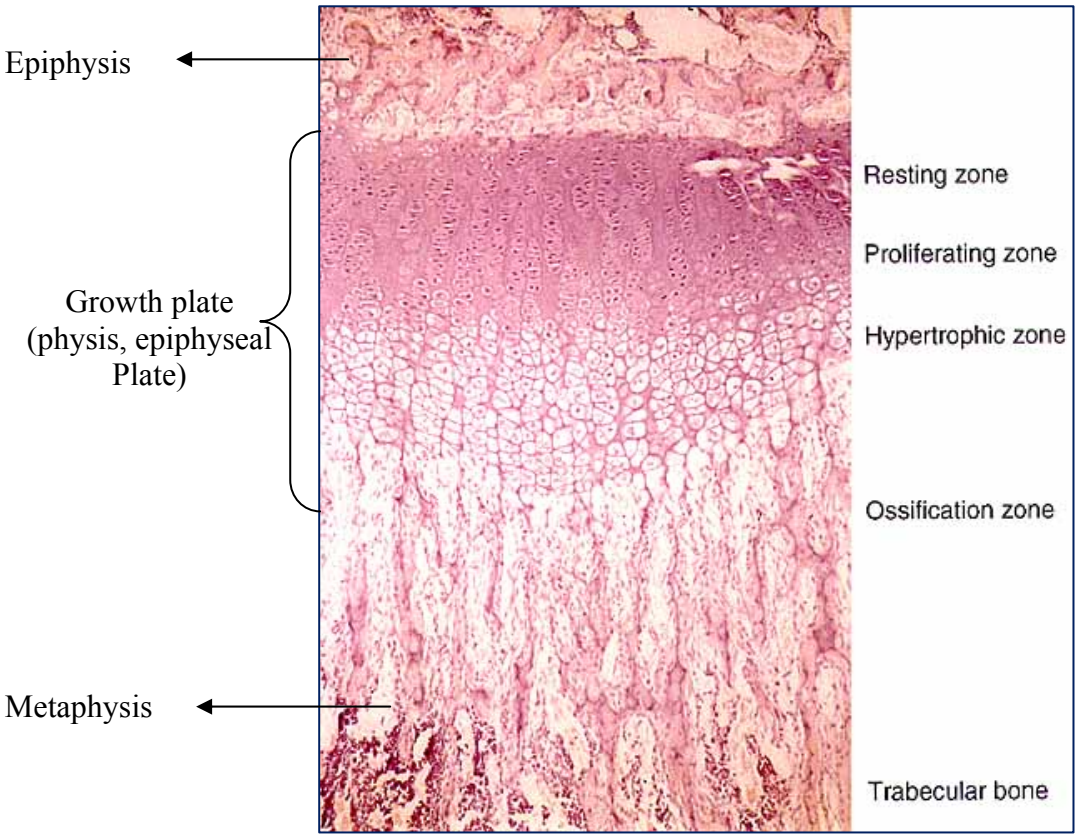
The reserve zone (RZ) is located at the epiphyseal end of the growth plate and contains quiescent chondrocytes, which could be called stem cells, too. This part of the growth plate contains a high ratio of extracellular matrix to cell volume and is essential for the orientation of chondrocytes of the underlying zone and therefore of great importance for unidirectional bone growth.[22]

Stem cells at the *proliferating zone (PZ)* produce large amounts of extracellular matrix proteins, divide excessively, have flattened shapes and arrange in columns. These stem cells synthesize mainly collagen type II and the proteoglycan aggrecan. The kind of zonal classification depends on the various authors, like Forriol and Shapiro deviate lightly from that classification. They refer to a *columnar zone* instead of a proliferative zone. Furthermore, they separate this columnar zone into two well-defined areas: the upper proliferating zone and the lower maturation zone. In the upper proliferating zone the flattened chondrocytes show multiple mitoses and in contrast, the lower maturation zone shows chondrocytes containing plenty of rough endoplasmic reticulum and the matrix synthesis takes place.[19] The columns of chondrocytes have a specific structure and are aligned in parallel to the longitudinal axis of the bone and longitudinal septa of cartilage matrix separate them from each other. Each column has various numbers of cells between ten and twenty, depending on the current mitotic activity of the column. In the lower part of the proliferative zone, chondrocytes lose their proliferating ability, cells stop dividing, and then increase in size and form to pre-hypertrophic and finally into fully differentiated round hypertrophic chondrocytes. The cells increase their intracellular volume five to ten-fold through a rise in intracellular organelles and an intracellular accumulation of glycogen. The hypertrophic cartilage (hypertrophic zone – HZ) is invaded by endothelial cells and is prepared for calcification. Hypertrophic chondrocytes stop with the synthesis of type II collagen but in contrast, they start to produce short-chain type C collagen and matrix vesicles. They act as the initial site of mineralization in the hypertrophic zone. In the zone of provisional calcification the mineralization of the longitudinal septa of the cartilage matrix occurs. Furthermore, the zone of vascular invasion at the metaphyseal end of the growth plate is of great importance for the invasion of osteoprogenitor cells, osteoblasts, chondroclasts, and osteoclasts. Hypertrophic cells stimulate the formation of blood vessels through secretion of VEGF, and further a vascular ingrowth in the metaphyseal end of the physis takes place. Finally, apoptosis of hypertrophic chondrocytes occurs in the mineralized zone. The various stages of chondrocytes differentiation are passed through within 24 hours, initiating with the germinal cells, next with the proliferating cell and the hypertrophic cell and eventually ending in the apoptotic cell.[18]

At the passage of epiphyseal plate and metaphysis, the main part, about 80 percent of the mineralized longitudinal septa are destroyed by chondroclasts. The remaining longitudinal septa function as templates on which osteoblasts deposit bone matrix. Therefore primary spongiosa is formed, consisting of a mineralized cartilage and bone formation. Step by step, all cartilaginous material is removed and secondary trabeculae appear, through bone resorption by osteoclasts and bone formation by osteoblasts.[18-20]

Matrix synthesis, cell proliferation and especially the mechanism of cell hypertrophy are essential for length growth of bones.[19, 20]

Figure 4: Haematoxylin and eosin stained section of the epiphyseal growth plate from the proximal tibia (two-week-old mouse).
The epiphyseal plate is located between the epiphysis and the metaphysis. The different zones of the physis represent the proliferation, differentiation, hypertrophy and apoptosis of chondrocytes (For details see text above)[23]



The longitudinal bone growth occurs primarily at the epiphyseal plate, which disappears after cessation of growth in human adolescents. The decrease of the proliferating and hypertrophic zones leads to vascular anastomoses between the epiphyseal and metaphyseal vessels and formation of bone bridges. The fusion of the epiphysis to the metaphysis leads to the termination of growth. Physeal closure starts about two years earlier in girls than in boys and depending on its location and on the specific bone occurs at different times.[19]

At the outside margin of the growth plate a ring with its fibres arranged in vertical, circumferential, and oblique directions can be found. The chondrocyte progenitor cells of the ossification groove of Ranvier cooperate to the latitudinal growth of the physis. The perichondrial fibrous ring of LaCroix connects with the periosteum of the bone and sustains the growth plate in case of tension, compression, or shear loads.[18]

2.5. Basics of Fracture Healing

Beside their metabolic function bones are important to protect organs and enable physical activity, which is carefully coordinated by muscles, that move bones within specialized joint structures. Furthermore, bone is uniquely capable of regenerating the original structure, the geometry and biomechanical competency after fracture, without resulting in the formation of a poorly organised tissue. A real restitutio ad integrum can be seen at fracture repair. For that reason, a correct fracture healing is important. Fracture healing is a complex, highly orchestrated, multistage biological process. This complex fracture healing process needs a well-coordinated interaction of vascular cells, skeletal precursor cells, immune and haematopoietic cells. Particular pathways of embryological skeletal development and skeletal growth recapitulate during the process of fracture healing.[24-26]

During fracture healing an enormous cell interaction occurs between the external soft tissue, like muscles, fat surrounding the injured bone, the cortical bone, the periosteum and the bone marrow, and the beginning endochondral and intramembranous bone tissues.[27] A series of different factors concerning the injured tissue, like the mobility of the fracture site, age, oxygen tension, nutrients, pH, extent of injury to hard and soft tissue and level of growth factors and hormones influence the extent of contribution of each individual tissue.[26, 28]

2.5.1 *Primary and Secondary Fracture Repair*

There are two different main types of fracture healing:

1. ***direct or primary cortical fracture repair***
2. ***indirect or secondary fracture healing***

Whereas the majority of fractures heals by a secondary regeneration process, because the major part of fractures is untreated or is left without a rigid fixation. *Secondary fracture repair* is characterized by the formation of a fracture callus. Endochondral and intramembranous ossification occur through this process and are induced by the incidence

of micromotions at the fracture site. These micromotions stimulate periosteal reaction and further, the process of callus generation. On the other side bone formation is inhibited by rigid fixation. In contrast, the *primary cortical fracture healing* is marked by the direct ingrowth of Haversian systems across the fracture line. This process does not require the formation of a callus. It occurs only when anatomic reduction and rigid immobilization for instance by an internal fixation are pre-existing. The cortex acts as the essential part in this process, whereas the bone marrow, the external soft tissues and the periosteum take a back seat.[24, 26]

The secondary fracture healing process can be divided into three main phases that overlap and cause the various tissue types to interact: **(see Figure 5)**

1. the *inflammatory* phase
2. the *reparative* phase
3. the *remodelling* phase

First of all the ***inflammatory phase*** describes the initial phase induced by a loss of integrity of the osseous structure. The formed haematoma appears immediately after the initiation of bone injury and plays a central role, due the ruptured blood vessels. It fills the fracture gap and contains a lot of fibrin and inflammatory mediators that attract mesenchymal cells. The major part of the haematoma contains leukocytes, macrophages, and lymphocytes, which are essential for the secretion of important proinflammatory cytokines. The differentiation of mesenchymal cells into chondroblasts, osteoblasts, and angioblasts are depend on certain proteins.
Vortkamp and co-workers (1998) sat up the idea that fracture repair is a recapitulation of the bone developmental process, because they have examined the hypothesis that the same molecules that regulate embryonic endochondral ossification are also expressed during the fracture healing process.[29]

The periosteum next to the site of fracture acts through an increase of proliferation of osteogenic precursor cells within 24 hours, which then differentiate into bone forming cells. The main object of the ***reparative phase*** is the *callus formation.* The periostal cells constitute the hard callus, which appears under the periosteal formative tissue within a few millimetres from the fracture site. They directly differentiate into osteoblasts, which lay

down bone by intramembranous ossification. Within two weeks the enhanced proliferation rate in the inner layer of the periosteum stops and thus indicates the reduction of periosteal response.[24, 30] On the other hand, in the centre of the fracture gap chondrocytes form the soft callus, which serves as a scaffold for woven bone formed by endochondral ossification within the first seven to ten days of fracture repair. The origin of the responsible cells for this endochondral cartilage formation has not been completely cleared. Some authors, like Gerstenfeld et al. claimed that either the regional adjacent periosteum, the surrounding muscles or the marrow space support mesenchymal stem cells and chondrogenic precursor cells which later then get chondroblasts.[31]

The hard and the soft callus together form the so-called provisional callus, which provides temporary stabilization and can be seen by the middle of the second week. On the one hand hard callus develops through intramembranous ossification and on the other soft callus is the result of endochondral ossification.[24, 30]

The next step is the preparation of the extracellular matrix for calcification. Therefore, chondrocytes of the soft callus begin to form into hypertrophic cells. Around two weeks after bone fracture, the mature hypertrophic chondrocytes get the main cell part in the chondroid callus and some areas start to calcify. Approximately five or six weeks later, almost the whole callus contains calcified cartilage, which is later resorbed by multinucleated chondroclasts. Blood vessels play a central key role in bone repair when they invade the tissue and thus, allow mesenchymal cells to invade too. These mesenchymal cells later differentiate into osteoprogenitor cells and then into osteoblasts. The callus contains calcified cartilage and newly formed woven bone after around six to seven weeks post-fracture.

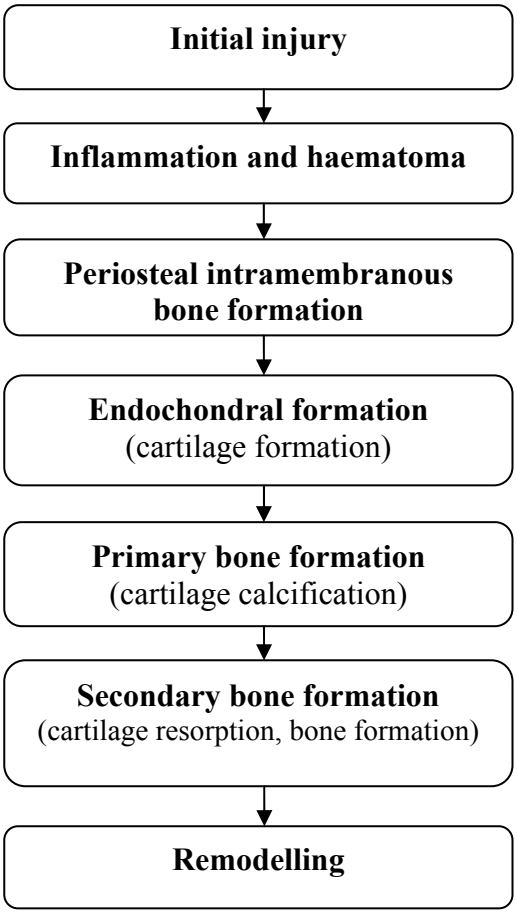
Finally, the ***remodelling phase***, can last months, even years in certain osseous structures, but it is the most important part for rigid stability. Rigid stability is necessary for full physical activity and occurs when lamellar bone is laid along the lines of stress and replaces the provisional callus. The most important cells of the remodelling phase are the osteoclasts and osteoblasts.[24, 30]

To sum up, it is known that bone healing is composed of two types of bone formation:

1. the endochondral bone (ECB) formation and
2. the intramembranous bone (IMB) formation

They appear in a symmetrical manner around the fracture line. The development of two circular centres of cartilage (ECB) shows that the fracture line in the damaged bone defines the spatial relationships of the morphogenetic fields during tissue regeneration. At the same time regions of intramembranous bone formation are at the proximal and distal ends and tapers inward toward the fracture line deep in the cartilage ring.[27]
Unfortunately, the identity and the origin of mesenchymal stem cells (MSCs) that start morphogenetic signals and play an important role in fracture healing are still unknown. The periosteum, the external soft tissue or both could be the origin of MSCs. Some studies have shown that morphogens recruit stem cells locally and induce them to differentiate, other studies that a pre-myogenic cell line can differentiate into chondrogenic or osteogenic cells when treated with BMPs.[27]

Figure 5: Different stages of secondary fracture healing.[32]



2.5.2 *Mechanical and Molecular Control of Fracture Healing*

A couple of soluble factors are essential for the control of the complex events proceeding during fracture healing. Every single step of these complex events has to be exactly regulated. Three groups of molecules are considered the main regulators in bone repair, although the knowledge about the exact regulatory pathways is not complete.

