The MHE Research Foundation

Wings of HOPE as we REACH for the CURE to Multiple Hereditary Exostoses

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Third International Multiple Hereditary Exostoses Research Conference Boston October 29- November 1, 2009

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THE THIRD INTERNATIONAL MULTIPLE HEREDITARY EXOSTOSES RESEARCH CONFERENCE

OCTOBER 29 – NOVEMBER 1, 2009
FOUR POINTS BY SHERATON BOSTON LOGAN AIRPORT

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PROGRAM

The scientific sessions will be held in the Suffolk Room.

THURSDAY, OCTOBER 29

19:00-22:00 **Welcome reception (Suffolk Room)**

FRIDAY, OCTOBER 30

7:30-8:30 *Continental Breakfast (Norfolk Room)*

8:30-8:40 **Opening Remarks**

Session 1: Overview and Clinical Presentation of MHE (Discussion Leader: Dror Paley)

8:40-9:00 **Dan Wells** (University of Houston)
Perspectives on the Genetics of Multiple Hereditary Exostosis

9:00-9:30 **Dror Paley** (Paley Advanced Limb Lengthening Institute)
Multiple Osteochondromas Treatment of the Lower Limb Deformities

9:30-10:00 **Scott Kozin** (Shriners Hospital for Children, Philadelphia)
MHE of the Upper Extremity

10:00-10:20 *Coffee Break*

Session 2: Human Genetics of MHE (Discussion Leaders: Wim Wuyts and Luca Sangiorgi)

10:20-10:50 **Wim Wuyts** (University of Antwerp)
Genetics of Multiple Osteochondromas: Overview of the Current Status

10:50-11:10 **Ivy Jennes** (University of Antwerp)
Characterisation of the Promoter Region of the Human Exostosin-1 Gene

11:10-11:40 **Luca Sangiorgi** (Rizzoli Orthopaedic Institute)
Genotype-Phenotype Correlation Study in 529 MO Patients: 'Protective' and 'Risk' Factors

11:40-12:00 **Elena Pedrini** (Rizzoli Orthopaedic Institute)
Osteochondroma Onset and Malignant Degeneration: Redefinition of EXT Gene Role

12:00-12:10 **Judith Bovée** (Leiden University)
Osteochondroma Formation: Haploinsufficiency or Two Hits

12:10-13:30 *Lunch (Norfolk Room)*

Session 3: Biochemistry, Chemistry, and Cell Biology of Heparan Sulfate (Discussion Leader: Marion Kusche-Gullberg)

13:30-14:00 **Marion Kusche-Gullberg** (University of Bergen)
EXT-Dependent Regulation of Heparan Sulfate Structure and Function

14:00-14:30 **Robert Linhardt** (Rensselaer Polytechnic Institute)
New Advances in Heparan Sulfate/Heparin Analysis and Biosynthesis

14:30-15:00 **Jeremy Turnbull** (University of Liverpool)
Elucidating the Functions of Heparan Sulfates: Towards Glycomics Strategies

15:00-15:20 **Cathy Merry** (University of Manchester)
Restoring Function to Heparan-Sulphate Deficient Cells

15:20-15:40 *Coffee Break*

Session 4: Studies Using Non-Mammalian Model Animals (Discussion Leader: Henry Roehl)

15:40-16:10 **Henry Roehl** (University of Sheffield)
Zebrafish as a Model for Multiple Hereditary Exostoses

16:10-16:40 **Rahul Warrior** (University of California, Irvine)
Developmental Regulation of Heparan Sulfate Proteoglycan Synthesis

16:40-17:10 **Joseph Yost** (University of Utah)
HS Fine Structure and FGF Signaling Pathways Converge at Cilia: Does Cilia Function Have a Role in HME?

17:10-17:30 **Malgorzata Wiweger** (Leiden University)
New Aspects of Multiple Osteochondromas – A Lesson from dackel (dak/ext2) Zebrafish Mutant

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19:30-22:30 Dinner (Suffolk Room)

SATURDAY, OCTOBER 31

8:00-9:00 *Continental Breakfast (Norfolk Room)*

Session 5: Developmental Biology of Bone and Cartilage (Discussion Leader: Henry Kronenberg)

9:00-9:30 **Henry Kronenberg** (Massachusetts General Hospital)
PTH/PTHrP Receptor Signaling is Required for Maintenance of the Growth Plate in Postnatal Life

