



## Research Group Development & Disease

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## Overview

The research group Development & Disease was established in October 2000. In August 2001 the group moved into the renovated laboratories on the 3rd floor. The group is part of and works in close collaboration with the Institute for Medical Genetics, which is located at the Campus Virchow of the Charité, Humboldt University, Berlin. The Institute for Medical Genetics provides clinical and diagnostic service for the Charité and the Berlin/Brandenburg area. Research at the Institute covers a broad spectrum of clinical and molecular analysis of genetic malformation syndromes and, as another major focus, tumor genetics. The combination of the basic science-oriented research group at the MPIMG with the more clinically oriented Medical Genetics group provides a unique opportunity for interaction. It has set the basis for many projects that focus on the molecular pathology of clinically defined conditions.

One of the fundamental questions in modern biology and medicine are the mechanisms by which the genotype determines the phenotype. Human genetics is a paradigm for this problem. In spite of our increasing knowledge about genetic diseases and the causative genes involved, we are frequently unable to predict the outcome, i.e. the phenotype or the course of a condition. Our focus is on the mechanisms by which the skeleton forms. The skeleton is a particularly useful system to study phenotype-genotype correlations because of the innumerable possibilities of phenotypic expression and the involvement of a limited number of cell-types (chondrocytes, osteoblasts and osteoclasts). The field of skeletal biology has expanded considerably in the last decade and has produced a number of breakthroughs that have led to a clearer understanding of skeletal development and function. Patterning genes such as the Hox-, Pax-genes control the overall bauplan of the skeleton and instruct mesenchymal cells where and how to differentiate into the skeletal anlagen. Sox9, Sox5, Sox6, and Runx2 have been identified as essential transcription factors that control the differentiation of determined precursor cells into chondrocytes or osteoblasts, respectively. Factors that control proliferation and differentiation such as the FGFs and their receptors and extracellular matrix proteins such as the proteoglycans or the

collagens are essential for proper growth of the skeleton before and after birth. Genes that control differentiation and function of osteoclasts are important for the regulation of bone resorption and homeostasis. The focus of our research is on the molecular basis by which the structure and function of the skeleton is regulated during vertebrate development. On a longer term, we want to understand the function of all relevant genes expressed in cartilage/bone and unravel their regulatory network.

## Results

To reach a better understanding of bone development and maintenance, we use three major approaches. First, we take a more comprehensive approach to identify and eventually characterize all relevant genes in this process. Second, we have established *in vitro* and *in vivo* systems to evaluate the function of selected genes, and third, we use a classic human genetics approach to identify novel disease related genes. Thus, our goal is to combine Human Genetics with functional genomics in order to understand pathology and normal development of the skeleton. Using this strategy, we have established the following projects:

### *Gene expression in bone/cartilage*

We have chosen the E14.5 mouse humerus as a model system for all further studies. At this stage, the humerus contains all cells and differentiation steps necessary for bone development, i.e. undifferentiated mesenchymal progenitor cells (perichondrium), undifferentiated, proliferating and hypertrophic chondrocytes (growth plate), osteoblasts (bone), invading blood vessels, and osteoclasts. Thus, with a single histological section through the E14.5 humerus, all of these differentiation stages can be captured at once in a two-dimensional system. We have extensively characterized this system histologically and by *in situ* hybridization to detect specific expression patterns of genes. Furthermore, we have conducted expression array analysis based on Affymetrix chip technology to gain information on the number and type of genes expressed. Based on this system we compare the expression of different mutants with the wt expression to identify regulated genes.

In order to get information on the expression on the cellular level, we have established an automated non-radioactive *in situ* hybridization methodology that allows for a relatively high throughput analysis of gene expression. Analysis of the first large scale expression studies shows multiple unique patterns that can easily be linked to certain cells types and differentiation steps and thus gives important information about gene function. This system has proved to be an invaluable tool for all further studies. Together with M.L. Yaspo, Dept Lehrach, MPIMG, we are investigating the expression of all chromosome 21 genes in our system in order to identify genes that have a role in cartilage/bone growth. It can be expected that the overexpression of one of these genes or a combination of them is responsible for the short stature and brachydactyly in trisomy 21. We have established a database with the expression patterns of the genes already studied to provide a tool for further analysis.