The three main groups are:

1. proinflammatory cytokines
2. members of the TGF-β superfamily
3. metalloproteinases and angiogenic factors[31]

2.5.2.1. Proinflammatory Cytokines

Like the name of proinflammatory cytokines tells by its own, they are expressed in the initial inflammatory phase of fracture healing. The initial inflammatory phase immediately follows bone injury and the whole process of fracture repair is initiated by the activation of the immune system and the expression of these cytokines.[33] Beside their first expression peak in the initial phase of bone repair a second one can be recognized later. Between these two expression peaks, they are reduced to very low levels. The first, as mentioned above, is recognized within 24 hours post fracture, the second appears at a very late stage of bone healing, called the phase of bone remodelling.[26, 34] The proinflammatory cytokines interleukin 1 (IL-1), interleukin 6 (IL-6), and tumor necrosis factor α (TNF-α) are essential for chemotaxis of other inflammatory cells, the facilitation of angiogenesis, and the increase of extracellular matrix production. Macrophages, inflammatory cells, further mesenchymal and osteoblastic cells located in the periosteum secrete these cytokines.[34]
The whole process of fracture healing includes two different types of resorption. The first occurs at the end of the endochondral period when mineralized cartilage has to give place to primary bone. That type of resorption shows elevated levels of M-CSF, RANKL, and OPG in contrast to low levels of the typical cytokines like IL-1α, IL-1β and IL-6. The second type of resorption takes place during the phase of remodelling and the formation of

secondary bone and seems to be dependent on the levels of IL-1, IL-6, and TNF-α. That type of resorption shows high levels of IL-1α, IL-1β and IL-6. Considering that expression pattern, the mechanism regulating cartilage resorption differs from that regulating bone resorption during the remodelling phase.[31]

Among the above-mentioned inflammatory cytokines TNF-α is additionally expressed at very high levels at the end of the period of cartilage resorption.[25, 31] Furthermore, TNF-α has a high-level expression in hypertrophic chondrocytes[34] and thus, takes up an exceptional position. TNF-α has the power to highly promote the process of fracture healing and take part in the recruitment of mesenchymal stem cells, in the stimulation of apoptosis of hypertrophic chondrocytes and the promotion of function of osteoclasts.[35]

2.5.2.2. TGF-β (Transforming Growth Factor β) Superfamily

A large group of functionally and structurally related polypeptides form the TGF-β superfamily. The group consists of at least 34 specific growth and differentiation factors.[26] The TGF- β superfamily includes the transforming growth factors themselves (TGF-β), the growth and differentiation factors (GDFs) and the bone morphogenetic proteins (BMPs). Those specific members of the superfamily act actively in fracture repair like BMPs 1-8, GDF-1, 5, 8, 10 or the TGFs-β1, -2, -3. TGF-β is secreted by thrombocytes, osteoblasts and chondrocytes and at least five isoforms have been identified so far. These isoforms have numerous functions for example induction of callus formation, stimulation and differentiation of mesenchymal stem cells, further the positive influence on the secretion of extracellular-proteins. However, the main function is observed during chondrogenesis and endochondral bone formation.[36] On the other side, BMPs for instance are produced by mesenchymal cells, osteoprogenitor cells, osteoblasts, and chondrocytes and have the capacity to regulate the differentiation of mesenchymal stem cells into the osteogenic and chondrogenic lineages. Furthermore, they lead to a stimulation of angiogenesis, to promotion of terminal differentiation of osteogenic and chondrogenic cells, and to enhancement of synthesis of VEGF and IGF.[26] The members of this superfamily show structural and functional relation to each other, but each shows a specific temporal expression pattern and has various functions during different stages of intramembranous and endochondral bone formation. Cho et al. showed the specific temporal expression patterns of TGF-β superfamily members during fracture repair, like

BMP-2 and GDF8 have a peak expression on day one post-fracture in the inflammatory phase, further BMP-2 shows another peak during the osteogenic phase. In the chondrogenic phase seven days after fracture an expression peak of GDF5, TGF-β2, and TGF-β3 is observed. This elevation suggests their potential role in cartilage formation. During the period of cartilage resorption and bone formation an elevation of BMP-3a, BMP-4, BMP-7, and BMP-8 is shown. The expression of BMP-5, BMP-6, GDF10, and TGF-β1 can be seen throughout the healing process.[37] The exact specific mechanism of each member is difficult to define until now, but it is known that they function in a network and do not act singly.[25]

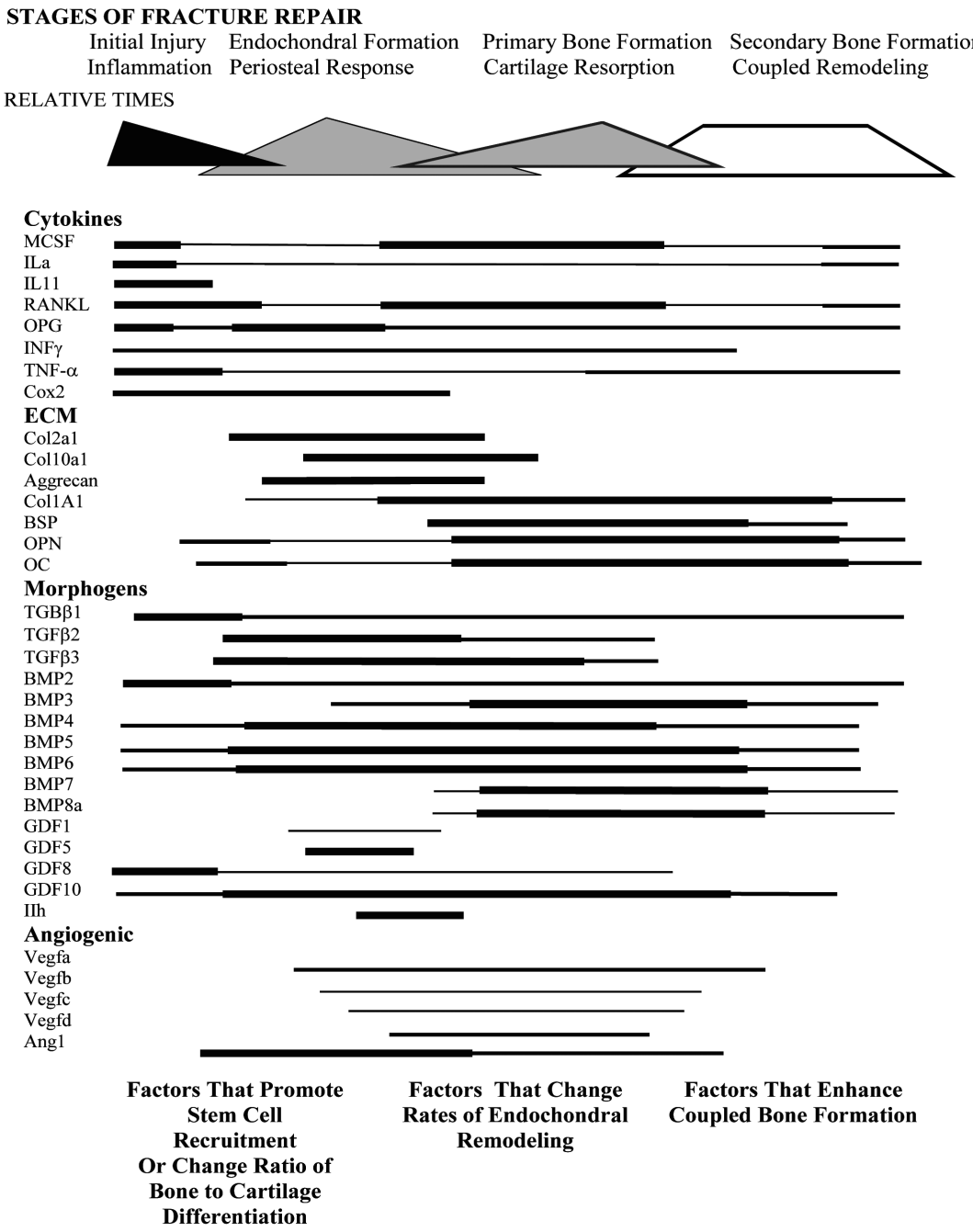
2.5.2.3. Metalloproteinases and Angiogenic Factors

Metalloproteinases and angiogenic factors constitute the third essential group of molecular factors of fracture healing. An adequate blood flow is one of the most important basic requirements for an optimal bone healing. The establishment of an adequate blood flow within the callus with the ingrowth of blood vessels allows the invasion of important cells. Those important cells like osteogenic precursor cells, osteoblasts, and osteoclasts can invade in the fracture site. At the end of endochondral ossification remodelling period metalloproteinases lead to a decrease of the extracellular matrix of cartilage and bone, further constitute the establishment for the invasion of blood vessels. To this day, two different classes of angiogenic factors and their receptors are known to regulate vascularisation during fracture repair. On the one hand there is the vascular endothelial growth factor (VEGF) family and on the other the angiopoietin family. The appropriate receptors for the VEGFs are the vascular endothelial growth factor receptors 1 and 2 (VEGFR) and for the angiopoietin family the Tie receptors. VEGFs are essential in the process of neo-angiogenesis as well as in stimulation of mitogenesis in vascular endothelial cells. There are five main groups known: VEGF-A, -B, -C, -D, and –E, whereas VEGF-A in scientific research is most investigated.[26, 31]

A recent study shows that the exogenous administration of VEGF during fracture healing has the capacity to enhance the fracture repair process.[38] Moreover, a couple of recent studies show that BMPs and VEGFs have an intimate relationship, because BMPs are able to stimulate the expression of VEGF by osteoblasts and osteoblast-like cells[39, 40] and further express VEGF related receptors.[41] In contrast to VEGF, angiopoietins do not

stimulate mitogenesis, but block apoptotic signals and thus, have the function as a survival factor for endothelial cells. Furthermore, they lead to a stabilization of cell-cell interactions of endothelial cells, to an activation of migration, and spreading of endothelial cells from pre-existent vessels.[42]

Figure 6: Schematic summary of the temporal expression patterns of molecular processes during fracture healing[25]



2.6. Normal Bone Formation

Bone growth in vertebrates takes place in two distinct processes, on the one hand the process of intramembranous and on the other the endochondral bone formation. The intramembranous bone formation is important for the development of craniofacial bones, like the jaw, and requires direct transformation of mesenchymal cells into osteoblasts. Whereas, the endochondral bone formation is not a direct process, but a process in which the condensation of mesenchymal cells leads to a development of cartilaginous templates, which are afterwards replaced by new bone. The long and short bones of vertebrates form through endochondral ossification. Postnatal, linear growth takes place at the growth plate, where cartilage is continuously replaced by bone. Besides of the control by autocrine, paracrine and endocrine factors, a normal growth plate function is essential for normal skeletal growth.[43]

2.6.1 *Endochondral Ossification*

During embryonic development, endochondral ossification forms mostly facial bones, the appendicular skeleton as well as the axial skeleton. At the beginning of this process, mesenchymal cells start to condense and differentiate into two distinct types of cells: chondrocytes and perichondrial cells. On the one hand, chondrocytes shape the cartilage elements and on the other perichondrial cells surround the cartilage model. First chondrocytes from the center of the cartilage elements develop through several steps of maturation from proliferating chondrocytes to non-proliferating hypertrophic cells. Usually during normal development, bone replaces only terminally differentiated chondrocytes. In parallel, the perichondrium differentiates into osteoblast-forming periosteum. Hypertrophic differentiation and periosteum formation have to work exactly together and the proliferative and hypertrophic zones are reduced to the growth plate. Proliferation and hypertrophic differentiation of chondrocytes in the small band, called growth plate is responsible for longitudinal growth of bones. Due to continuous replacement of hypertrophic cartilage by bone, the exact regulation of all these various steps of chondrocyte differentiation is essential for balancing growth and ossification of the skeletal elements.[44, 45]

2.7. Regulation of Longitudinal Growth at the Growth Plate

The regulation of normal longitudinal bone growth happens at three different levels:

1. *extrinsic factors* such as hormones
2. *intrinsic molecules*
3. *mechanical forces*

Extrinsic factors provide systemic control, intrinsic molecules provide a local regulation of bone growth and furthermore, mild tension and mild compression increases growth. Besides, mechanical forces, like severe compression would inhibit length growth.[20]