9:30-10:00 **David Ornitz** (Washington University School of Medicine)
FGF Signaling in Skeletal Development and Repair

10:00-10:20 **Patrick Allard** (Harvard Medical School)
Understanding the Role of Ihh and Proteoglycans Interaction during Bone Development

10:20-10:40 *Coffee Break*

10:40-11:10 **Michael Underhill** (University of British Columbia)
Regulation of Osteogenesis by the Retinoic Acid Signaling Pathway

11:10-11:40 **Olena Jacenko** (University of Pennsylvania)
Altered Matrix at the Chondro-Osseous Junction Leads to Defects in the Hematopoietic Stem Cell Niche

11:40-12:00 **Kyle Kurek** (Children's Hospital, Boston)
Metachondromatosis: Expanding the Clinicopathologic Spectrum

11:50-1:30 *Lunch (Norfolk Room)*

Session 6: Role of Heparan Sulfate in Bone Development / Novel MHE Mouse Models (Discussion Leader: Maurizio Pacifici)

13:30-14:00 **Maurizio Pacifici** (Thomas Jefferson University)
Mechanisms of Exostosis Formation in Mouse Models of HME

14:00-14:30 **Kevin Jones** (University of Utah)
Osteochondromagenesis: Somatic Loss of Heterozygosity Modeled via Cre-Mediated Inversion of the Second Exon of Ext1 in Chondrocytes

14:30-15:00 **Andrea Vortkamp** (University of Duisburg-Essen)
Molecular Characterization of Osteochondroma Development in Mice

15:00-15:20 **Kazu Matsumoto** (Burnham Institute for Medical Research)
Stochastic Conditional Knockout of Ext1 Reveals an Unexpected Relationship between Biallelic Inactivation of the Gene and the

Development of Multiple Exostoses

15:20-15:40 *Coffee Break*

Session 7: Role of Heparan Sulfate in Non-Skeletal Tissues (Discussion Leader: Jeffrey Esko)

15:40-16:10 **Jeffrey Esko** (University of California, San Diego)
Do Mutations in EXT1 or EXT2 Affect Non-Skeletal Tissues?

16:10-16:40 **Hudson Freeze** (Burnham Institute for Medical Research)
Deficient Heparan Sulfate and N-Glycosylation Contribute to Protein-Losing Enteropathy in Humans and Mice

16:40-17:10 **Yu Yamaguchi** (Burnham Institute for Medical Research)
Deficiency of Heparan Sulfate in Excitatory Neurons Causes Autism-like Behaviors in Mice

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19:30-22:30 *Dinner Banquet (Suffolk Room)*

SUNDAY, NOVEMBER 1

8:00-8:30 *Continental Breakfast (Suffolk Room)*

Session 8: Biology of MHE and Related Bone Disorders (Discussion Leader: Benjamin Alman)

8:30-9:00 **Benjamin Alman** (University of Toronto)
Gli2 and p53 Cooperate to Regulate IGFBP-3–Mediated Chondrocyte Apoptosis in the Progression from Benign to Malignant Cartilage Tumors

9:00-9:30 **Pancras Hogendoorn** (Leiden University)
Primary Cilia Organization Orchestrating Cell Polarity in the Growth Plate and its Loss in Osteochondroma

9:30-10:00 **Frederick Kaplan** (University of Pennsylvania)
Osteochondromas & the FOP Metamorphogene

10:00-10:30 **Judith Bovée** (Leiden University)
On the Clinical Manifestation and the Genetics of Ollier Disease

10:30-11:15 **Meeting Summary and General Discussion** – Discussion Leaders: Yu Yamaguchi and Maurizio Pacifici
Remarks from the MHE Research Foundation – Sarah Ziegler