### *Genetic and functional analysis of hereditary skeletal phenotypes*

*(A) Hand malformations.* Hand malformations are caused by defects in patterning genes. Brachydactyly, a special form of hand malformation, refers to shortening of the hands/feet due to absent or small fingers/toes. Our group has contributed to unravel the genetic basis of several brachydactylies. To investigate the molecular pathology of these conditions we have established model systems in the mouse (transgenic, knock out, spontaneous mutations), the chick embryo (overexpression using retroviral systems) and *in vitro* (micromass). Our results show that the genes involved in the pathogenesis of brachydactyly are part of a molecular network regulating early chondrocyte differentiation and joint formation.

We were able to show that specific mutations in the ROR2, a receptor tyrosine kinase, result in brachydactyly type B (BDB), a human limb malformation syndrome with hypoplasia/aplasia of distal limb structures. The mutations identified in BDB patients are predicted to result in truncation of the receptor, either before or after the tyrosine kinase. Our studies aim at the functional analysis of Ror2 during development. In order to recapitulate this disease phenotype we have expressed the BDB-mutations in the chick embryo system. In collaboration with P. Knaus, Institute of Physiological Chemistry, University of Würzburg, we were able to show that Ror2

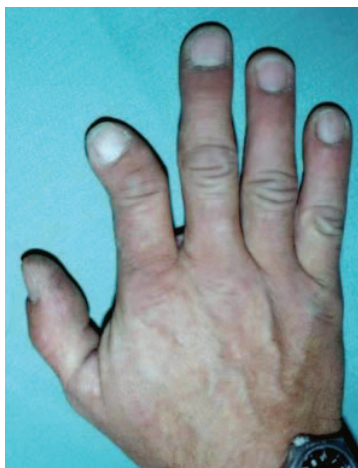


interacts with the BMP-pathway through a negative feed back loop, and that Ror2 is cartilage-inductive through Smad-independent pathways. Using the yeast two-hybrid-system we have identified components of the intracellular signaling cascade of Ror2 that give novel insights into the signal transduction pathway of this receptor. RNA-profiling using Affymetrix chip technology has identified a number of regulated genes in E14.5 humeri of Ror2<sup>-/-</sup> mice.

In previous studies we have identified mutations in the transcription factor HOXD13 as the cause of human synpolydactyly. The mutation is an in-frame expansion of an alanine-coding repeat in the 5'-region of the gene. To get further insight into the mechanisms of the mutation we are investigating the mouse mutation *spd*, which carries the identical alanine-expansion in *Hoxd13*. We were able to show that the brachydactyly observed in *spd* mice (and humans) is due to the persistent expression of *Hoxd*-genes resulting in a) a reduced rate of chondrocyte proliferation, b) a block in chondrocyte differentiation, and c) a lack of phalangeal joint formation. Using the cre-loxP system we are selectively inactivating *Hox*-genes from the 5' D-cluster in order to dissect the different functions of *Hox* genes during development of the limb skeleton. *In vitro* experiments using *Hoxd13* with different alanine repeat expansions have revealed a novel mechanism by which these mutations are likely to function. Expansion of the repeat beyond a certain threshold results in the accumulation of misfolded protein outside of the nucleus. Furthermore, the mutated protein prevents wt protein from entering the nucleus, possibly explaining the dominant nature of the condition. This mechanism may explain the molecular pathogenesis of other alanine-expansion diseases as well and could thus provide the basis for a new mutational mechanism. Together with V. Kalscheuer, Dept.

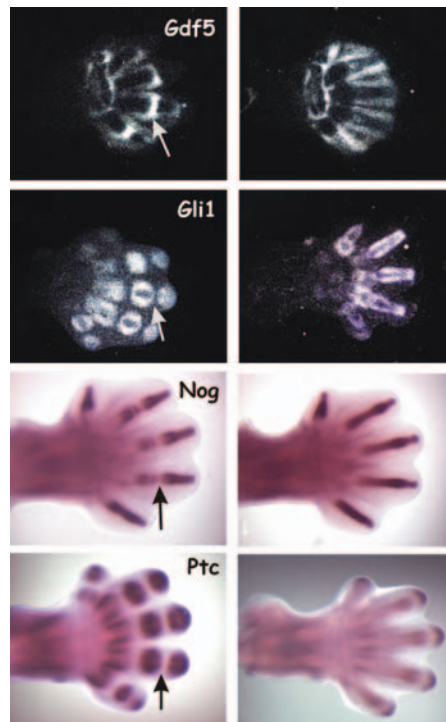
Ropers, MPIMG, patients with translocations 5' and 3' of the HOXD-cluster have been investigated in order to identify regulatory elements that are disrupted by the translocations.