Table 1: Major extrinsic and intrinsic factors controlling the growth plate[19]

EXTRINSIC FACTORS	INTRINSIC FACTORS
Growth hormone	Indian hedgehog (Ihh)
Insulin-like growth factor I/II	Parathyroid hormone-related protein (PTHrP)
Thyroid hormone	Bone morphogenetic proteins (BMPs)
Vitamin D3	Fibroblast growth factors (FGFs)
Glucocorticoids	Vascular endothelial growth factor (VEGF)
Sex hormones (oestrogen, androgen)	Matrix Metalloproteinases (MMPs)

Local ***paracrine factors***, such as bone morphogenetic proteins **(BMPs),** Wnts, fibroblast growth factors (FGFs), hedgehog proteins and retinoids, act in concert with ***systemic factors***, such as growth hormone (GH), thyroid hormone (T3, T4), oestrogen and androgen (sex hormones), vitamin D3 and glucocorticoids, in controlling bone development, remodelling, and partially in fracture healing. Furthermore, they define shapes and sizes of bones. For instance, systemic hormones like oestrogen cooperate with local BMP and influence osteoblast function.[43]

Bones allow adjacent muscles to move them, they move within specialized joint structures, and they protect adjacent organs, and for that reason it is so important, that the sizes and shapes of bones are carefully coordinated to allow efficient movement.[46]

2.8. Major intrinsic factors

Bone morphogenetic proteins (BMPs) are essential at every stage of endochondral bone formation. BMP-2, -3, -4, -5 and -7 are expressed in the perichondrium of long bones, BMP-2 and -6 are produced by hypertrophic chondrocytes, and BMP-7 is synthesized by proliferative chondrocytes too. Furthermore, they are essential for the mesenchymal condensation in early limb buds. To sum up BMPs stimulate the proliferation of chondrocytes and thus leads to an increase of the length of proliferating columns. Besides, they increase the expression of Ihh by pre-hypertrophic chondrocytes and cause a delay of terminal differentiation of hypertrophic chondrocytes.[19, 46, 47]

Fibroblast growth factors (FGFs) bind to fibroblast growth factor receptors (FGFRs) and further affect chondrocyte proliferation and differentiation. The fibroblast growth factor receptors constitute a family of four major transmembrane tyrosine kinase receptors. FGF signalling works antagonistically to BMP signalling at each level and has a negative effect on chondrocytes proliferation, leads to an acceleration of terminal differentiation of hypertrophic chondrocytes, decreases the length of proliferation columns and suppresses Ihh expression.[46, 47]

Vascular endothelial growth factor (VEGF) is synthesized by hypertrophic chondrocytes and is absolutely essential for the ingrowth of new vessels into the epiphyseal plate. Resting and proliferating chondrocytes do not express that intrinsic molecule. Vascular endothelial cells are the targets of VEGF, and are stimulated to proliferation, migration, and finally formation of new blood vessels. Furthermore, since the

receptor for VEGF has been found on hypertrophic chondrocytes an autocrine role is speculated, but it is not clear how VEGF affects chondrocytes directly.[18, 19]

2.9. BMP and Ihh/PTHrP Signalling Interaction

Two secreted factors, Indian Hedgehog (Ihh) and parathyroid hormone-related protein (PTHrP) form a negative feedback loop modulating the onset of hypertrophic differentiation during endochondral ossification. Through a feedback loop between these two factors, the size of the proliferating pool is regulated. Indian Hedgehog (Ihh), a member of the Hedgehog family of signalling molecules, is required for several points of endochondral ossification. Parathyroid hormone-related protein is located in the periarticular region of the developing cartilage elements. Besides, it has been shown that BMPs are potential interactors of the Ihh/PTHrP feedback loop, but BMP signalling does not work as a secondary signal of Ihh to induce PTHrP expression or to delay the beginning of hypertrophic differentiation.[48]

There are three main functions of BMP signalling in regulating proliferation and hypertrophic differentiation of chondrocytes: [48]

1. BMP and Ihh signalling have to act in parallel for maintaining a normal proliferation rate
2. BMP has an important role in modulating the expression of Ihh
3. BMP signalling delays the process of hypertrophic differentiation itself and this function is independent of the Ihh/PTHrP pathway[48]

Indian Hedgehog (Ihh) activates a transmembrane protein, called Smoothened (Smo) through binding to its receptor Patched-1 (Ptc-1) and finally starts a signal cascade that leads to specific gene activation. Through this gene activation, or gene expression, Ihh plays a key role in normal bone development by directly stimulating chondrocytes proliferation, differentiation and osteoblast differentiation. Furthermore, Ihh leads in a PTHrP dependent manner to a delay of chondrocyte hypertrophy. Ihh is expressed by pre-hypertrophic[49] and early hypertrophic chondrocytes.[46]

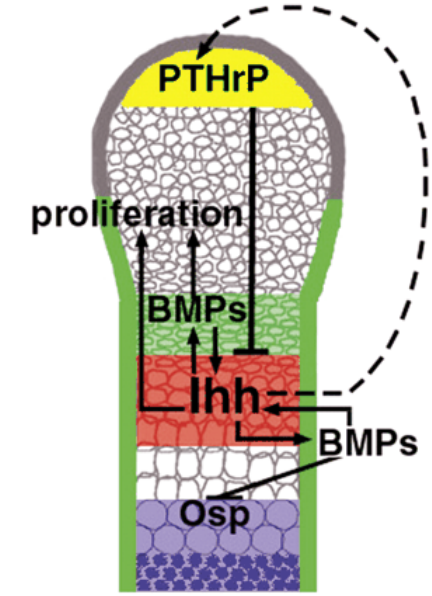
The PTH/PTHrP receptor is required for the PTHrP signalling and is located at high levels in the transitional region where the change from proliferating into hypertrophic chondrocytes takes place, by pre-hypertrophic/ early hypertrophic chondrocytes, and at low levels throughout the growth plate by proliferating chondrocytes.[50-52] A series of adult and foetal tissues synthesizes this auto/paracrine factor and it binds and stimulates the same G-protein-coupled receptor that is used by PTH, the calcium-regulating hormone. Perichondrial cells and chondrocytes in the periarticular region of long bones synthesize PTHrP and through PTHrP signalling the further maturation of proliferating and pre-hypertrophic chondrocytes is inhibited. Moreover, PTHrP keeps the proliferating chondrocytes in the proliferative pool.[18, 46, 47]

It is well established that Ihh signalling induces the expression of several BMP genes and that both act in parallel to enhance chondrocyte proliferation.[53, 54]

BMP signalling increases the expression of Ihh by pre-hypertrophic chondrocytes, and further increases both the proliferation of chondrocytes and the length of proliferating columns of chondrocytes. FGF, Ihh/Pthlh, and BMP represent the three main groups of signalling molecules that regulate bone development in parallel or antagonistic pathways. Hormones, growth factors, cytokines and vitamins influence their expression or signalling pathways. Nevertheless, the interaction of these molecules is poorly understood and needs to be further investigated.[46]

As told in the text above the interaction of Ihh and PTHrP can be seen as a negative-feedback loop. PTHrP works on the receptor of proliferating chondrocytes and keeps them in the proliferating pool. By the delay of hypertrophy, PTHrP inhibits the production of Ihh by pre-/hypertrophic chondrocytes. When PTHrP is not sufficiently able to stimulate the proliferating chondrocytes longer, they get hypertrophic and initiate to secrete Ihh. Inversely, Ihh leads to a stimulation of chondrocytes proliferation and to an increase of the production of PTHrP by cells of the periarticular region.[18, 46, 47] **(see Figure 7)**

Figure 7: Schematic model of feedback loops between Ihh, PTHrP, FGFs, and BMPs in the growth plate [48]



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2.10. Main Parts of the Bone

There are three different main parts of the bone:

1) the mineral component
2) the collagenous matrix component
3) the growth factor component

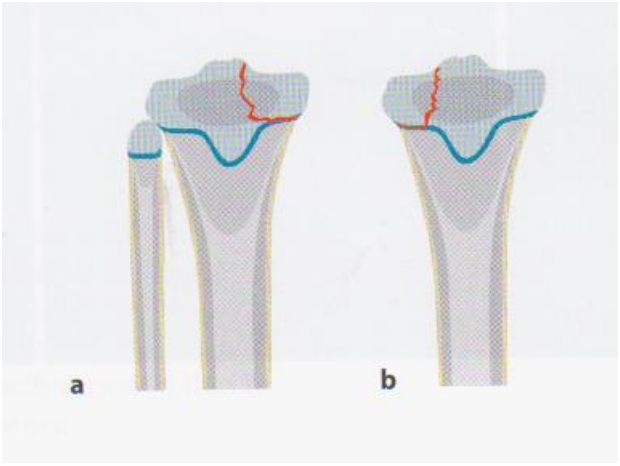
The mineral component gives the bone its structural integrity and stability. The growth factor component includes the BMP activity and can be extracted after the bone has been demineralised. When the growth component is implanted subcutaneously in a rat, for instance, you can see the different stages of bone healing. At the beginning, the implant area is infiltrated by undifferentiated cell types, like mesenchymal cells. After about four to seven days, these undifferentiated cells differentiate into chondrocyte. So after around one week the undifferentiated mesenchymal cells differentiate systematically to mature chondrocytes, which later calcify. By day ten new bone formation begins when the

cartilage intermediate is reduced. At the end, there is a development into normal remodelling bone tissue, which is complete with osteoblasts and osteoclasts. Osteoblasts are essential in the process of forming bone and in contrast, osteoclasts resorb the bone tissue.[55]

2.11. Basics of Growth Plate Injuries

The epiphyseal plate in long bones is the most fragile structure in a child's developing long bone. Because of that reason trauma injury located near to the epiphysis is a common occurrence. The main reason of fractures of the epiphyseal plate is when the mechanical loading placed on the long bones exceeds the mechanical strength of the physis.[6] Trauma injuries to the growth plate that include the entire vertical destruction of the epiphyseal plate can result in significant problems for the development of a long bone. Particularly Salter's type III and type IV are common in young children and involve the entire vertical destruction of the growth plate. **(see Figure 8)**

Figure 8: Classification of epiphyseal fractures of the proximal tibia. These typical fractures in young children bones are classified by Salter Harris.
Picture a shows a Salter Harris III fracture
Picture b shows a Salter Harris IV fracture
(Weinberg, Tscherne, 2006, Unfallchirurgie im Kindesalter Band II, S.704, Abb. 21.29 a-b)[10]



In fact, the epiphyseal plate has limited ability to regenerate and these injuries are often repaired by bony tissue at the injury site, a phenomenon often called bone bridge formation. The process of bone bridge formation at the injured site leads to structural disorganization of the epiphyseal plate and further disrupts endochondral longitudinal bone growth.[8] Further this process results in significant skeletal complications like limb length discrepancy and angulation deformity.

As just mentioned, cellular and molecular mechanisms for the bony repair of the injured epiphyseal plate up to now, remain unclear. Previous studies have shown sequential injury responses leading to the bony bridge formation at the injury site after using a growth plate drill-hole injury model in young rats. The drill-hole injury model in young rats simulates or causes a Salter's type IV-like injury and the sequential injury responses are called the inflammatory, fibrogenic, and osteogenic responses and further the bony bridge remodelling/maturation. Inflammatory response occurs typically after the growth plate injury with an infiltration of inflammatory cells peaking on day 1 and a decrease on day 3. Afterwards from day 3 to day 7 an infiltration and proliferation of mesenchymal cells can be observed at the epiphyseal plate injury site. Further, some of these infiltrated and proliferated mesenchymal cells differentiate into osteoblasts.[8]
After day 7, a formation and enlargement of bony trabeculae at the injury site can be observed. From day 14 onwards to day 25, maturation of bony bridge follows with formation of bone marrow between bone trabeculae. A previous study has shown up regulated expression of some growth factors and cytokines at these different phases of bony repair, but the way these cellular healing responses are regulated remains largely unknown.[56]

3. BONE MORPHOGENETIC PROTEINS (BMP)

Marshall R. Urist discovered bone morphogenetic proteins (BMP) in 1965. Urist detected that when BMPs are implanted subcutaneously they are able to induce ectopic bone formation. BMP is a genetically produced substance that is considered one of the major groups of morphogenetic factors that helps bone to regenerate by inducing certain types of connective tissues and other unspecialized cells to become bone cells. Single molecules, like BMP-2 are capable of inducing the formation of new cartilage and bone when implanted ectopically.[57]

Urist implanted the homogenous HCl decalcified diaphyseal bone into a pouch in the belly of either the rectus abdominus, quadriceps, or erector spinae muscles of rabbits, rats, mice, and guinea pigs and the result was an ectopic bone formation. He first showed that the demineralised matrix of bone graft was capable of induction of de novo bone formation. In purifying the active component of the demineralised bone graft, researchers were led to discover a family of regulatory molecules known as bone morphogenetic protein. In more than twenty years of experimentation, Urist proved that BMP could be used to build living bone tissue around surgical pins and screws used to repair broken hips, shoulders and other bones.[57]

Since that time, there has been an ongoing effort to develop an ideal osteoinductive bone graft substitute. Although millions of fractures occur annually, and the majority heal satisfactorily, on the one hand 5% to 10% go on to delayed union or non-union and on the other hand there are paediatric growth disturbances induced by diaphyseal fracture or physeal lesions. BMPs may be able to improve bony healing in these conditions and perhaps enhance the healing of fractures that otherwise heal satisfactorily. During the last years, BMPs have been produced in highly purified form through using rDNA technology.