11:15 Departure

Session 1

Overview and Clinical Presentation of MHE

Friday, October 30, 2009

8:40 – 10:00

Perspectives on the Genetics of Multiple Hereditary Exostosis

Dan Wells

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The first description of multiple exostoses in several members of the same family is generally accredited to Boyer in 1814. Since then close to 1000 reports have been published describing a hereditary form of multiple exostoses. The first comprehensive genetic description of the disease in modern times was done by Solomon, 1964 who analyzed 42 cases. In this study, Solomon established Multiple Hereditary Exostoses as autosomal dominant syndrome with essentially 100% penetrance. Initial evidence for the chromosomal location of MHE came from its association with Langer-Giedion Syndrome (tricho-rhino-phalangeal syndrome type 2). In addition to other phenotypes, individuals with LGS had multiple exostoses identical to those seen in MHE. Various reports, summarized by Bulher and Malik, 1984, associated LGS with deletions in 8q24.1. Formal conformation of this genetic location came in 1993 by Cook *et al*., who performed a genetic linkage analysis on 11 families with MHE. A side result of this study indicated that there was at least one other chromosomal location that could cause MHE. This was quickly located on chromosome 11 by Wu *et al*. 1994. In 1995, the EXT1 gene was positionally cloned and sequenced (Ahn *et al*., 1995) followed by the identification of the EXT2 gene (Stickens, 1996; Wuyts 1996). Although the two genes showed some structural similarities, their protein sequences gave few clues as to their function. The first indications of function of the EXT genes came from a series of papers in 1998 (McCormick *et al*., 1998; Lin *et al*., 1998; Lind *et al*., 1998; Simmons *et al*., 1999). McCormick *et al*. (1998) lead the way somewhat by accident. Using an eloquent cell culture technique to look for genes involved in the biosynthetic pathway of GAGs, they identified EXT1 as an ER-resident type II transmembrane glycoprotein that altered the synthesis and display of cell surface heparan sulfate glycosaminoglycans (GAGs) and Lind *et al*. (1998) further showed EXT2 had similar capabilities. A suggestion as to how heparan sulfate GAGs function in abnormal bone growth came with the discovery of the *Drosophila* EXT1 homolog, *tout velu* (*ttv*). Bellaichie *et al*. (1998) showed that *ttv* was required for the diffusion of hedgehog in *Drosophila*. Lin *et al*. (1999) use EXT1 deficient mice to show that heparan sulfate GAGs affected the binding of hedgehog to the cell surface and disrupted the expression of a number of embryonic markers. Over the past 10 years, research on MHE appears to have focused on three main areas, tumoragenesis, endochondral bone growth, and model systems (Hecht, *et al*., 2002; Han *et al*., 2004; Koziel *et al*., 2004; Hilton *et al*., 2005; Stickens *et al*., 2005; Hameetman *et al*., 2007; Kitagawa *et al*., 2007; Clement *et al*., 2008). Some key aspects of these studies will be discussed in light of the where the field is currently and what it will be its focus as we move into the next decade. Some old questions need to be put to rest (such as "loss of heterozygosity" and the "genesis of tumors") while others need to be explored more deeply (such as "is this just a bone disease?").

Multiple Osteochondromas Treatment of the Lower Limb Deformities

Paley, D.

Paley Advanced Limb Lengthening Institute, 901 45th Street Kimmel Building West Palm Beach, FL 33407. e-mail: dpaley@lengthening.us.

There are a variety of problems related to the exostoses of Hereditary Multiple Osteochondromas. The majority of these problems relate to bothersome bony protrusions with their affect on surrounding joints, muscles, tendons, nerves, blood vessels and skin. Osteochondromas can also affect growth plates and lead to limb deformities and length discrepancies. The focus of this talk will be on the limb deformities and discrepancies secondary to the multiple osteochondromas.

Treatment of the Lower Limb Deformities

Femero-acetabular impingement

The best way to treat this problem is 'safe' surgical dislocation of the hip according to the technique of Ganz from Switzerland. This safeguards the circulation of the femoral head avoiding avascular necrosis of the femoral head. The osteochondromas can be resected under direct vision and the femoral head templated with a spherical template to acertain if the femoral head is spherical. The range of motion of the hip greatly improves after this surgery. It can be combined with a varus osteotomy using a blade plate for fixation.

Valgus Knee Deformity (Knock knee deformity)

This deformity is usually in the upper tibia. There is usually a large osteochondroma involving the upper end of the fibula. The fibular osteochondroma often tethers or envelops the peroneal nerve. This is a very important nerve that is responsible for controlling the muscles that pull the foot up and out. Injury to this nerve results in a drop foot (inability to pull the foot up). Correction of the valgus deformity of the upper tibia requires an osteotomy (bone cut) of the upper tibia. All osteotomies of the upper tibia to correct valgus stretch the peroneal nerve even in patients without HME. In patients with HME and a fibular exostosis the nerve is very tethered and stretched even before surgery. The nerve can actually be inside the bone if the osteochondroma envelops it. Therefore to correct the deformity safely the nerve must first be found above the fibula and decompressed around the neck of the fibula. The osteochondroma of the fibula should be resected If the upper fibular growth plate is considered to be damaged beyond recovery then a segment of the fibula should be removed so that the two ends of the fibula do not join together again to prevent re-tethering of the tibia. Only after all of this is performed can an osteotomy of the tibia be carried out safely to correct the valgus deformity. The valgus deformity can either be corrected all at once or gradually. Correcting it all at once is usually performed by taking out a wedge shaped piece of bone and then closing the wedge to straighten the tibia. This can be fixed in place with a metal plate or with an external fixator. Gradual correction is carried out by minimal incision technique to cut the bone. The correction is achieved by use of an external fixator. This is a device that fixes to the bone by means of screws or wires that attach to an external bar or set of rings. Adjustment of the external fixator slowly corrects the deformity. This opens a wedge instead of closes a wedge of bone. This has the advantage of adding length to the leg which if the leg is short already is advantageous. This type