The mouse mutant short digits (*Dsh*) has a similar phenotype as human brachydactyly type A1, but has no mutation in Indian hedgehog (*IHH*), as its human counterpart. We have extensively studied this mutant and were able to show that *Dsh* is allelic with Sonic hedgehog (*Shh*), a secreted signaling molecule with a central role during development. Using a positional cloning approach, we are investigating the region around *Shh* for regulatory mutations. In addition, we have carried out extensive studies to characterize the mechanism of brachydactyly in *Dsh*/<sup>+</sup> embryos. The results suggest that *Dsh* is caused by a regulatory mutation affecting *Shh* expression and a mutation in a second gene responsible for the brachydactyly phenotype.



Brachydactyly type A2 caused by a mutation in the bone morphogenetic protein receptor 1B (*BMPRI1B*).

Through national and international collaborations we have been able to study several families with brachydactyly. Using a positional cloning approach, we were able to identify the gene for brachydactyly type A2, a condition characterized by shortening of the index finger. Two mutations were identified in the bone morphogenetic protein (BMP) receptor 1B, a serine threonine receptor kinase known to play an essential role in several developmental processes. The mutations result in a dominant inactivation of the receptor, as shown by *in vitro* experiments due to a lack of Smad activation. Overexpression of the mutant receptors in chick embryos results in a brachydactyly similar to the human phenotype. Several other families with limb brachydactyly phenotypes have been ascertained that are currently being mapped.



Section (top) and whole mount (bottom) *in situ* hybridization of E13.5 hands of normal (left) and *Dsh* (right) mice. Gene expression is indicated by the presence of white (top) or brown (bottom) color. The developing joints between the phalanges, as labeled by the presence or absence (arrows) of gene expression, are disturbed in the mutant.

*(B) Other skeletal conditions.* In a previous study we were able to show that Runx2 is essential for the differentiation of precursor cells into osteoblast and for the differentiation of chondrocytes. Using *in vivo* approaches including transgenic mice and overexpression in chick embryos as well as an *in vitro* micromass system we were able to show that Runx2 is a) not sufficient to



Preparation of a chick skeleton with cartilage staining blue and bone staining red. Overexpression of a transcription factor identified in the Runx2 screen results in severe bending of the tibia and a retardation of bone formation (right side).

induce bone and b) a positive regulator of chondrocyte differentiation. In a complimentary approach, we are using RNA-profiling techniques to isolate regulated genes that are differentially expressed in the E14.5 humerus of Runx2<sup>-/-</sup> vs. wt mouse embryos. The comparison of gene expression has revealed approx. 80 regulated genes. All of these genes have been evaluated for their expression patterns using the automated *in situ* hybridization system. Through this screen we were able to identify a large number of so far unknown bone-expressed genes. Interesting candidates are being evaluated for their function and regulation. Together with the Dept. Vingron, MPIMG, Runx target genes are identified by searching promotor sequences for Runx binding sites. Candidates are tested by *in situ* hybridization analysis and quantification of mRNA levels in wt vs. Runx2<sup>-/-</sup> mice.

Recently, we are able to identify mutations in the membrane transporter Ank as the cause of craniometaphyseal dysplasia (CMD), a dominantly inherited skeletal dysplasia with increased bone formation and density (sclerosis). Ank has been shown to transport inorganic pyrophosphate (PPi) from the cytoplasm to the extracellular space. We use the ank/ank mouse as a model to study bone homeostasis and have established *in vivo* and *in vitro* assay systems to monitor bone density/degradation.

### Identification of disease genes

An important part of our project includes the clinical evaluation and ascertainment of individuals and families with hereditary conditions. During the past year, the clinical genetics unit at the Charité has been restructured and now provides an excellent tool for this purpose. In collaboration with P. Nürnberg, MDC, several other conditions with skeletal dysplasia or decreased bone density have been mapped. In one, the causative gene defect has been identified (SED Omani type). Mapping is an extremely powerful approach to identify disease genes but in the great majority of cases no large pedigrees are available. Together with the Dept. Ropers, MPIMG, we are establishing a center for array CGH (comparative genome hybridization). This technology will enable us to screen genome wide for deletions at a very high resolution, opening up new avenues in the identification of genotype-phenotype correlations.