The first clinical use of bone morphogenetic proteins in fracture repair was by Urist and his colleagues at the University of California at Los Angeles, where bone morphogenetic protein composites were used in difficult healing processes or non-unions. The exact identification of these osteoinductive molecules would lead to an enormous benefit in therapeutic treatments for a variety of bone defects, like non-unions, fractures, and periodontal disease.[58]

BMPs, also called growth and differentiation factors (GDFs) are known key regulators of bone formation and repair. Except of BMP-1, they are members of the transforming growth factor beta (TGF-β) family of paracrine factors, which activate heterodimeric receptors with serin / threonine kinase activity and regulate multiple steps of endochondral ossification and bone formation in embryonic development and postnatal physiological function. They regulate the proliferation, differentiation, and apoptosis of various types of cells and organs and act to promote osteoblast differentiation.[59]

Some BMPs have synonyms. For instance, BMP-3 is called Osteogenin or BMP-7 and BMP-8 are named Osteogenetic Proteins (OP-1 and OP-2). BMP mediates patterning and growth of many tissue types during embryogenesis and organogenesis.[59]

BMPs can be divided into some subgroups, on the one hand with BMP-2 and BMP-4 and on the other with BMP-5, BMP-6 and BMP-7. BMP-5, -6, -7 are an average of about 90 percent identical, and the two others about 92 percent identical.[55]

BMPs regulate osteoblast function and differentiation through recruiting osteoblast precursors and later stimulating their osteoblast differentiation. When BMP-2, BMP-3, BMP-4, BMP-6 and BMP-7 are implanted subcutaneously in rats they all induce ectopic bone formation [60] BMPs start their signalling at the cell surface and not intracellular, and they are attached to certain receptors, called BMPR. The BMPRs are called type-IA- type-IB- and type-II-receptors. Nevertheless, there is an interaction with two distinct serine/threonine kinase receptors. On the one hand there is a type I receptor (50-55kDa) and on the other hand a type II receptor (more than 75kDa).[55, 59]

BMP ligand binding induces a receptor dimerization, and the type II receptor transphosphorylates the type I receptor. Furthermore, an activation of intracellular Smad (Sma and Mad) protein leads to a transmission of the BMP signal. Eight Smad proteins have been identified, that play essential roles in mammals in intracellular signalling. The type I receptor phosphorylates directly the receptor Smads (R-Smads), consisting of Smad1, Smad2, Smad3, Smad5, and Smad8 and then establish complexes with he Co-Smad, Smad4. These complexes move from the surface into the nucleus, where they bind to the regulatory regions of the target genes and regulate their expression.[61]

TGF-ß family also includes activins, inhibins, and growth and differentiation factors (GDFs). Around 16 types of BMPs have been identified in humans and the majority shows a high percentage of amino acid sequence homology. BMP-1 is a member of the astacin family of metalloendopeptidases.[27, 62]

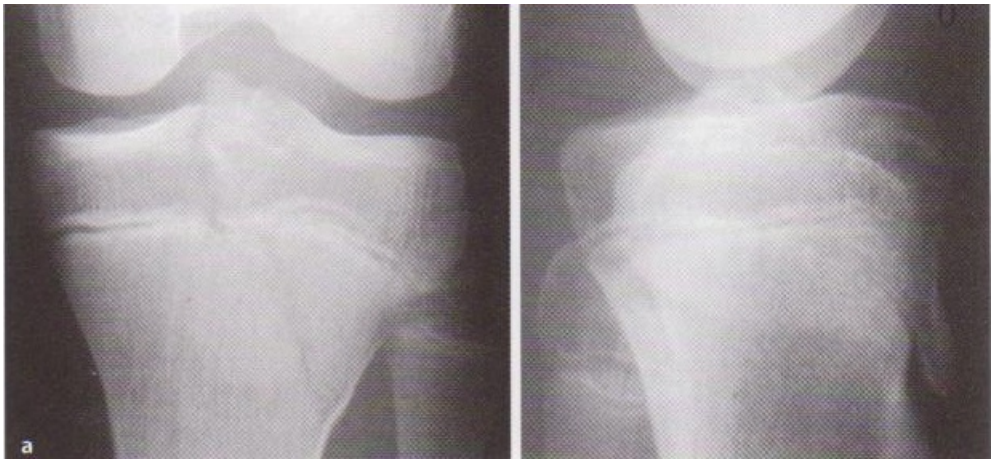
Besides bone formation, BMPs are important for the development of organs such as lung, heart, kidney, eyes, and gonads.[63] BMPs are found in many other regions of the developing embryo, for instance BMP-2 in the heart and in whisker follicles, BMP-6 in brain, spinal cord, and skin and BMP-3 in the brain.[55] During embryogenesis, certain BMPs are expressed in the condensing mesenchyme, which later forms cartilage templates and furthermore it is known that in the lack of certain BMPs, specific systems fail to develop, following in embryonic defects, like distinct cardiac defects or lethality. Postnatally, they are localized at the epiphyseal plate in resting, proliferating and pre-/hypertrophic chondrocytes, as well as in the perichondrium/periosteum. Considering that working in concert BMPs coordinate various patterns of endochondral bone formation.[48, 52-54, 64-67]

As mentioned above BMPs are known important regulators for bone formation and repair and for that reason it could be hypothesized that they also have important roles in regulating growth plate injury responses and bony repair. They have been shown to activate proliferation, migration, and differentiation of mesenchymal cells into osteogenic and chondrogenic lineages.[68] Particularly, BMP-2, -4, and -6 have the essential role to control the rate of proliferation and chondrocyte maturation and the stimulation of collagen production within longitudinal bone growth of young bones.[69]

Furthermore, BMPs have the essential role of mediating the migration of progenitor cells, proliferation of mesenchymal cells, differentiation of osteogenic cells, and bone remodelling and for that reason they are important regulators of bone formation. For instance, BMP-2 and -7 lead to an induction of osteoblast differentiation of mesenchymal cells and to a stimulation of mineralized bone matrix formation.[70-72] As mentioned above, BMPs are also strong regulators of bone fracture repair. They are involved in the stages of endochondral formation – cartilage formation – and intramembranous bone formation, and later followed by bone formation and remodelling.[31]

Although BMPs are essential known key regulators of bone formation and bone fracture healing, their exact potential role in the bony repair of injured epiphyseal plate cartilage, like Salter's type IV for instance, remains unclear. **(see Figure 9)**

Figure 9: Salter Harris IV fracture of the proximal tibia of a fourteen year old boy[10] (Weinberg, Tscherne, 2006, Unfallchirurgie im Kindesalter Band II, S.708, Abb. 21.33.a)



3.1. Bone Morphogenetic Protein's Expression in Fracture Healing

Like written above fracture healing includes a complex interaction of many local as well as systemic regulatory factors. In the nineties, the presence of several bone morphogenetic proteins and their receptors in the process of fracture repair could be demonstrated. At the early stages of fracture repair, just a small number of primitive cells show an expression of BMPs in the area of fracture callus. During the process of endochondral ossification, the expression of BMPs and their receptors constitute an enormous increase, particularly in primitive mesenchymal and chondrocytic cells. During the maturation of the cartilaginous component of the callus, the number of primitive cells decreases and further this process is accompanied by a decrease in the presence of BMP expressing cells. While the osteoblasts initiate to lie down woven bone on the chondroid matrix, these cells show an expression of BMPs and their receptors. However, as lamellar bone substitutes the primitive woven bone the expression of BMPs decreases. In the areas of the callus undergoing intramembranous ossification within several days after the fracture, periosteal cells and osteoblasts constitute an intense expression of BMPs and their receptors. Again, when mature lamellar bone substitutes the woven bone, the intense expression of BMP shows a decrease. These observations and the awareness of the powerful osteoinductive capacities of BMPs propose

that different bone morphogenetic proteins play essential roles during fracture healing / repair and development.[73]

4. BONE MORPHOGENETIC PROTEIN-2 (BMP-2)

Like mentioned above BMP-2 has the capacity to play an essential role in the development of bone and cartilage. It is involved in different pathways, like the hedgehog pathway and the TGF-β signalling pathway. Further, it plays an important role in the regulation of cardiac cell differentiation and epithelial to mesenchymal transition. Bone morphogenetic protein – 2 is an example of a typical BMP structure:

4.1. Molecular Aspects of BMP-2

"It is translated as a 396-amino-acid preproprotein that contains a 19-amino-acid signal sequence for targeted secretion, a 263-amino-acid proregion, and a 114-amino-acid mature segment." Within the mature region, you can identify seven cysteins and one N-linked glycosylation recognition site. The important functional protein consists of a homodimer that is linked by two disulfide bridges, and the observed mass is about 18 kDa. Using ribonuclease protection analysis showed that BMP-2 has an early peak in expression on day 1 of fracture healing. This suggests that BMP-2 may be the most upstream mediator in de cascade of BMP expression.[27]

4.2. Medical Uses of BMP-2

BMP-2 has the capacity to the induction of ectopic bone formation when it is implanted into muscles or other soft tissues. It plays an important role in triggering osteoblasts differentiation and in up-regulation of the expression of most genes encoding osteoblastic phenotype-like proteins in vitro.[61]

Systemic and local paracrine factors, like BMPs are essential for skeletal development and skeletal growth and further play an important role at different stages. BMP-2 has important roles in mesenchymal condensation and cartilage differentiation. Like at an early stage,

BMP signalling causes a prechondrogenic condensation of mesenchymal cells and at a late stage, BMP-2 stimulates chondrocyte proliferation and hypertrophy of the growth plate, called growth plate chondrogenesis. Through stimulation of these cells, the proliferation and hypertrophy of chondrocytes of the so-called epiphyseal plate, BMP-2 accelerates longitudinal bone growth. BMP-2 is localized in the posterior limb bud early in embryogenesis where it contributes in the pattern formation of the extremities and is essential in facilitating the commitment of the condensing mesenchyme undergoing chondrogenesis. Altogether BMP-2 stimulates longitudinal bone growth, growth plate chondrogenesis and an over-expression of an active BMP-R in chondrocytes induces acceleration of their differentiation toward hypertrophic chondrocytes.[69, 74, 75]

M.J. Perry et al. showed that in hypertrophic chondrocytes the immunolabelling for BMP-2 is present and in some late proliferative chondrocytes too. Furthermore they observed that the immunoreactivity for BMP-2 changed between hypertrophic chondrocytes with casual cells being prominently labelled.[43]

Besides, Wozney and Co. have shown that rhBMP-2 induces ectopic bone formation. In all models, they have investigated like the rat ectopic bone formation assay, the rat, sheep, and dog segmental defect models rhBMP-2 induces bone. In addition to it they have found that even though the rat femoral-, the sheep femoral- and the dog mandibular - defect model have significantly different sizes and metabolic rates the amount of BMP-2 needed and the time required to produce bone are similar in all animals. The events around BMP-2 in vivo are quite complex and it is possible that the main function of the BMPs is to start the first step of the differentiation of mesenchymal cells into chondrocytes and later in vivo other cells, growth factors and processes. BMP could induce the initial steps of the cascade of bone formation. After implantation of BMP in vivo, the course of events was differentiation of mesenchyme, chondrogenesis, hypertrophy, maturation of cartilage, removal of hypertrophic cartilage, and then osteogenesis. “However, with large amounts of BMP, osteogenesis can be seen concurrently with chondrogenesis.”[55]

The treatment of multi-potential cells with large doses of rhBMP-2 leads to an induction of alkaline phosphatase and a cAMP response to PTH, both markers of osteoblasts. Besides, rhBMP-2 considerably induces the expression of BGP mRNA, called bone gla protein or osteocalcin. The induction of bone gla protein is enhanced by the presence of 1,25

dihydroxy-vitamin D3 and is likely the one specific marker of the mature osteoblast phenotype. Altogether, cells can be stimulated to differentiate into the osteoblastic phenotype or to increase the expression of osteoblastic phenotypic markers. To sum up, BMP-2 activates cells that participate in bone repair and that phenomenon has been shown in vivo in a variety of species, as well as in cultured cells from both, the chondroblastic and the osteoblastic lineages.[55]

Mammalian NF-κB is a group of transcription factors including seven members, for example p65 or c-Rel which facilitates growth plate chondrogenesis and longitudinal bone growth by activating BMP-2. For instance, an over-expression of NF-κB p65 in cultured chondrocytes accelerates chondrocyte proliferation and differentiation and prevents apoptosis. The inhibitors of NF-κB reduce the expression of BMP-2 in cultured growth plate chondrocytes, the over expression of p65 increases it.[76]

Noggin is a BMP antagonist and neutralizes the stimulatory effects on chondrocyte proliferation and differentiation, as well as its anti-apoptotic effect.[76]

Very early in development both BMP-2 and BMP-4 are found in the apical ectodermal ridge of the limb-bud and the realization that those two morphogens are the mammalian homologues of the Drosophila *dpp* was the first evidence for a role in embryogenesis. Like the *dpp,* they may play an important role for either delivering positional information in the developing limb bud or interpreting it. Later in development BMP-2 is observed in prevertebrae, in the tooth bud and the interdigital mesenchyme of the limb where the cartilaginous condensation occurs.[55] Nevertheless, some people report BMP-2 and not BMP-4 is seen in the developing tooth bud[77], others report that BMP-4 is also expressed. Furthermore both proteins are found in the more mature perichondrium, periosteum, and in odontoblasts later in development. Besides, BMP-2 occurs in many other regions of the developing embryo like in the heart and in the whisker follicles.