of external fixator is also used for limb lengthening. Therefore if there is a LLD the angular correction can be performed simultaneous with lengthening. Gradual correction is safer than acute (all at once) correction for correction of the valgus deformity.

Another way to address the valgus knee deformity without addressing limb length discrepancy is hemi-epiphyseal stapling of the growth plate. This is perhaps the most minor procedure possible and involves insertion of one or two metal staples on the medial side (inside) of the growth plate of the upper tibia. The metal staple straddles the growth zone on the medial side preventing growth of the medial growth plate while permitting growth on the lateral side. This allows the tibia to slowly autocorrect its alignment. It is a very slow process and may require several years. Once the tibia is aligned the staple can be removed permitting resumption of growth from the medial side. There is a small risk of damaging the medial growth plate which could lead to a varus bowing deformity of the tibia. Stapling can also be used in the distal tibia to correct the ankle deformity.

Valgus deformity of the ankle
Patients complain of walking on the outer border of the foot. Viewed from behind this posture of the foot is very apparent. This deformity is often well tolerated. The lower end of the tibia tilts outwards towards the fibula. The lower end of the fibula is the lateral malleolus. It is important for stability of the ankle. Since the fibula grows less than the tibia the lateral malleolus is often underdeveloped leading to lateral shift of the talus (ankle bone). This can eventually lead to arthritis of the ankle. Lateral tilt of the ankle joint is compensated by the subtalar joint (joint under the ankle) by inversion of the foot (turning of the foot in). Since this is a longstanding process the subtalar joint becomes fixed in this position of compensation for the ankle joint. Therefore if one tries to fix the ankle joint tilt completely the foot will end up tilted inwards and the patient will be standing on the outer border of the foot. Therefore one either has to accept the valgus ankle or correct it together with the subtalar joint fixed deformity. This is best done with a circular external fixator (Ilizarov device). This correction involves gradual correction of a minimally invasive osteotomy of the lower tibia and fibula together with distraction (pulling apart) of the subtalar joint contracture.

Flexion deformity of the knee
This deformity is usually related to tethering or locking of the soft tissues around the knee by distal femoral or proximal tibial osteochondromas. The treatment involves resection of the offending exostosis and lengthening of the hamstring tendons if needed.

Flexion deformity of the hip/subluxation of the hip/valgus upper femur
This is treated by resecting the offending osteochondroma of the femoral neck. This hip capsule has to be opened to access these. At the same time to reduce the hip subluxation (hip coming out of joint) a varus osteotomy of the upper femur should be done (bending the femur inwards towards the joint). The bone can be fixed either by an internal metal plate or an external fixator.

MHE of the Upper Extremity

Kozin, SH

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Hereditary multiple exostoses (HME) is an inheritable disorder of enchondral bone growth. HME is inherited in an autosomal dominant pattern with high penetrance and variable expressivity. Cartilaginous exostoses, grow from the physes of long bones and from the pelvis, ribs, scapula, and vertebrae. The most common upper extremity sites of involvement are the, humerus, scapula, distal radius and ulna, elbow, and hands. Approximately 1/2 of all patients have forearm involvement.

A clinical "bump search" of all long bones is indicated if HME is suspected. Shoulder, elbow, forearm, wrist, and digit range of motion should be measured. Radiographs should be obtained of any part of the extremity where osteochondromas are suspected or that have decreased range of motion. Serial measurements of forearm rotation are used to document progressive forearm deformity.

Most exostoses are asymptomatic and do not require removal. Exostoses may cause local discomfort, nerve or tendon impingement, decreased range of motion, and longitudinal and angulatory growth abnormalities. Several reports of adults with untreated forearm deformities due to HME indicated that they maintained function and were comfortable with their appearance. These reports question the role of aggressive surgical treatment to maintain or improve function.

