



Molecular control of skeletal development



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Summary

The aim of my research is the analysis of the signaling network controlling embryonic bone formation. Using mouse mutants and an organ culture system for embryonic limb explants we have for the first time integrated three signaling systems, the *Ihh*/PTHrP, BMP and FGF signaling systems, into a common control network. These investigations led to a new understanding of the molecular origins of Achondroplasia, which results from activated FGF signaling. They furthermore identified the BMP signaling pathway as a new target to treat Achondroplasia.

To understand signal propagation in the growth plate we have started to investigate the interaction of *Ihh* with the extracellular matrix. We found that heparan sulfates sequester *Ihh* signals, strongly indicating that *Ihh* can act as a long range signal. In addition these studies revealed activated *Ihh* signaling as the likely cause for the development of the human 'Hereditary Multiple Exostoses Syndrome'. We have started to extend our studies on signal interactions and signal transport to transcription factors regulating downstream gene expression.

Current state of research in the field and significance

The vertebrate skeleton is a complex organ necessary for the survival and the quality of vertebrate life. This is reflected in the large number of inherited disorders characterized by malformations of the skeleton. Moreover, age related bone diseases affecting for example bone stability (Osteoporosis) or the joint cartilage (Osteoarthritis), have become a new focus of scientific research. The aim of my laboratory is to decipher the control mechanisms regulating embryonic bone formation. We hope that such an understanding will not only lead to new insight into developmentally derived skeletal disorders but will ultimately result in new ways to treat adult bone diseases by reactivating the embryonic program *in vivo* or in stem cell cultures.

Most of the bones of the skeleton are formed by endochondral ossification, a multistep process in which a cartilage skeleton is initially formed, that is later replaced by bone. Although endochondral ossification has been extensively studied on a morphological level, the various signaling systems regulating this complex process and their interactions are just being elucidated. We are concentrating our analysis on the early steps of endochondral ossification, when chondrocytes proliferate and differentiate into hypertrophic chondrocytes, which are subsequently replaced by bone (Figure 1). As longitudinal growth of endochondral bones is dependent on the proliferation and hypertrophic differentiation of chondrocytes, the tight regulation of these two steps is crucial to balance growth and stability of the bones.

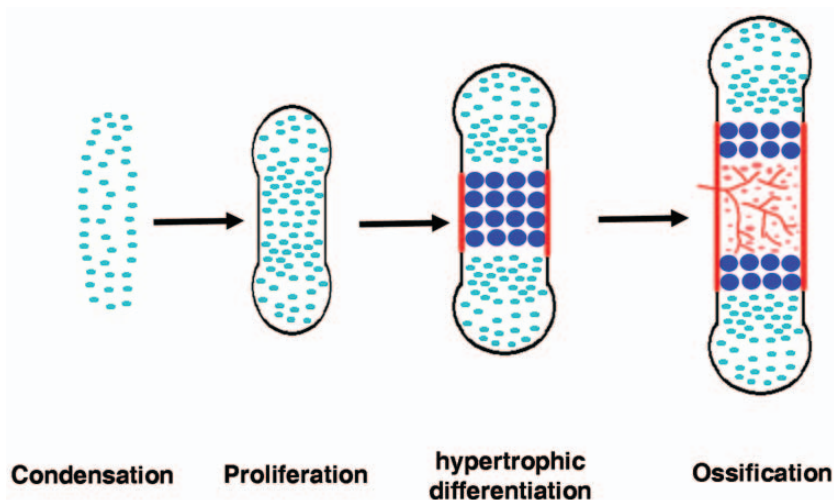


Figure 1: Endochondral Ossification - mesenchymal cells condense and differentiate into chondrocytes, which form cartilage elements, the precursors of the later bones (turquoise). The cartilage elements are surrounded by a layer of fibroblastic cells, the perichondrium (black). Starting from the center of the cartilage anlagen chondrocytes differentiate into a hypertrophic chondrocytes (blue). The hypertrophic region is invaded by blood vessels (Red) osteoclast and osteoblasts, which start to replace the hypertrophic chondrocytes by bone (red) and bone marrow (red).

Results

Interaction of signaling systems controlling chondrocyte differentiation

My previous work on Ihh has uncovered a first important feedback loop in which Indian hedgehog (Ihh) expressed in the differentiating chondrocytes and Parathyroid Hormone related Protein (PTHrP) expressed in the periarticular chondrocytes interact to regulate the onset of hypertrophic differentiation. To integrate the Ihh/PTHrP signaling system with that of other signaling pathways regulating chondrocyte differentiation we have established a culture system for embryonic limb explants. This system allows the epistatic analysis of different signaling systems by co-treatment of explants with combinations of growth factors and by utilizing limbs of various mutants as source for the explants. We have for the first time integrated three signaling systems, that of Ihh/PTHrP, FGFs and



BMPs, into a common control network (Figure 2). We demonstrated that BMP and FGF signals antagonize each other in regulating at least three distinct steps of chondrocyte development. They regulate (1) chondrocyte proliferation independent of the Ihh/PTHrP system, (2) the onset of hypertrophic differentiation by acting upstream of the Ihh/PTHrP system and (3) the process of hypertrophic differentiation independent of Ihh/PTHrP (Minina et al. 2001, 2002).