4.3. BMP-2 during Development

Local morphogens like Bone morphogenetic protein-2 (BMP-2) are essential for the regulation of proliferation and condensation of mesenchymal cells during embryonic development. Condensation plays the key role in the development of skeletal and other mesenchymal tissues and occurs when dispersed cells get together to differentiate into a single cell type as for instance cartilage, bone, muscle, tendon, kidney, and lung. Condensation constitutes the earliest stage during organ formation when cell/tissue-specific genes show an up-regulation. BMPs have the capacity to activate Pax-2, Hoxa-2 and Hoxd-11 among other genes which subsequent regulate growth of condensation. Those "Hox genes" indirectly ensure cell adhesion and further with other transcription factors modulate the proliferation of cells within condensation. When Noggin, a BMP-2 antagonist, inhibits BMP signalling the growth of condensation stops and the next stage of skeletal development, called cell differentiation follows.[78]

Bone morphogenetic proteins have the important role to lead to induction of the differentiation of cells of the osteoblastic lineage and further to enhancement of the function of the osteoblasts. Growth factors display a regulation through binding proteins. Especially, BMP-2 shows a negative feedback-mechanism. It leads to an expression of an antagonist, called Noggin, by transcriptional mechanisms. Further, Noggin leads to stimulatory effects of BMPs on DNA and collagen synthesis and alkaline phosphatase activity in osteoblast enriched cells.[79]

The study/findings of Gazzerro et al. demonstrated that BMPs induce Noggin mRNA and protein levels in skeletal cells by transcriptional mechanisms, and furthermore that Noggin causes an inhibition of the effects of BMPs in osteoblasts. BMPs cause an induction of Noggin and it appears to be a mechanism to limit BMP effects in bone.[79]

4.4. BMP-2 during Fracture and Bone Healing

It is known that fracture healing includes a complex interaction of many local and systemic regulatory factors. Bone morphogenetic proteins are the most potent osteoinductive proteins known and the immunohistochemical analysis respectively experiments of Bostrom et al. allowed a description of the distribution pattern of BMP-2 and BMP-4, the physiologic presence, localization, and chronology of BMPs during fracture healing in 1985. This study displayed that all types of cells which play important roles in osteogenesis show BMP-2 respectively BMP-4 positive expression. At early stages of fracture repair, only a minimum of primitive cells showed a positive staining in the fracture callus. During intramembranous fracture healing, periosteal cells next to the fracture gap and osteoblasts surrounding new formed bone showed an intense positive immunohistochemical sign. During the process of endochondral ossification, primitive mesenchymal cells, precursors of chondrocytes and chondrocytes showed a positive immunohistochemical expression of BMP-2 and BMP-4. When the cartilaginous component of the callus started to mature, it was accompanied by a decrease in the number of primitive cells as well as a decrease in both the intensity and the number of positive stained cells. While osteoblasts initiated to lay down woven bone on the chondroid matrix, these osteoblasts showed an intense positive staining. The positive immunohistochemical detection of BMP decreased with the increase of ossification in both ossification mechanisms, namely the intramembranous and the endochondral ossification. As the mature lamellar bone replaced the woven bone, the intense positive staining decreased. These observations and the the strong osteoinductive capacities of bone morphogenetic protein, constitute that bone morphogenetic protein 2 and 4 are essential regulators of cell differentiation during fracture healing. [80]

Tsuji et al. demonstrated that BMP-2 is an important and necessary component of the signalling cascade that regulates fracture healing. Mice deficient of the possibility to produce BMP-2 showed spontaneous fractures in their limb bones that unexpectedly did not resolve in time. The earliest steps/stages of fracture repair appear to be blocked in bones deficient of BMP-2. In fact, BMP-2 activity is needed for the initiation of fracture healing/repair, although it is dispensable for bone formation. Although, in bones lacking BMP-2 other osteogenic stimuli are still existing, they are not able to compensate for the

absence of BMP-2. BMP-2 constitutes an essential endogenous mediator required for fracture healing.[81]

4.5. Use of BMP-2 in the Stimulation of Fracture Healing

Govender et al. performed a prospective, controlled, randomized study of four hundred and fifty patients (BESTT = BMP-2 Evaluation in Surgery for Tibial Trauma) about the treatment of open tibial fractures with recombinant human bone morphogenetic protein-2. Like in other open fractures of long bones, the treatment of open fractures of the tibial shaft often creates complications like delayed union or non-union. The aim of this study was to demonstrate and to evaluate the safety and efficacy of the use of recombinant human BMP-2 (rhBMP-2; dibotermin alpha). In the European Union recombinant human BMP-2 is permitted as implantation-kit "Dibotermin alpha". The use of recombinant human BMP-2 should accelerate the repair of open shaft fractures of the tibia and reduce the need for secondary intervention. The results of this study showed an accelerating fracture and wound-healing effect in open tibial shaft fractures. Further, the findings of that investigation demonstrated a reduction of the infection rate in patients. In addition to it, the use of rhBMP-2 treatment showed the reduced need for secondary interventions after open fractures. In conclusion, the rhBMP-2 treatment was safe and significantly better than the standard of care. The outcome was better in reducing the frequency of secondary interventions and the invasiveness of the procedures, in advancing fracture and wound-healing, and further in reducing the complications like the infection rate in patients with an open tibial shaft fracture.[82]

5. MATERIAL AND METHODS

A rat model was used to explore the BMP-2 expression at the growth plate after setting a transepiphyseal drill-hole injury to the proximal tibial physis. The Austrian Federal Ministry of Science and Research approved all animal experiments.
The analysis of BMP-2 spatial distribution in epiphyseal plate tissue from injured and uninjured control bones was performed by immunohistochemistry.
The animal experiments, the following tissue preparation, as well as the immunohistochemical process were performed at the special laboratories of the Center of Medical Research of the Medical University of Graz (ZMF).

5.1. Material

All used materials and devices for the realization of my thesis are part of the infrastructure of the Center for Medical Research and the Institute for Biomedical Research (IBF) of the Medical University of Graz (MUG).

5.2. Animal Experiments

Seventy male Sprague-Dawley rats at the age of five weeks weighing 100-120 g were obtained from the Division for Laboratory Animal Science and Genetics in Himberg (Medical University Vienna, Core Unit for Biomedical Research). The rats received special forage (pellets of the company ®Sniff) for rodents ad libitum and conventionally clear drinking water. After delivery, the Sprague-Dawley rats were housed in the animal laboratory of the Institute for Biomedical Research (IBF) of the Medical University of Graz, Rosseggerweg 48, 8036 Graz, Austria during the experimental period. After one week, experimental animals were subjected to the surgery in the operating room of the IBF. The laboratory animals were randomly distributed into two main groups, the experimental and the control group. These two main groups were divided into several subgroups (see

Table 2). Sprague-Dawley rats of the experimental group sustained a transepiphyseal drill-hole injury of the left tibia.

Table 2: Animal distribution into groups

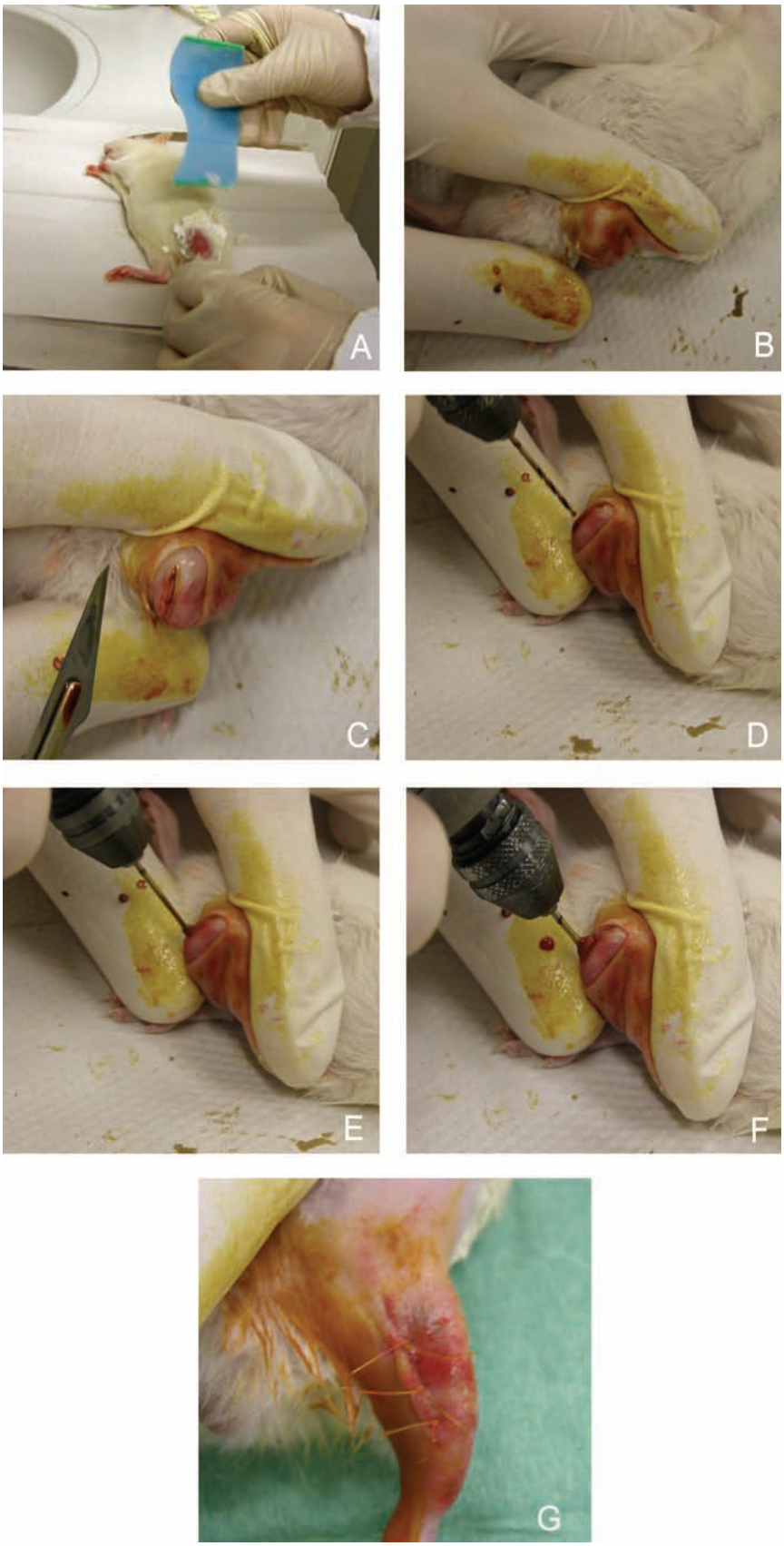
Groups	Number of Rats	Procedure	Day of Euthanasia
Group1 (control group)	10	Without setting a lesion	Day 0
Group 2	10	Lesion on day zero	Day 1
Group 3	10	Lesion on day zero	Day 3
Group 4	10	Lesion on day zero	Day 7
Group 5	10	Lesion on day zero	Day 14
Group 6	10	Lesion on day zero	Day 28
Group 7	10	Lesion on day zero	Day 82

5.2.1 The Setting of the Transepiphyseal Drill-hole Injury

Before surgery, the rodents of the experimental group were moved to the operating room of the IBF. The rats of the experimental group were subjected to a transepiphyseal drill-hole injury of the left tibia. The control group did not sustain any lesion. Anaesthesia was started by Furane 2% ® insufflations (Abbot Laboratories Ltd., Kent, England). The narcosis was continued via subcutaneous injection of 0.1ml/100g body weight FDD-narcotic solution (Fentanyl® 100µg; Midazolam Delta® 2.0mg; Domitor® 1.0mg). Then the operating field, the left posterior limb, was shaved using Veet® depilatory cream (Reckitt Benckiser Austria GmbH, Vienna) **(See Figure 8 - A)**. Further, washing with sterile Betaisodona®-solution was performed **(See Figure 8 - B)**, and further we longitudinally incised the skin infrapatellar for about 1.5 cm long by a scalpel. After preparation of the region around the knee joint a small longitudinal incision of the patellar ligament in full knee flexion was performed **(See Figure 8 - C)**. In full knee flexion position, a hole measuring 1.2 mm in diameter was drilled from proximal towards distal through the intercondylar area into the medullar cavity, causing a centered growth plate lesion. After the entrance in the medullary cavity of the tibia, a loss of resistance and an extravasation of bone marrow was recognized **(See Figure 8 - D-F)**. After disinfection for

another time with Betaisodona®-solution, the skin was sutured by simple interrupted knots with Vicryl® 5-0 suture **(See Figure 8 - G)**. This technique became evident as a simple and rapid method to cause a standardised growth plate lesion without affecting bone stability.

Figure 10: The process of setting a transepiphyseal drill-hole injury to a left tibia of Sprague-Dawley rats.[9]



5.2.2 *Postoperative nursing*

Immediately after wound closure, anaesthesia antidote and the pain killer Caprofen (Rimadyl®, Pfizer Corp. Austria GmbH, Vienna; 1ml Rimadyl® diluted with 49ml NaCl 0.9%, of that 0.4ml/100g body weight) was injected intraperitoneally and subcutaneously, respectively.

Table 3: Antidots for FSS Narcosis

AGENT	TRADEMARKS	MANUFACTURER
Atipamazol	Antisedan®	Pfizer Corp. Austria GmbH, Vienna
Naloxon	Narcanti®	Torrex Chiesi Pharma GmbH, Vienna
Flumazenil	Anexate®	Roche Austria GmbH, Vienna
NaCl 0.9%	NaCl-Fresenius®	Fresenius Kabi Austria GmbH, Graz

Afterwards the Sprague-Dawley rats rested on a warming plate, with a temperature about 38°C until their awakening. Further, the rats were returned to their litters for nursing and doing activity ad libitum.

In the first postoperative week, painkiller Caprofen (Rimadyl®) was injected in the same dose as intra-operative subcutaneously to ensure analgesia. In the second week post surgery, the subcutaneous injections of Caprofen (Rimadyl®) were discontinued. Instead 250 mg Metamizol (Novalgin drops®, Sanofi-Aventis GmbH, Vienna) drops were added to the drinking water. Pain medication was stopped after 2 weeks.