Upper Extremity Surgery: Osteochondromas may cause visible local pain, deformity, and growth disturbance. Malignant transformation is uncommon. Local pain, caused by impingement of the osteochondroma on surrounding tissue, is a frequent indication for exostosis removal, and is effectively relieved by excision. If forearm rotation is blocked by an exostosis, removal will often improve motion.

Forearm osteochondromas frequently cause a length discrepancy between the radius and ulna. The radius becomes longer than the ulna and accepts the entire forearm load, resulting in radial bowing, radial tilting, and possible radial head dislocation. The increased radial inclination and lack of ulnar support positions the wrist into ulnar deviation and causes the carpus to "slip" toward the ulna.

Early osteochondroma removal to retard or prevent progressive growth disturbances is controversial. Hemiepiphyseal stapling of the radial side of the distal radius retards radial growth and allows correction of the radial articular angle and ulnar length discrepancy with growth. Ulnar lengthening may be performed in a single stage or using gradual distraction osteogenesis. Differential lengthening combined with angular correction can be used to reduce the radial head, because restoration of normal forearm anatomy may result in spontaneous radial head reduction. In patients with an isolated distal ulnar lesion, freeing the soft tissue tether between the ulna and ulnar sided carpus and distal radius may prevent radial head dislocation. The dislocated radial

head may become painful in the adolescent; resection relieves pain and removes the associated prominence. Radial head resection is delayed until skeletal maturity, because removal of the radial head in the growing child may cause cubitus valgus or proximal radial overgrowth. Creation of a one-bone forearm may be used to salvage a severely disorganized forearm.

In adults, an indication for HME excision is suspected malignant transformation. Symptoms and signs of malignant transformation include local pain and growth of an osteochondroma after skeletal maturity. Radiographic changes include internal lytic areas, erosion or destruction of the adjacent bone, and/ or presence of a soft tissue mass containing irregular calcifications. Malignant transformation, however, is quite rare in the upper extremity.

Session 2

Human Genetics of MHE

Friday, October 30, 2009

10:20 – 12:10

Genetics of Multiple Osteochondromas; Overview of the Current Status

Wim Wuyts, PhD

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Multiple osteochondromas (MO; Hereditary multiple exostoses-HME) is an autosomal dominant bone disorder characterized by the presence of osteochondromas (exostoses) on the long bones. This condition is genetic heterogeneous with at present two proteins known to be mutated in MO patients: Extosin 1 encoded by the EXT1 gene and Extosin 2 encoded by the EXT2 gene. Both EXT genes belong to a larger gene family of EXT-EXT-like (EXTL) genes which encode glycosyltransferases involved in the adhesion and/or polymerization of heparin sulphate (HS) chains at HS proteoglycans (HSPGs). EXT1 is located at 8q24.11-q24.13 and comprises 11 exons, while EXT2 is located at 11p12-p11 and contains 16 exons.

To date, an EXT1 or EXT2 mutation is detected in 70-95% of affected individuals and various mutation detection protocols have been developed to screen both EXT genes. Mutation data gathered during the last 15 years is available and can be consulted at the online Multiple Osteochondromas Mutation Database (Modb; http://medgen.ua.ac.be/LOVD/home.ph). Currently, the MOdb lists over 900 variant entries of which more than 550 are unique. It provides a global overview of mutation distribution, frequency and nature of the identified mutation.

A small percentage of patients does not show a mutation with standard mutation detection techniques including DHPLC, direct sequencing and MLPA. These are in general sporadic patients and extended analysis has shown that at least a fraction of these patients may have mosaic mutations.

There is great variation in the phenotypic manifestation of MO in various patients. It has been suggested that part of the interfamilial variation is due to the EXT mutation, with EXT1 patients showing more severe clinical spectrum than EXT2 patients. However, as also intrafamilial variation is observed also other factors may play a role and the identification of such other genomic variants is an ongoing challenge.

Characterisation of the Promoter Region of the Human Exostosin-1 Gene

Ivy Jennes[1], Ines Cilissen[1], Monia Zuntini[2], Luca Sangiorgi[2] and Wim Wuyts[1]

[1]Department of Medical Genetics, University and University Hospital of Antwerp, Belgium. [2]Department of Medical Genetics, Rizzoli Orthopaedic Institute, Bologna, Italy. e-mail: ivy.jennes@ua.ac.be.