### Molecular biology of fracture repair

In the event of injury, bones heal by generating new bone rather than by scar tissue. Recent studies have provided evidence that skeletal regeneration as it occurs in fracture repair is similar to embryonic bone development. In this project we intend to systematically evaluate and categorize genes that are expressed during the early phase of fracture repair. To do this we use the controlled fracture of the sheep tibia as a model and callus from these fractures as a source of material. We have established a cDNA library of the fracture tissue. In collaboration with the Dept. Lehrach, MPIMG, we will use this library as a basis for a comprehensive study of genes involved in fracture repair.

### Evolution of the skeleton

Cartilage/bone and haematopoiesis evolved in a multi-step process in early chordata evolution. In mammals there are only three runt-transcription factors. Whereas Runx2 is essential for bone development, Runx1 is of crucial importance for haematopoiesis. Runt genes appear to be particularly useful to analyze in which way gene duplications are related to the evolution of new characters. The aim of the project is to analyze the number, structure and expression of runt genes in branchiostoma, hagfish, lamprey and sharks. According to our phylogenetic analyses two runt gene duplications occurred in early chordata evolution.



## Goals

An important future goal is to expand our knowledge of pathogenetic pathways by studying factors that modify a phenotype known to be caused by a certain mutation. Our approach to this problem will include extensive studies of downstream factors that are regulated by a certain mutation in order to identify interacting genes. For an effective functional testing of genes we will have to optimize our systems of gene/mutation testing. A complementary approach will be to systematically identify all genes that are relevant for the formation of the skeleton. Systematic *in situ* hybridization will greatly enhance our knowledge about gene function in this system. It will thus be of utmost importance to obtain a sufficient amount of data in this system.

## General information

### *Selected Publications 2001-2003*

**Kornak U & Mundlos S** (2003). *Genetic disorders of the skeleton: a developmental approach*. Am J Hum Genet 73(3): 447-74

Lehmann K, Hecht J, **Stricker S**, Sammar M, Meyer B, **Süring K**, Majewski F, Tinschert S, Müller D, Knaus P, Nürnberg P & **Mundlos S** (2003). *Mutations in Bone morphogenetic protein receptor 1B cause brachydactyly type A2*. PNAS USA (in press)

**Schwabe GC**, Trepczik B, **Süring K**, **Brieske N**, Tucker AS, Sharpe PT, Minami Y & **Mundlos S** (2003). *The Ror2 knock out mouse as a model for the developmental pathology of autosomal recessive Robinow syndrome*. Dev Dyn (in press)

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Stock M, Schafer H, **Stricker S**, Gross G, **Mundlos S** & Otto F (2003). *Expression of galectin-3 in skeletal tissues is controlled by Runx2*. J Biol Chem 278(19): 17360-7

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Türkmen S, Gillessen-Kaesbach G, Meinecke P, Albrecht B, Neumann LM, Hesse V, Palanduz S, Balg S, Majewski F, Fuchs S,

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**Albrecht AN**, **Schwabe GC**, **Stricker S**, Boddrich A, Wanker EE & **Mundlos S** (2002). *The synpolydactyly homolog (spdh) mutation in the mouse — a defect in patterning and growth of limb cartilage elements*. Mech Develop 112(1-2): 53-67

**Stricker S**, **Fundele R**, **Vortkamp A** & **Mundlos S** (2002). *Role of Runx genes in chondrocyte differentiation*. Dev Biol 245(1) : 95-108

Kruger M, Mennerich D, Fees S, Schafer R, **Mundlos S** & Braun T (2001). *Sonic hedgehog is a survival factor for hypaxial muscles during mousedevlopment*. Development 128(5): 743-52

Nürnberg P, Thiele H, Chandler D, Hohne W, Cunningham ML, Ritter H, Leschik G, Uhlmann K, Mischung C, Harrop K, Goldblatt J, Borochowitz ZU, Kotzot D, Westermann F, **Mundlos S**, Braun HS, Laing N & Tinschert S (2001). *Heterozygous mutations in ANKH, the human ortholog of the mouse progressive ankylosis gene, result in craniometaphyseal dysplasia*. Nat Genet 28(1): 37-41