5.2.3 *Euthanasia*

Depending on the groups, euthanasia was performed at different points of time. Euthanasia was performed on day 1, 3, 7, 14, 28, and 82 after setting a transepiphyseal lesion. The rats were narcotised through an insufflation of Furane®. In narcosis, euthanasia was performed by intracardial Thiopental-sodium injection (Thiopental Sandoz®, Sandoz GmbH, Kundl, Austria). After this procedure the left and the right tibia were harvested, and further cautiously dissected from soft tissue, like muscle, fat and other connective tissue. Immediately after harvesting, the bones were shock-frozen and stored in liquid nitrogen until processing for immunohistochemical staining.

5.2.4 *Medication*

The below-mentioned table **(Table 4)** shows the complete medication used for the study of my thesis.

Table 4: Medication List

ACTIVE AGENT	TRADENAME	MANUFACTURER
Fentanyl	Fentanyl®	Janssen-Cilag GmbH, Neuss, Germany
Midazolam	Midazolam Delta®	Deltaselect GmbH, Dreireich, Germany
Medetomedin	Domitor®	Pfizer Corp. Austria GmbH, Vienna
Naloxon	Narcanti®	Torrex Chiesi Pharma GmbH, Vienna
Flumazenil	Anexate®	Roche Austria GmbH, Vienna
Atipamazol	Antisedan®	Pfizer Corp. Austria GmbH, Vienna
NaCl 0.9% (Physiological Saline Solution)	NaCl-Fresenius®	Fresenius Kabi Austria GmbH, Graz
Caprofen	Rimadyl®	Pfizer Corp. Austria GmbH, Vienna
Metamizol	Novalgin® Drops	Sanofi-Aventis GmbH, Vienna
Polyvidon-Jod	Betaisodona® Solution	Mundipharma GesmbH, Vienna
Thiopental-sodium	Thiopental Sandoz®	Sandoz GmbH, Kundl, Austria
Isofluran	Furane®	Abbot Laboratories Ltd., Kent, England

5.3. Immunohistochemistry

Target of this thesis was to show the spatial distribution of BMP-2 expression in epiphyseal plate tissue from drill-hole injured and uninjured control bones. After harvesting the tibiae from operated and control animals, the immunohistochemistry was performed on frozen slides (Cryo-slides). Commercially available antibody against BMP-2 was obtained from Abcam®.

5.3.1 *Immunohistochemical Staining of BMP-2*

Immunohistochemical staining of BMP-2 was performed on specific cryo-sections.
The procedure of immunohistochemistry was done as written in the *immunohistochemistry labelling protocol* below. Before staining, the slides - frozen sections - were divided into several groups **(Table 5)**:

Table 5: Several groups of frozen sections

DAYS	LEFT TIBIA	RIGHT TIBIA
Day 1	5	1
Day 3	5	1
Day 7	5	1
Day 14	5	1
Day 28	5	1
Day 42	5	1
Day 82	5	1

5.3.2 *Immunohistochemistry labelling protocol*

DAY 1:

1. Description of the slides (Number and Primary Antibody with pencil)
2. Take one control slide for each secondary antibody
3. Take 10µ Cryo slides (frozen tissue sections) out of freezer (-20°C) and store it 60 minutes
4. Draw circles around each section with DAKO PAP pen – hydrophobic and dry it five minutes
5. 25 minutes rehydration in PBS (phosphate buffered saline) Tween (Davos)
6. 15 minutes: 0.3% H_2O_2 / PBS wash
7. 3 x 2 minutes PBS Tween wash; spill the first PBS solution and refill with PBS Tween
8. Cover each slide with the 1:20 diluted goat-serum (about 100µl each section) for 60 minutes
9. Knock the plates on the table and do not wash them!
10. Make sure that the DAKO PAP pen is still fixed around the sections
11. Application of the primary antibody (diluted 1:100, about 100µl each section) and put the slides in the freezer (at 4°C) over night
12. Cover the control slide just with the DAKO diluent! Be careful!

DAY 2:

1. Take the slides out of the freezer and make sure that the DAKO PAP pen is still fixed
2. 3 x 2 minutes PBS Tween washing
3. Application of the secondary antibody BA1000 1:600 diluted (100µl each section): 30 minutes in a box with lid on
4. In the meantime prepare ABC complex (ABC Staining System: 20µl Avidin, 20 µl biotinylated in 1 ml PBS Tween): has to store at least 30 minutes and at a maximum of 60 minutes in the freezer (at 4°C) in a box with lid on
5. 3 x 2 minutes PBS Tween washing
6. Application of ABC complex (100µl each section): 30 minutes in a box with lid on
7. 3 x 2 minutes PBS Tween washing
8. Application of DAKO AEC+ for 13 minutes (incubate in the dark!) – Attention: it's poisonous and you have to wear gloves for your own safety!
9. Stop the reaction in Aqua dest. and change it one time
10. Counterstaining the tissue sections with "Mayer's Haemalaun solution" (Merck) – staining of nuclei
    - Filter the haematoxylin – the solution can be reused
    - Place the slides into the haematoxylin solution for 15 seconds
11. Wash in tapped water (need to be a bit alkaline), changing the water several times – "blueing"
12. Wash in Aqua dest. and change the containers one time
13. Mounting in waterproof Kaiser's glycerol gelatine (Merck)

5.3.3 *Chemicals for Immunohistochemistry*

Table 6: Chemicals

LABEL	PARTICULARS
0.3 % H_2O_2 / PBS	100ml PBS + 1ml 30% H_2O_2
ABC complex	1 ml PBS Tween + 20 µl Avidin + 20 µl Biotinyl. HRP at least 30 min storing at 4°C with lid on
Ammonia water*	1 lit Aqua dest + 1 ml 25% ammonia solution
Goat serum	950 µl PBS Tween + 50 µl Normal goat Serum 1:20 diluted
Hydrogen Peroxide	30% (Merck KGaA)
Kaiser's glycerol gelatine	Waterproof mounting medium: 40g Gelatine, 210 ml distilled water, 250 ml glycerol, 5ml phenol
Mayer's Haemalaun	pH 2.26 (Gatt-Koller GmbH)
PBS Tween	1 lit PBS + 1 ml Tween 20
PBS	Phosphate buffered saline pH 7.2-7.3, 10 000 ml; containing reagents: sodium chloride, disodium hydrogen phosphate, potassium dihydrogen phosphate
Primary Antibody	Abcam 14933 1+100 1 µl Antibody + 99 µl DAKO Diluent Rabbit IgG (polyclonal)
Secondary Antibody BA 1000	599 µl PBS Tween + 1 µl BA 1000 (Anti-Rabbit biotinylated 1:600)

* Products were kindly provided by the Center of Medical Research of the Medical University of Graz.

5.4. Rabbit polyclonal to BMP-2 (abcam14933) [83]

Immunogen:	synthetic peptide: corresponding to amino acids 45-60 of BMP-2
Species reactivity:	reacts with Human, Mouse and Rat (not yet tested in other species)
Positive control:	WB: neonatal brain lysate
Cellular localization:	secreted
Clonality:	polyclonal
Isotype:	IgG
Purity:	immunogen affinity purified
Form:	liquid
Concentration:	0.50 mg/ml = 500µg/ml; (1:100 = 5µg/ml; 1:500 = 1µg/ml; 1:50 = 10µg/ml)
Storage instructions:	Store at +4°C short term (1-2weeks)
Storage buffer:	the antibody is provided in antibody stabilization buffer containing phosphate buffer, pH 7.4, glycerol, stabilizing proteins and preservatives. The exact buffer contents are considered proprietary.[83]
Relevance:	BMP-2 is a member of the transforming growth factor-beta (TGF-β) superfamily of secreted growth factors. Its structure consists of a disulfide-linked homodimer and induces bone and cartilage formation. Furthermore, BMP-2 is essential in cardiac morphogenesis and is expressed in various tissues like lung, spleen, brain, liver, prostate, ovary and small intestine. The 26-kDa protein is the functional form of BMP-2 and consists of two identical 114 amino acid polypeptide chains linked by a single disulfide bond.[83]

To sum up, BMPs play an important role in fundamental events in early embryonic development, organogenesis and adult tissue homeostasis like already mentioned above in my thesis.[83]

BMP-2 is a rabbit IgG and was used in a dilution of 1:100, that means 5 µg/ml and all Isotype control staining on bones were until 5µg/ml completely negative. **(see Figure11 and 12)**

Figure 11: IHC of BMP-2 cryo-slides: BMP-2 isotype control staining (IgG): completely negative

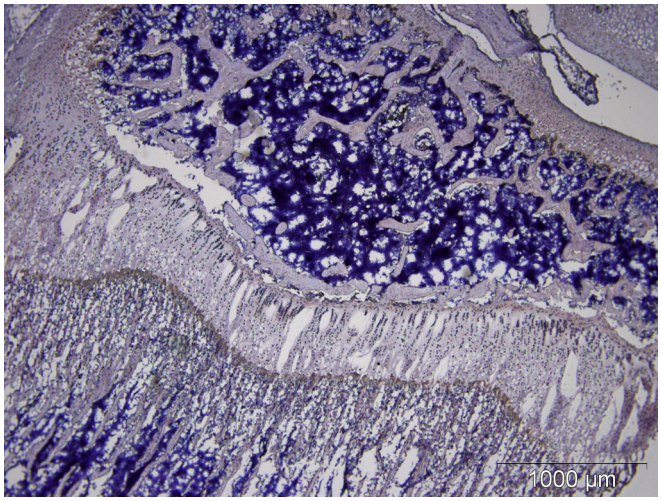
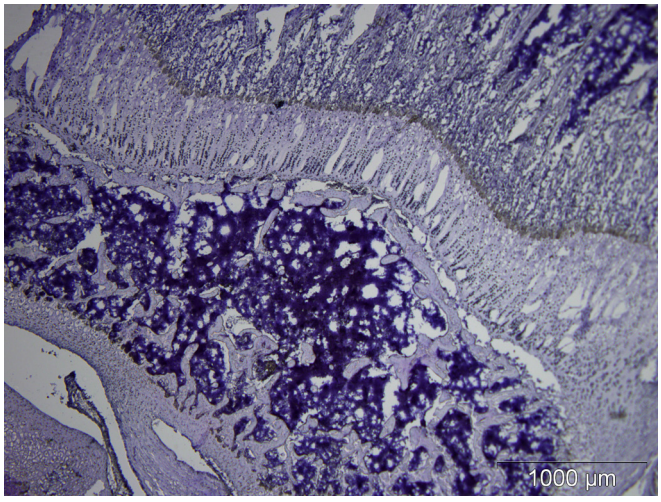


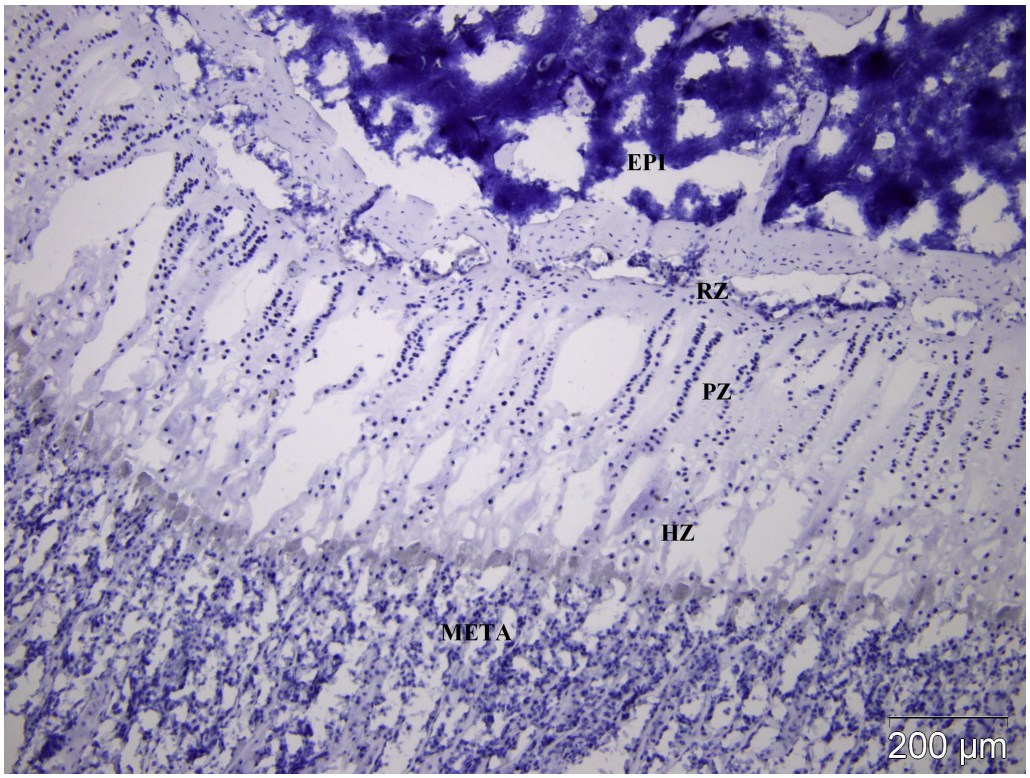
Figure 12: IHC of BMP-2 cryo-slides: BMP-2 negative control with a diluent: completely negative



6. RESULTS

The growth plate has three main parts: the resting zone (RZ), the proliferating zone (PZ) and the hypertrophic zone (HZ). **(see Figure 13)** In the so-called resting zone, stem-like cells differentiate into fast dividing chondrocytes of the proliferative zone. Proliferating chondrocytes terminally differentiate into the non-dividing chondrocytes of the hypertrophic zone. Our analysis showed that bone morphogenetic protein 2 (BMP-2) expression changed during the differentiation program. Immunohistochemistry (IHC) was performed to localize expression of BMP-2 at the growth plate drill-hole injury site.