Mutations in Exostosin-1 (*EXT1*) or Exostosin-2 (*EXT2*) cause multiple osteochondromas (MO). *EXT1* and *EXT2* are both tumour suppressor genes that encode proteins that function as glycosyltransferases in the biosynthesis of heparan sulphate (HS). At present, very little is known about the regulation of the EXT genes. Mutations in the *EXT1* gene are responsible for 47-64% of the MO families. To elucidate the transcriptional regulation of *EXT1*, we isolated and characterized the *EXT1* promoter region.

Theoretical analysis of the 10 kb upstream of the *EXT1* start codon was performed with promoter prediction programs TSSG, TSSW, FPROM, BDGP, Promoter 2.0 Prediction Server and Web Promoter Scan. These programs showed presence of a CpG island containing CG and CAAT boxes but no TATA box, which is characteristic for a housekeeping gene. Two potential functional promoter regions were identified, located respectively ~2.650 bp and ~900 bp upstream of the start codon.

To confirm the correct promoter region experimentally, overlapping PCR fragments in the 10 kb putative *EXT1* promoter region were generated and cloned in the pGL4.72 Luciferase Reporter Vector. After transfection in Human Embryonic Kidney cells, promoter activity was determined by performing luciferase assays, which located the actual core promoter within the 560 bp fragment containing the predicted promoter sequence at ~-900 bp. Further fine mapping located the minimal core promoter within in a fragment of 350 kb.

Subsequently, the promoter region was analysed for protein binding capacities with transcription binding prediction programs AliBaBa2, Cister, TFsearch, TFSiteScan and TESS. This analysis revealed various putative transcription factor binding sites. Additional analysis revealed the presence of two polymorphic G/C SNP's in which the presence of a cytosine destroys the binding site of a predicted transcription factor. Subsequently, new luciferase assays were designed to test whether these SNP's might indeed influence the *EXT1* promoter activity. They revealed a promoter activity that was up to 37% lower in CC genotypes compared to GG genotypes. Consequently, we identified both SNP's as primary modifiers that might explain part of the clinical variation observed in MO patients.

To test this hypothesis, both SNP's were characterised in 269 MO patients from 2 populations (148 patients from mixed ethnic origin from the Antwerp research group and 121 patients from Italian origin from the Bologna research group). The clinical features evaluated included the presence of deformities and complications, patients' stature and the number of osteochondroma and osteochondroma sites. First of all, significant association was observed between the frequency of a cytosine in the first SNP location and short stature in patients ($\leq$ P25) ($p<0,05$). Secondly, the presence of a cytosine in the first SNP location in trans with the *EXT1* mutation showed significant association with the presence of deformities ($p<0,05$). Finally, analysis of the

first SNP location in *EXT2* associated patients revealed association with the presence of deformities ($p<0,02$), short stature in patients ($\leq$ P25) ($p<0,02$) and the number of osteochondroma sites ($p<0,05$). These observations are the first indication for a genotype-phenotype association for promoter SNP's in *EXT1*.

Genotype-Phenotype Correlation Study in 529 MO Patients: 'Protective' and 'Risk' Factors

Pedrini E.[1], Jennes I.[2], Tremosini M.[1], Mordenti M.[1], Parra A.[1], Pignotti E.[3], Wuyts W.[2], and Sangiorgi L.[1]

[1]Department of Medical Genetics, Rizzoli Orthopaedic Institute, Bologna, Italy. [2]Department of Medical Genetics, University and University Hospital of Antwerp, Belgium. [3]Section of Statistics, Rizzoli Orthopaedic Institute, Bologna, Italy. e-mail: luca.sangiorgi@ior.it.

Multiple Osteochondroma (MO [MIM 133700]) is a genetic disorder characterized by a large spectrum of related EXT mutations and a wide clinical heterogeneity. To evaluate if the severity of disease and the risk of malignant degeneration are linked with a specific genetic background we performed a genotype-phenotype correlation study analyzing the larger MO case study ever collected. To estimate the constancy of what observed and substantiate the statistical significance of the data we compared results coming from two different European MO reference centers (the department of Medical Genetics of Bologna, IOR and University Hospital of Antwerp).