These investigations led furthermore to a new interpretation of the molecular origin of achondroplasia, the most common form of human dwarfism, which results from activated FGF signaling. In contrast to the established model that activated FGF signaling in Achondroplasia delays hypertrophic differentiation we could demonstrate that it in fact accelerates this process, a finding of high importance for the development of specific treatment strategies. Building on our signaling network we could consequently demonstrate that BMP signaling rescues the reduced regions of proliferating and hypertrophic chondrocytes in a mouse model for achondroplasia implicating manipulation of the BMP signaling system as a new target to treat Achondroplasia (Minina et al., 2002).

This work has demonstrated that our explant system provides a unique, powerful tool for dissecting the regulation of chondrocyte differentiation. Accordingly, we have started to extend this control network by integrating further secreted factors like TGF- β s, Wnts and others. Preliminary results indicate that TGF- β , like FGFs, accelerate hypertrophic differentiation. In addition we have started to explore techniques to introduce siRNA into the organ cultures, a technique, which - if successful - will enable us to rapidly analyze the function of newly identified genes before generating mouse models.

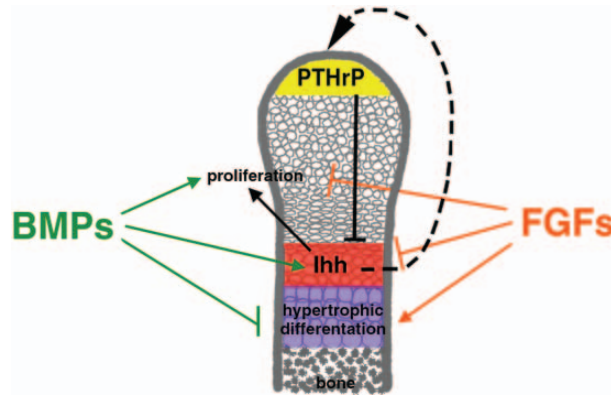


Figure 2: Ihh/PTHrP, BMP und FGF signals interact to regulate chondrocyte differentiation.

Signal propagation in the growth plate

As described above a large number of growth factors, each produced in a discrete location, interact to regulate chondrocyte proliferation and differentiation. To understand how these signals relate to one another one must have an understanding of how their respective ranges of action are determined. To this end, we have started to investigate the role of the extracellular matrix (ECM) in Ihh propagation. The glycosyl transferase Ext1 is one of the key enzymes for the synthesis of heparan sulfates (HS). Mutations in Ext1 in human result in benign bone tumors and short stature (Heritable multiple exostoses' (HME)). We are analyzing a gene trap mouse line carrying a hypomorphic allele of Ext1, which leads to reduced HS synthesis. These mice are characterized by delayed hypertrophic differentiation of chondrocytes. Analysis of the Ihh/PTHrP system revealed an activation of Ihh signaling. Correspondingly, treatment of limbs in culture with heparin restricts Ihh signaling in wild type and mutant animals and blocks PTHrP expression. In contrast FGF signaling seems to be not affected at the stages analyzed (Koziel, in preparation).

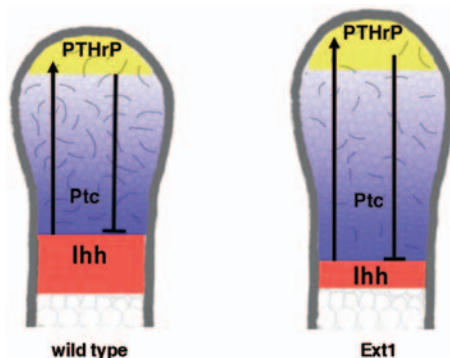


Figure 3: Heparansulfates restrict Ihh signaling.

Several important conclusions can be drawn from these experiments: 1) Although the Drosophila homolog of Ext1 has been shown to be necessary for hedgehog transport Ext1 dependent HS in mice seem to restrict Ihh signaling and might thus regulate the establishment of an Ihh signaling gradient. 2) In contrast to the current model, which predicts a secondary mediator, our experiments strongly indicate that Ihh travels through the growth plate to directly induce PTHrP expression. Culture experiments, demonstrat-

ing that neither of the predicted signals, BMPs or TGF- β s, can activate PTHrP expression in absence of Ihh signaling support this result (Minina et al., 2001; Kreschel, unpublished). 3) Activated Ihh signaling acting either on neighboring chondrocyte or on the flanking perichondrium is the most likely cause for the development of exostoses in human. These investigations have for the first time addressed the role of the ECM in Ihh distribution and have resulted in a new understanding of Ihh acting as a long range signal. We are planning to extend these studies to other growth factors that depend on interactions with HS. Because of its size the developing bone seems to be a very sensitive model to investigate long distance signaling events, and results from such investigations might be applicable to other organs.