Figure 13: Immunohistochemistry of a growth plate of a proximal tibia (negative control staining). RZ (Resting Zone), PZ (Proliferating Zone), HZ (Hypertrophic Zone), EPI (Epiphysis), META (Metaphysis)



Immunohistochemistry (IHC) showed that the epiphyseal injury gap was filled by a haematoma on day 1 and 3 and by cartilage on day 7 (Collagen II positive, and Collagen I negative tissue shown by another study of our research team of the Department of Pediatric and Adolescent Surgery – Medical University of Graz under the control of PD Dr. Weinberg).

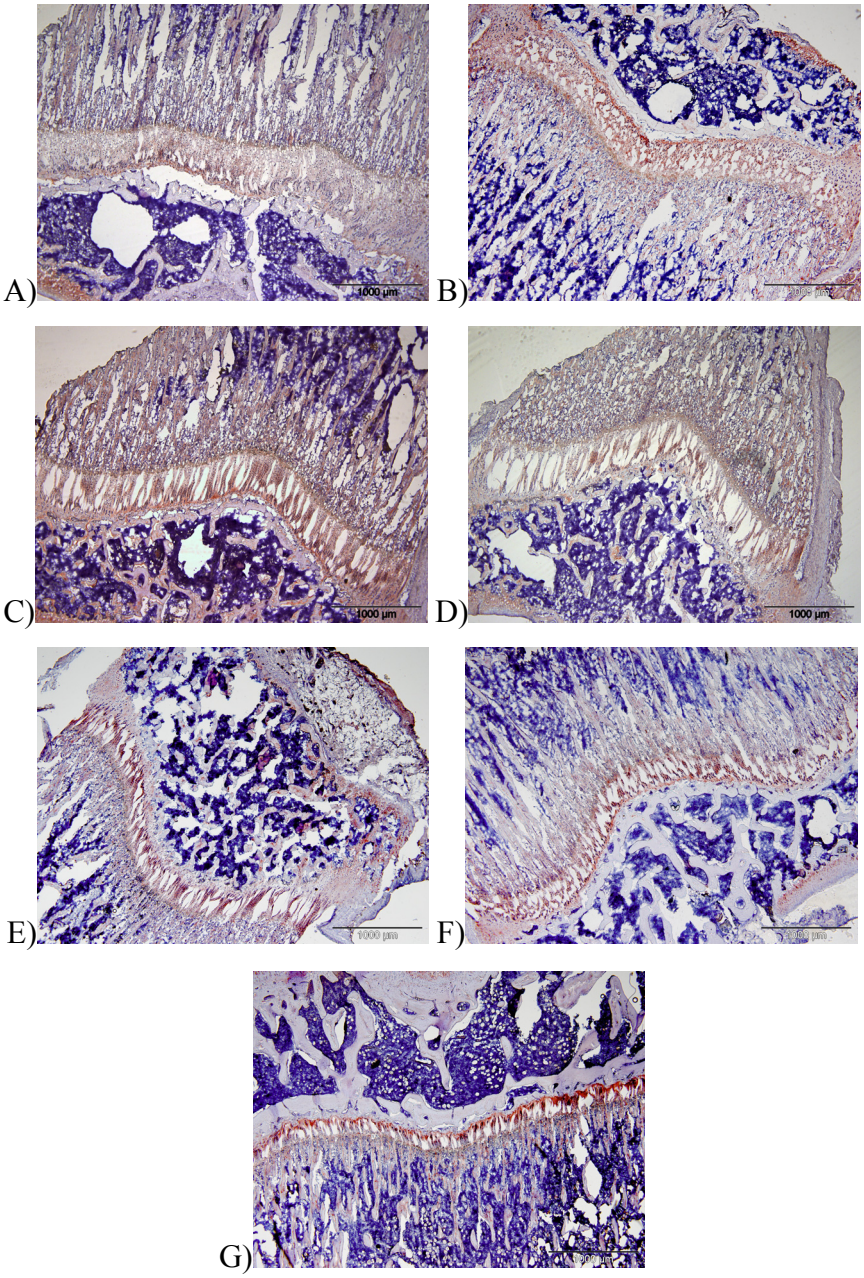
By day 14 some bony filaments were crossing the growth plate at the lesion site. On day 28 osseous trabeculae with sinusoids in between were clearly documented. These osseous structures were getting denser with time and were still detectable on day 82 at the lesion site.

Immunohistochemistry (IHC) analyses were done to examine and localize expression of BMP-2 at the injured growth plate, in contrast to the uninjured growth plate of the other tibia.

Positive immunostaining of BMP-2 was found in the proliferative and hypertrophic chondrocytes at the uninjured normal growth plate of the counter-leg on each day. The positive staining got denser and denser during the evaluated period. On day 82 the most intense immunostaining could be seen in the proliferative zone. Furthermore, positive staining was found in the osteoblasts lining the bone trabeculae at the metaphysis, and in the fibrous mesenchymal cells. **(see Figure 14)**

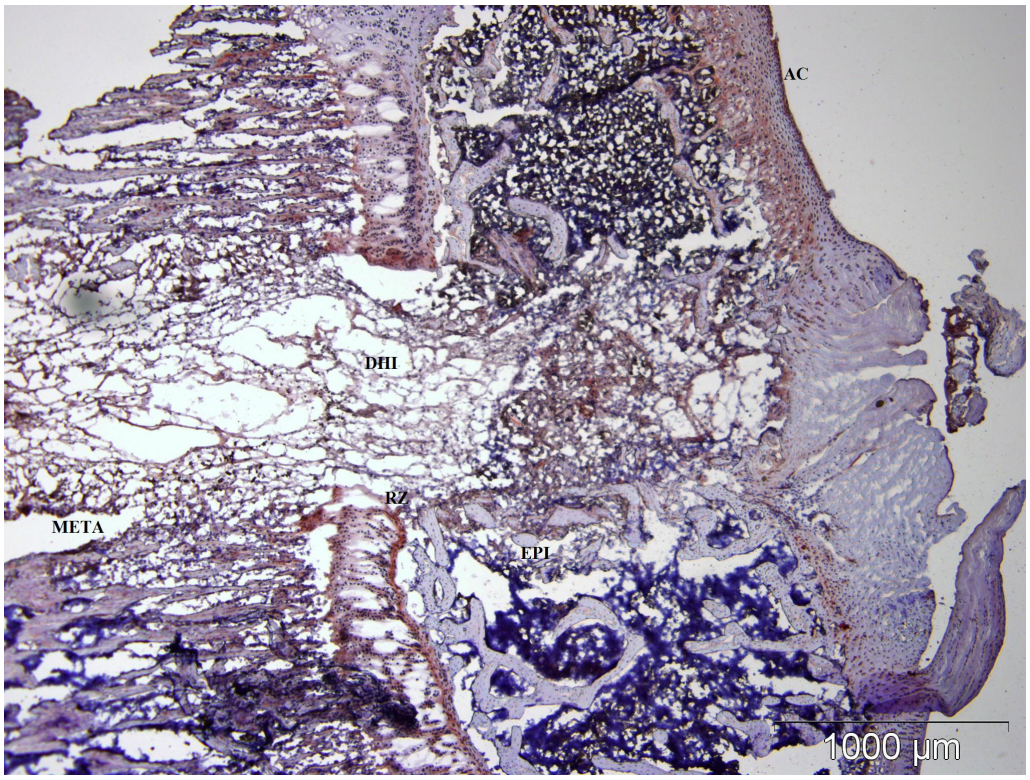
Figure 14: Positive Immunostaining of BMP-2 at the uninjured growth plate of the counter leg

A) day 1; B) day 3; C) day 7; D) day 14; E) day 28; F) day 42; G) day 82



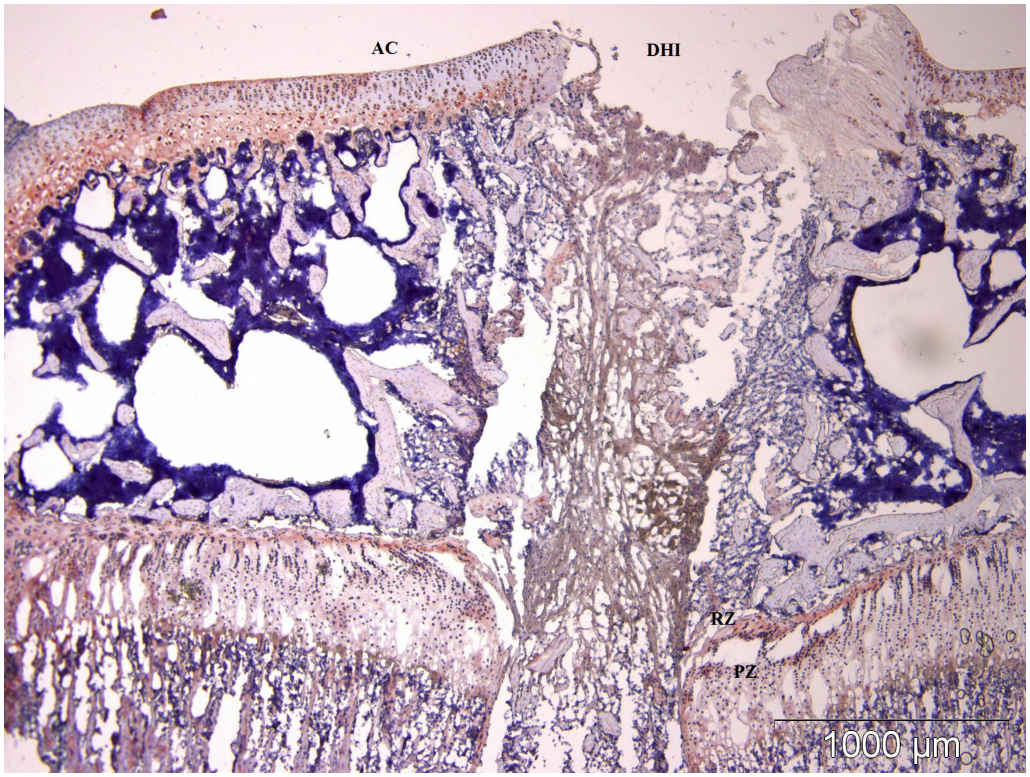
Consistently, at day 1 at the beginning of inflammatory response, nearly no positive BMP-2 staining was observed among the inflammatory cells. Only chondrocytes of the resting zone and cells along the drill-hole injury showed a little positive staining. **(see Figure 15)**

Figure 15: BMP-2 positive immunostaining on day 1 of chondrocytes of the resting zone
RZ (Resting Zone), META (Metaphysis), EPI (Epiphysis), AC (articular cartilage)



At the beginning of fibrogenic response at day 3, BMP-2 positive immunostaining was observed in the resting zone and in some proliferative chondrocytes of the growth plate. **(see Figure 16)**

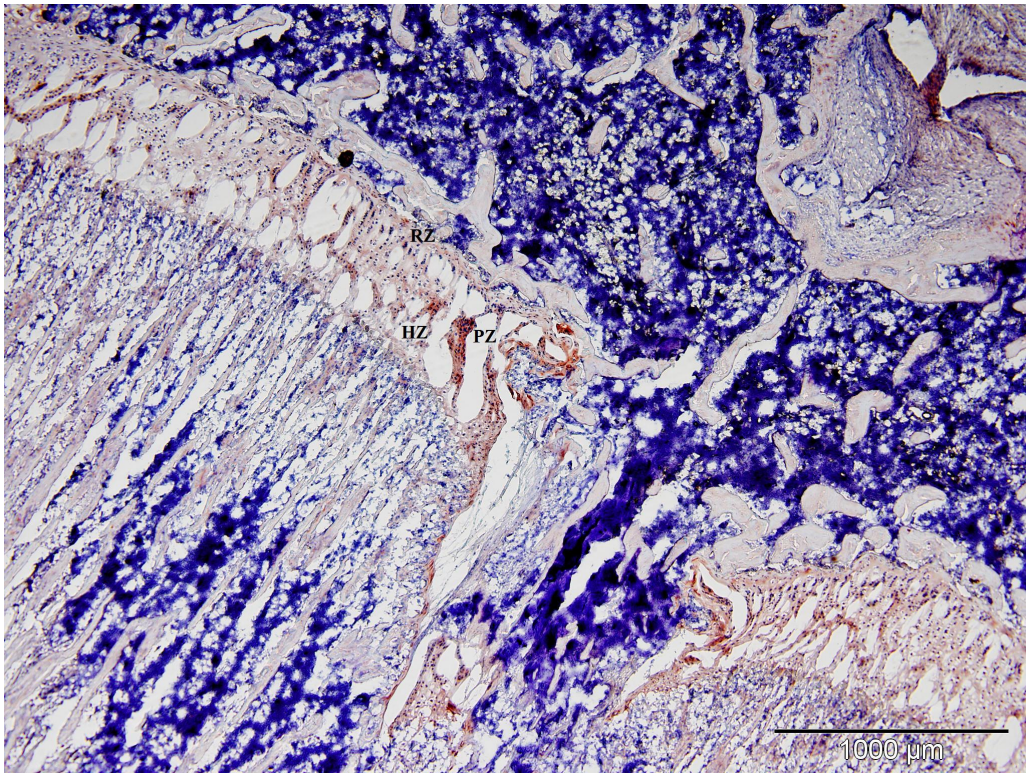
Figure 16: BMP-2 positive immunostaining on day 3: light positive staining in RZ (Resting Zone) and PZ (Proliferating Zone)
AC (articular cartilage), DHI (drill hole injury)



At day 7, a lot of mesenchymal cells and osteoblasts were highly stained positive for BMP-2. The resting, proliferative and hypertrophic zones were stained positive, whereas proliferative chondrocytes showed the highest staining. Further, denser positive immunostaining was observed in cells next to the drill-hole injury, compared to cells more lateral of the epiphyseal plate.