Clinical parameters were evaluated using a new clinical classification repeatable and easy to apply, based on deformity and functional limitations caused by lesions. The study was performed on a total of 529 MO patients (405 from IOR and 124 from Antwerp) ranging from 2 to 83 years old and representing 344 probands with their available affected relatives. The presence of mutations in EXT1 (8q24) or EXT2 (11p11-12) genes was investigated with a combined DHPLC/MLPA protocol screening with a direct sequencing of all abnormal profile. Statistical analyses were performed considering the two case studies both separately and on the whole; all collected data were then analyzed to define ‘protective’ and ‘risk’ factors related with mild or severe clinical presentations.

Molecular analyses revealed 344 EXT1 and 132 EXT2 mutations; no disease causing mutation was found in 51 patients. This study confirmed the predominant role of EXT1 in defining a severe clinical phenotype characterized also by short stature. Adverse clinical presentation is associated also with sporadic cases and male gender. Interestingly, patients without EXT1/2 detected mutations are significantly related to mild phenotypes, with a stature closely approximating to the average population height. Malignant transformation was observed in 26 patients; there is no evidence of any association between chondrosarcoma occurrence and EXT1 mutations, number of lesions and the severity of disease. Interestingly, malignant degeneration mainly occurred in patients with a positive family history (only 2 were classified as sporadic cases).

The comprehensive study, beside the intrinsic limitations of multicentric revisions, shows that trends observed in the IOR has been confirmed in the Antwerp patients substantiating the statistical significance of the data; this leads to consider MO information obtained in this study as probable constant features of disease and therefore could play a relevant role in the clinical approach to MO patients (providing information to be offer during genetic counselling, calibrating follow-up program for each group of patients and helping in defining appropriate clinical treatment) except for prevention of malignant transformation where a regular screening

is still highly recommended. Since individuals with identical mutation often show quite different phenotypes, the presence of other factors which can modulate the clinical presentation of MO is evident; therefore, another goal of this study consists in the definition of clinical and genetic homogeneous group of patients where appropriate studies could be performed in order to clarify this feature and better characterize the pathogenesis of the disease.

Osteochondroma Onset and Malignant Degeneration: Redefinition of EXT Gene Role

Monia Zuntini[1*], Elena Pedrini[1*], Alessandro Parra[1], Marco Alberghini[2], Federica Sgariglia[1], and Luca Sangiorgi[1]

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Osteochondroma is the most common of the benign tumors of the bone. It could presents as a single lesion, solitary osteochondroma (SO) with an incidence of 1-2%, whereas it occurs as multiple lesions in the context of multiple osteochondroma disease (MO, incidence of 1/50000). The most severe complication of osteochondroma is the malignant transformation into secondary peripheral chondrosarcoma (CHS); it is estimated to be rare in SO (<1%) whereas it occurs in 1-5% of MO patients. Even if both conditions have been associated with mutations in EXT1 and EXT2 genes, the molecular mechanism involved in osteochondroma onset and malignant progression is still contradictory.

To evaluate whether a second mutational hit is required for the development of solitary/multiple osteochondromas and/or their malignant degenerations, we investigated 65 tissue samples, including 46 osteochondromas (35 MO and 11 SO) and 17 peripheral chondrosarcomas (12 derived from MO and 5 from SO) with 2 recurrences, for the presence of both point mutations and big rearrangements in EXT1/EXT2 genes; mutational screening was performed with a combined DHPLC/MLPA protocol including also direct sequencing of all abnormal profiles and quantitative Real Time qPCR to validate MLPA results. Analyses were performed also on corresponding constitutional blood samples. For 5 patients we considered more resections from different affected skeletal sites.

6 out of 11 SO samples showed the presence of point or big mutations in EXT1 gene whereas 5 showed no EXT mutations; no samples with two mutational hit were observed. Mutational screening of 35 MO samples confirmed all the presence of the germ-line mutation found in constitutional DNA; 30 samples (86%) showed only this heterozygous mutation, while a second mutational hit was found in 5 tissues. Analyzing malignant degeneration, 3 out of 5 SO-related chondrosarcomas showed no additional somatic mutation, while in 2 primary tumour resections the loss of one copy of EXT1 or EXT2 gene was detected; the corresponding recurrence of this latter sample showed the loss of both EXT2 alleles. 5 out of 12 CHS resections derived from MO showed the presence of a second EXT1/2 somatic mutational hit; in the only available recurrence the heterozygous germline mutation was found at homozygous status due to the loss of the wt allele.