**Schwabe GC**, Tinschert S, Buschow C, Meinecke P, Wolff G, Gillessen-Kaesbach G, Oldridge M, Wilkie AO, Komec R & **Mundlos S** (2001). *Distinct mutations in the receptor tyrosine kinase gene ROR2 cause brachydactyly type B*. Am J Hum Genet 67(4): 822-31

### Books

**Mundlos S** (2000). *Skeletal morphogenesis*. Methods Mol Biol 136: 61-70

**Mundlos S & Olsen BR** (2002). *Defects in skeletal morphogenesis*. In: Royce PM & Steinmann B (eds), *Connective Tissue And Its Heritable Disorders*, Wiley Liss, Chap.23, Part V: 993-1023

**Mundlos S** (2003). *Konntatale anatomische Entwicklungsstörungen*. In: Lentze MJ, Schaub J, Schulte FJ, Spranger J (eds), *Pädiatrie, Grundlagen und Praxis*, Springer Verlag, Chapt. V, 31: 278-284

**Mundlos S** (2003). *Genetics of bone Disease and Skeletal Disorders*. In: Ganten D & Ruckpaul K, eds., *Encyclopedic Reference of Genomics and Proteomics in Molecular Medicine*, Springer Verlag (in press)

**Mundlos S** (2003). *Molekulare Ursachen von Fehlbildungen des Skeletts bei Neugeborenen*. In: Ganten D & Ruckpaul K, eds., *Molekularmedizinische Grundlagen von fetalen und neonatalen Erkrankungen*. Springer Verlag (in press)

**Mundlos S**, ed. (2003). *Genetik der Skelettdysplasien*, Z Med Genetik (in press)

### Co-operations

*Function of Ror2 in chondrocyte differentiation*, with Prof. Sebald, PD Dr. Knaus, Institute of Physiological Chemistry, University of Würzburg

*Signaltransduction of Ror2*, with Prof. W. Birchmeier, Max-Delbrück-Center of Molecular Medicine (MDC), Berlin-Buch

*Down stream targets of Runx2*, with Dr. F. Otto, Dept. of Haematology and Oncology, University of Freiburg

*Mechanisms of brachydactyly in humans*, with Dr. M. Warman, Dept. of Genetics and Center for Human Genetics, Case Western Reserve University, Cleveland, Ohio, USA

*Mapping of disease genes*, with PD Dr. P. Nürnberg, Mikrosatellitenzentrum, MDC, Berlin-Buch

*Mouse models for brachydactyly*, with Dr. D. Chan, University of Hong Kong

*Sheep fracture model. Mechanotransduction in bone*, with Prof. Dr. G. Duda, Charité, Dept of Surgery, Humboldt University of Berlin

*Mapping of disease genes. Expression patterns of cartilage specific genes*, with Dr. D. Cohn, Dept of Genetics, UCLA, USA

*Mechanisms of Gdf5 function*, with Dr. J. Pohl, BioPharm, Heidelberg

*Mapping of disease genes*, with K. Kjaer, Dept of Medical Genetics, University of Copenhagen, Denmark

*ANABONOS EU-project*, with Prof. S. Ralston, Institute of Medical Science, University of Aberdeen

*Function of NF1 in bone*, with Prof. L. Parada, Center for Developmental Biology, University of Texas Southwestern Medical Center at Dallas, USA

### External funding (MPIMG projects)

*Establishment of Array-CGH-Comparative genome hybridization* (EFRE - EU, 1/03-12/05)

*Klinische Forschergruppe TP 9: Molecular mechanism of fracture healing* (DFG, 1/02-12/03)

*Identification of therapeutic relevant genes for cartilage/bone formation through analysis of a mouse model for cleidocranial dysplasia* (Fritz-Thyssen-Stiftung, 3/03-2/05)

*SFB 577, TP A4: Craniometaphyseal dysplasia (CMD) – Clinical Variability and Pathogenic Pathways* (DFG, 7/01-6/04)

*SFB 577, TP A6: HOXD-gene. Molecular Pathology and Embryology of HOXD-related Limb Malformations* (DFG, 7/01-6/04)

*Neurofibromatosis type 1 as a bone dysplasia? Analyzing the role of neurofibromin in maintenance and development of the skeleton* (US-Army, 3/03-3/03)