At day 14, when newly formed bone trabeculae matured, a positive immunostaining was observed, but to a less extent compared to day 1, 3 and 7. Just few cells of the resting zone showed a slight positive staining. In the whole hypertrophic zone (HZ) hardly any positive immunostaining was observed in contrast to cells of the proliferative zone (PZ). Especially, cells of the growth plate next to the drill-hole injury showed a denser positive staining compared to cells of the epiphyseal plate, which were lateral of the injury. In addition to it, mesenchymal cells along this drill-hole injury were abundantly stained positive for BMP-2. On day 14 cells of the resorption zone of the metaphysis did not show any positive BMP-2 immunostaining. **(see Figure 17)**

Figure 17: BMP-2 positive immunostaining on day 14: cells of the HZ next to the drill hole injury
HZ (Hypertrophic zone), PZ (Proliferating zone), RZ (Resting zone)

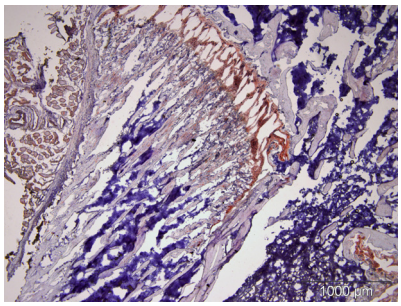
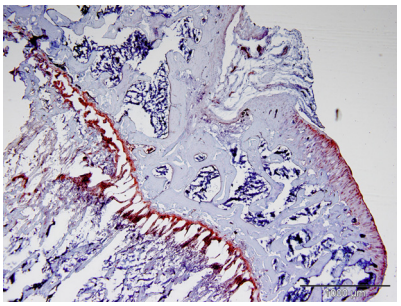


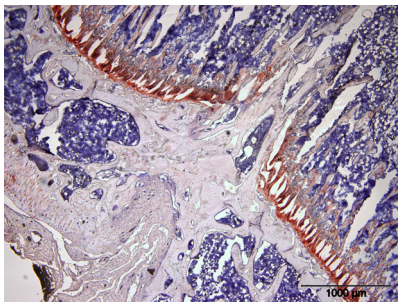
Positive staining of BMP-2 was also observed in some bone-marrow-derived infiltrating mesenchymal cells on day 3 at the beginning of the fibrogenic response.

Further, during the osteogenic response on day 7 and during the bone bridge maturation at day 14 BMP-2 immunostaining in mesenchymal cells and differentiated osteoblasts surrounding the formed bone were observed too.

At day 28, 42 and 82 nearly the whole height of the growth plate showed an abundant immunostaining, whereas cells of the proliferative zone showed the highest positive staining. These findings suggest, that BMP-2 plays an important role in the beginning, when early inflammatory response begins, in the fibrogenic and osteogenic phase and further in the bone formation/maturation, and further in bone bridge remodeling in the injured growth plate. **(see Figure 18)**

Figure 18: BMP-2 positive immunostaining of the injured tibia
A) day 28, B) day 42, C) day 82

A)  B) 

C) 

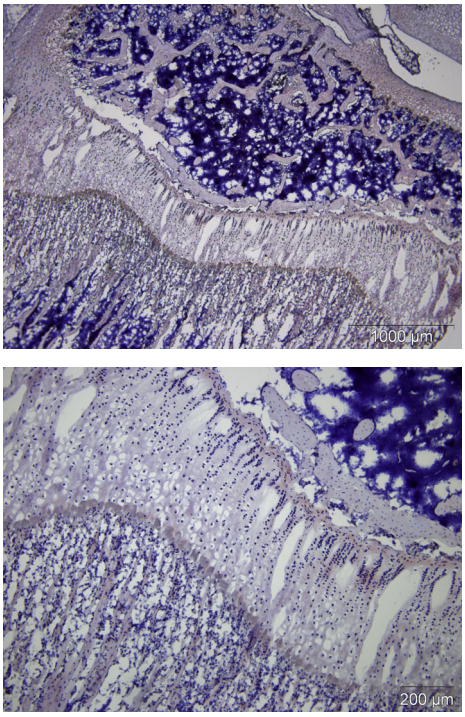
In contrast to day 7 and 14, the cells of the metaphysis on day 28 showed limited BMP-2 positive staining.

Further, chondrocytes of the articular cartilage were all consistently positive stained on day 1, 3, 7, 14, 28 and 82.

Another interesting observation was that the height of the growth plate decreased from day one to day 82. This phenomenon was seen in both, in the right uninjured and in the left injured tibia and could demonstrate the regular length growth in bones.

As negative controls for immunostaining, staining procedures with the addition of the rabbit IgG (from Sigma® and DAKO®) in various concentrations or 1% BSA/PBS instead of primary antibodies were used, which resulted in negative staining. (see Figure 19)

Figure 19: IHC of BMP-2 cryo-slides: Negative controls for immunostaining: use of rabbit IgG instead of primary antibody



6.1. Tables of BMP-2 Immunostaining Results

Table 7: Results of BMP-2 Immunostaining of the right uninjured tibia

RIGHT TIBIA	RESTING ZONE	PROLIFERATING ZONE	HYPERTROPHIC ZONE	RESORPTION ZONE
DAY 1	–	+	-	+
DAY 3	–	+ + +	+	+
DAY 7	–	+ + +	++/-(in the lowest one third)	+ +
DAY 14	–(+)	+ + +	-/+	+ +
DAY 28	–	+ + +	-/+	+/-
DAY 42	+	+ +	+(+)	+/-
DAY 82	+	+ + +	+(+)	++

Table 8: Results of BMP-2 immunostaining of the left injured tibia

LEFT TIBIA	RESTING ZONE	PROLIFERATING ZONE	HYPERTROPHIC ZONE	RESORPTION ZONE
DAY 1	+	+	-	+
DAY 3	+	+(–)	-	(–)+
DAY 7	-	++	-	+
DAY 14	-	++	-	-/+
DAY 28	+	++	++	+
DAY 42	++	+++	+++	+(-)
DAY 82	++	+++	+(+)	++

7. DISCUSSION

The epiphyseal plate in long bones is the most fragile structure in a child's developing long bone. Because of that trauma injury to the epiphysis is a common occurrence. Reason for fractures of the epiphyseal plate is when the mechanical loading placed on the long bones exceeds the mechanical strength of the physis.[6]

Trauma injuries to the growth plate that include the entire vertical destruction of the epiphyseal plate, can result in significant problems for the development of a long bone. Particularly Salter's type III and type IV are common in young children and involve the entire vertical destruction of the growth plate.[8]

Fact is that the epiphyseal plate has limited ability to regenerate and these injuries are often repaired by bony tissue at the injury site, a phenomenon called bone bridge formation. The process of bone bridge formation at the injured site leads to structural disorganization of the epiphyseal plate and further disrupts endochondral longitudinal bone growth.[8] This process results in significant skeletal complications like limb length discrepancy and angulations deformity.

BMPs, especially BMP-2 are well known to have a central role in the regulation of endochondral ossification and of osteogenesis during bone fracture repair. In this study, the hypothesised involvement and essential role of BMP-2 in the drill-hole injury-induced inflammatory, fibrogenic, osteogenic, and remodelling phases in the repair process of the physeal injury were examined by immunohistochemistry (IHC) at various points of time (day 1, 3, 7, 14, 28, 82).

Numerous studies have shown that bone morphogenetic proteins play an essential role in bone fracture healing. After bone injury, BMP-2 was indicated to be an early response gene.[37]

Furthermore, BMP-2 is an important mediator of bone marrow-derived mesenchymal cell migration and differentiation of osteoblasts potently stimulating the formation of new bone.[70, 71, 80] Bone morphogenetic proteins are known important regulators for bone formation and repair and for that reason it could be hypothesized that BMPs might play an important part in regulating epiphyseal plate injury responses and bony repair. In fact the potential roles of BMPs in the bony repair of injured epiphyseal plate cartilage remain unclear until now. [21]

In organ cultures, BMP-2 leads to stimulation of resting zone chondrocytes to proliferate and to stimulation of proliferative zone chondrocytes to hypertrophy. In higher concentration an extensive hypertrophic differentiation in the epiphyseal plate is observed. In contrast, using a BMP antagonist, called Noggin, treatment in that organ culture results in inhibition of proliferation of resting zone chondrocytes and hypertrophy of proliferative zone (PZ) chondrocytes.[69]

This study examined the expression of bone morphogenetic proteins through immunohistochemical (IHC) analysis in a rat drill-whole growth plate injury model to investigate their potential involvements in regulating the different growth plate injury responses, which lead to bony repair at the injury site.

One reason for the abundant staining of BMP-2 at the beginning of the osteogenic response on day 7 could be that BMP-2 plays an important role in stimulating the differentiation of osteoblasts and further stimulating bone formation at the drill-hole injured growth plate.

As described in the results above, positive staining of BMP-2 was also observed in some bone-marrow-derived infiltrating mesenchymal cells on day 3 at the beginning of the fibrogenic response. Further, during the osteogenic response on day 7 and during the bone bridge maturation at day 14 BMP-2 immunostaining in mesenchymal cells and differentiated osteoblasts surrounding the formed bone was observed too. These results suggest that BMP-2 may play an important role not only in the early inflammatory phase, but also in bone formation and maturation at the growth plate injury site.

Furthermore at day 28, 42 and 82 cells of the proliferative zone showed the highest positive staining, whereas nearly the whole height of the growth plate showed an abundant immunostaining.

These findings suggest, that BMP-2 plays an important role in the early phase of the inflammatory response, in the fibrogenic and osteogenic phase and in the bone formation/maturation, and in bone bridge remodeling in the injured growth plate.

Besides, in this study we used tibial bones instead of femurs to analyse growth plate reactions to a physeal lesion, set by a transepiphyseal drill-hole injury. The proximal tibial physis has the advantage of being flat in contrast to the femoral growth plate.

An interesting feature of this study was the finding that BMP-2 was deregulated in both the injured left tibia epiphyseal plate and the uninjured right contra-lateral growth plate. This apparent “cross-talk” between the two legs could be mediated by mechanical stress to the

contra-lateral uninjured leg because it has a greater weight-bearing role after fracture. It can be speculated that this mechanism represents a natural means of limiting leg length disturbances, meaning that under usual circumstances when the uninjured limb is immediately exposed to an increase of weight-bearing stress, with associated survival advantage through preservation of mobility. But, the question is if you can say it in that way, because if on the one hand BMP-2 causes a bone bridge formation at the drill-hole injured epiphyseal plate, which represents a limiting leg length disturbance and on the other hand BMP causes a stimulating leg length disturbance in the uninjured tibial growth plate. Consequently, the usual organized mechanism of growth would be deregulated! The extent of the growth disturbance and the extent of leg length difference could occur by this phenomenon.

In this study, an up-regulation of BMP-2 is observed in the drill-hole injured leg as well as in the uninjured contra-lateral tibia. Another hypothesis could be that the up-regulation of BMP-2 in the uninjured leg symbolizes just the expression of growth of bones in young rats. The epiphyseal plate of rats never shows a closure, so that could be the reason for the BMP-2 expression. If the expression respectively the positive immunostaining of BMP-2 in legs of control animals show the same staining, this would suggest a normal expression of BMP during growth.

Further, a systemic mechanism could be the reason for the increased BMP-2 expression in both legs, the one with the drill-hole injury and the other without any injury. A growth plate injury of just one leg could influence the whole body via systemic mechanisms, especially the epiphyseal plate of the counter-leg.

In summary, this current study showed induction and localization of BMP-2 at the growth plate injury-induced inflammatory response. This finding suggests that BMP-2 may play an important role in regulating the inflammatory response itself or on the other hand may be a mediator of that response in regulating downstream healing events. In addition, BMP-2 was observed in infiltrated mesenchymal cells and osteoblasts at the physeal injury site during fibrogenic and osteogenic responses suggesting that BMP-2 is involved in early bone bridge formation by inducing ossification.[21] Further, at the bone bridge maturation and remodelling phase BMP-2 was up-regulated and positively stained nearly in all zones of the growth plate. If this phenomenon is observed in the control animal too, it could indicate just usual growth.

In this study, immunohistochemistry (IHC) was used to localize changes in BMP-2 immunoreactivity in a drill-hole injured tibia in contrast to an uninjured tibia. A possible

future study could use an experimental group with young rats, which do not have any injury to look for normal BMP-2 expression in growth plates in growing bones of young rats. Further, a such study should be accompanied by taking blood samples of the young rats on day 1, 3, 7, 14. 28 and 82, to analyze if there is a systemic reaction after a growth plate injury in one leg. Actually, this idea is already part of the designed study, but this examination is not able to establish a systemic effect of an epiphyseal fracture, because this process shows only inflammatory mediators in the blood. This study shows only inflammatory mediators in blood as a systemic reaction and serves only for classification of inflammatory stages.

This provides a basis for future studies focusing on the role of BMP-2 in the molecular pathology of bone growth disturbances with the final goal of preventing growth discrepancies in children with injured growing bones.

To sum up this study shows a spatial differential expression of BMP-2 during various stages of bony repair of the injured growth plate. This suggests that BMP-2 has a critical and potential role in regulating the cellular events leading to the formation of bone bridges at the injured growth plate. The study gives evidence that BMP-2 is involved in the regulation of the healing process of a physeal injury as well as in fractures.

8. FIGURELIST

9. TABLELIST

10. LIST OF LITERATURE

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