Transcription factors acting downstream of Ihh

To understand how signals regulate gene expression it is necessary to investigate the downstream transcription factors. Zinc finger transcription factors of the Gli family, like Gli3, act downstream of hedgehog signaling. We have started to analyze the specific roles of Gli3, which can act as an activator and repressor, in regulating chondrocyte proliferation and differentiation.

Trich-rhino phalangeal-syndrome affects craniofacial and skeletal development in human. During cartilage development the underlying gene *Trps1*, a GATA zinc finger transcription factor is expressed partially overlapping with PTHrP and *Ptc*. (Kunath et al. 2002). First analyses of *Trps1* null mice indicate a delay in hypertrophic differentiation. After a detailed analysis of the *Trps1*^{-/-} phenotype it will be highly interesting to investigate a possible interaction of the Ihh signaling system and *Trps1*.

Identification of new genes regulating endochondral ossification

The experiments described above are focused on the analysis of known genes. However, we expect that a large number of genes regulating bone development has not been identified yet. Using PCR based subtraction approaches we have identified more than 20 genes with specific expression patterns in the developing skeletal elements. One of the most interesting candidates is highly conserved and exclusively expressed in the developing bone. We have started to generate gain and loss of function mutants hoping that these will give new insight into how Ihh regulates the ossification process.

Another gene, *PERP*, which is expressed overlapping with *Ihh*, has originally been isolated as a target of p53. We found *PERP* expression overlapping with both, p53 and p63, indicating that *PERP* might act downstream of both genes. Accordingly we found *PERP* expression reduced in the skin *p63*^{-/-} mice (Lintermann, in preparation).

Four-jointed (*Fj*) is a type II transmembrane protein, which is widely expressed in the mouse embryo including the central nervous system, joints and tendons (manuscript in preparation). We have deleted *Fjx* in mice but did not detect a phenotype on a 129SvEv background. We are therefore planning to reinvestigate the phenotype on a C57B6 background.

Gene expression profiling

To be able to analyze gene expression in a broader way we have started to carry out complex hybridizations on cDNA chips (Affimetrix). We plan to establish gene expression profiles of limb cultures, in which different signaling pathways have been manipulated. In addition to isolating new cartilage specific genes, we hope to identify groups of genes that react to different signals in similar ways. Recognizing such groups will extend the understanding of the signaling network and facilitate the integration of new candidates in the future.

Interaction of positional information and bone differentiation

From a developmental perspective, a key question that is still poorly addressed is how patterning of the skeleton is linked to the process of bone formation. Most differences between skeletal elements arise by differential growth after the initial cartilage anlagen are laid down. Thus the signals that regulate bone formation are likely points at which positional information might act to regulate the shape of the bones. Towards



this end I plan to analyze mouse mutants in which the skeletal anlagen form normally, but certain elements fail to develop the proper final bone structure as for example the Hox mutant *ulnaless*. We have started to analyze the specific steps at which bone formation is disturbed by gene expression analysis *in situ* and on chips. Subsequently we will try to rescue the phenotypes by manipulating the affected downstream signaling systems.

Goal

The goal of my laboratory is to identify the network of signaling systems regulating embryonic bone formation. I plan to understand the specific function of each of these signals and to place them into the context of the control network of genes regulating skeletal development. In addition to the interaction of signals we will concentrate our studies on the role of the ECM on the distribution of growth factors and on the regulation of gene expression by downstream transcription factors. We will further use gene expression analysis to identify the majority of genes regulating chondrocyte differentiation and to investigate gene regulation in a global way. In the long run we will extend these studies to mechanisms translating positional information into a bone pattern. The combination of the experimental approaches used should result in an in depth understanding of the basic mechanisms of bone formation and ultimately lead to new insight into the molecular origins of bone diseases.

General information

Publications 1998-2003

Zhou H, Weskamp G, Chesneau V, Sahin U, **Vortkamp A**, Horiuchi K, Chiusaroli R, Hahn R, Wilkes D, Fisher P, Baron R, Manova R, Basson CT, Hempstead B & Blobel CP (2003). *Essential role for ADAM19 in cardiovascular morphogenesis*. Mol Cell Biol (in press)

Kunath M, Lueddecke H-J & **Vortkamp A** (2002). *Expression of *Trps1* during mouse embryonic development*. Mech Dev 119S: 117-120.

Minina E, **Kreschel C**, Naski MC, Ornitz DM & **Vortkamp A**. (2002). *Interaction of FGF, *Ihh/Pthlh* and BMP signaling integrates chondrocyte proliferation and hypertrophic differentiation*. Dev Cell 3: 439-449

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Minina E, **Wenzel M**, **Kreschel C**, Karp S, Gaffield W, McMahon AP & **Vortkamp A** (2001). *BMP and *Ihh/PTHrP* signaling interact to coordinate chondrocyte proliferation and differentiation*. Development 128: 4523-34

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Shan Z, Nanda I, Wang Y, Schmid M, **Vortkamp A** & **Haaf T** (2000). *Sex-specific expression of an evolutionarily conserved male regulatory gene, *DMRT1*, in birds*. Cytogenet Cell Genet 89: 252-7

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Pathi S, Rutenberg JB, Johnson RL & **Vortkamp A** (1999). *Interaction of *Ihh* and BMP/*Noggin* signaling during cartilage differentiation*. Dev Biol 209: 239-53

Vortkamp A, Pathi S, Peretti GM, Caruso EM, Zaleske DJ & Tabin CJ (1998). *Recapitulation of signals regulating embryonic bone formation during postnatal growth and in fracture repair*. Mech Dev 71: 65-76.

Oral presentations on conferences 1998-2003

5th EMBL Mouse Molecular Genetics Meeting. Heidelberg (2003)

Gordon Conference: Cartilage Biology and Pathology. Ventura, USA (2003)

1st Wittgenstein Conference. Lucca, Italy (2002)

2nd European Conference on Bone Morphogenetic Proteins. Zagreb, Croatia (2002)

14th International Congress of Developmental Biology. Kyoto, Japan (2001)

Basic and Applied Research in Skeletal Tissue Engineering: Perspectives. Camogli Genova, Italy (2001)

Belgische Vereniging voor Biochimie en Moleculaire Biologie. Antwerpen, Belgium (2001)

3. MSD Kolloquium "Seener Gespräche". Bad Sarow (2000)

Deutsche Gesellschaft für Genetik: Genetik der Entwicklung. München (1999)

Sulzer Surlej Meeting on Cartilage Biology. Surlej Silvaplana, Switzerland (1999)

4th International Skeletal Dysplasia Meeting. Baden Baden (1999)

Ernst Schering Research Foundation, Workshop 29, Of Fish, Fly Worm and Man: Lessons from Developmental Biology for Human Gene Function and Disease. Berlin (1999)

EMBO workshop on Skeletal Development. Heidelberg (1998)

Molecular Signaling in Development, Cell Differentiation and Proliferation. Tokyo, Japan (1998)

Teaching

Practical course and lecture *Biologie für Mediziner*, Humboldt University Berlin, SS 2003 (2x3SWS), SS 2001 (2x3SWS), WS 2000/01 (1x3SWS), SS 2000 (2x3SWS), WS 1999/00 (1xSWS)

State doctorate (Habilitation)

Andrea Vortkamp, *Molekulare Kontrolle der Skelettentwicklung* (submitted April 2003)

Theses

Eleonora Minina: *Interaction von Ihh/Pthlh, BMP and FGF signaling in regulating chondrocyte proliferation and differentiation*. PhD Thesis, FU Berlin (February 2002)

Markus Wenzel: *Identifizierung neuer Zielgene im Indian-Hedgehog Signalweg*, PhD Thesis, FU Berlin (March 2003)

Averhoff, P.: *Analyse der Aufgabe von EXT1 als potentieller Mediator des Hedgehog-Signales während der Chondrozyten-differenzierung im sich entwickelnden Embryo*. Diploma Thesis, Freie Universität Berlin, 2001

Appointments, scientific honors & memberships

Speaker of the Independent Junior Research Groups of the Max Planck Society (since 1999)

Member of the German Society for Developmental Biology

Member of the International Society for Developmental Biology

Organization of scientific events

Symposium der Selbständigen Nachwuchsgruppen

- October 14, 1999, Heidelberg
- October 19, 2000, Berlin
- October 18, 2001, Berlin
- October 17, 2002, Berlin

External funding

SKELNET- *German Skeletal Dysplasia Network* (in BMBF Rare diseases program, funding since 10/2003)

Analysis of Ext1 and its potential role in propagating Ihh signaling during endochondral ossification. (DFG Vo/620-4-1, since 2002)

'Schwerpunkt Molekulare Dysmorphogenese' *Interaction of FGF and Ihh signals in regulating chondrocyte differentiation during embryonic endochondral ossification*. (DFG Vo/6-2, 2000-2001)

Public relations

Minima et al. featured in:

Wenn Knochen nicht mehr wachsen, Max Planck Forschung aktuell, 2002(4):10-11

Von Knochen und Knorpeln, Spektrum der Wissenschaft 6/2003:22-24