Our results show the absence of a second mutational hit in most of analyzed MO and SO samples suggesting that their growth may not necessarily require two EXT1/2 genetic alterations, which seems to be a common prerequisite for malignant transformation and tumour progression. All these evidences lead to the hypothesis of the presence of molecular mechanisms alternative to EXT inactivation in MO pathogenesis, as mutations/polymorphisms in EXT regulatory

sequences, post-transcriptional regulation pathways or involvement of other genes not belonging to EXT family.

Osteochondroma Formation: Haploinsufficiency or Two Hits?

Christianne MA Reijnders [1], Cathelijn JF Waaijer[1], Andrew Hamilton[7], Sander Dijkstra[2], John Ham[5], Egbert Bakker[3], Karoly Szuhai[4], Marcel Karperien[6], Pancras CW Hogendoorn[1], Sally E Stringer[7], and Judith VMG Bovée[1]

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Multiple osteochondromas (MO) is an autosomal dominant disorder caused by germline mutations in EXT1 and/or EXT2, whereas solitary osteochondroma is a non-hereditary lesion. EXT is involved in heparan sulfate biosynthesis. We investigated the controversial issue whether osteochondromas arise via the classical two-hit model for tumor suppressor genes or via haploinsufficiency.

Design: An *in vitro* 3D chondrogenic pellet model was used to compare heterozygous mesenchymal stem cells (MSCs)($EXT^{wt/-}$) of MO patients with normal MSCs and the corresponding tumor specimens (presumed $EXT^{-/-}$). EXT mutations and mRNA expression levels were assessed. HS chain length and structure of normal and heterozygous MSCs in monolayer culture was determined. Immunohistochemistry was performed on heparan sulfate (HS), heparan sulfate proteoglycans (HSPGs)(SDC2-4, perlecan, CD44v3), and HS dependent signaling pathways: TGFbeta/BMP (phosphosmad-1, phosphosmad-2, PAI-1), Wnt (beta-catenin) and PTHLH (PTHR1, bcl2).

Results: Germline EXT1 and EXT2 mutations were present in MO patients (6/8). We demonstrated a second hit in the EXT genes in 5 out of 8 osteochondromas (both solitary and hereditary). MSCs with a heterozygous EXT mutation are identical to wildtype MSCs with regard to HS chain length and structure, *in vitro* chondrogenesis and the expression of EXT and EXT downstream signaling molecules.

Conclusion: In conclusion, since I) a heterozygous EXT mutation does not affect chondrogenesis, heparan sulfate and downstream signaling pathways and II) we show a second hit in the majority of osteochondromas our results refute the haploinsufficiency theory and strongly support the two-hit model for osteochondroma formation.

Session 3

Biochemistry, Chemistry, and Cell Biology of Heparan Sulfate

Friday, October 30, 2009

13:30 – 14:00

EXT-Dependent Regulation of Heparan Sulfate Structure and Function

Cecilia Österholm[1], Marta Busse[2], Almir Feta[1] and Marion Kusche-Gullberg[1]

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Heparan sulfate is a complex polysaccharide that plays an important role in several cellular processes, including normal fetal development, wound healing and inflammation. Heparan sulfate chain elongation has been ascribed to a hetero-oligomeric complex of EXT1 and EXT2. Mutations in either EXT1 or EXT2 have been linked to the human disorder, hereditary multiple exostoses (HME), characterized by the formation of cartilage-capped bony outgrowths at the end of the long bones.

The individual functions of EXT1 and EXT2 in heparan sulfate chain elongation are currently unknown. EXT1 alone has the capacity to elongate heparan sulfate chains *in vitro*. The level of EXT2 protein modifies the catalytic properties of EXT1 but the role of EXT2 in heparan sulfate chain elongation is not clear. To understand the individual roles of EXT1 and EXT2, we have overexpressed the proteins or reduced their levels in mammalian cell systems and studied the effects of these manipulations on heparan sulfate structure. Our findings indicate that the levels of EXT1 and EXT2 influence heparan sulfate chain elongation.

Heparan sulfates are ubiquitously expressed in all tissues, where they function as adhesion molecules and co-receptors. Thus, they modulate cell-matrix interactions and growth factor signaling. We have previously shown that mouse embryonic fibroblasts with a gene trap mutation in *Ext1* have substantially reduced heparan sulfate chain length. We have now used these fibroblasts to investigate the functional consequences of the *Ext1* mutation for heparan sulfate-dependent growth factor signaling and for cell interactions with the extracellular matrix. Our results indicate that shorter heparan sulfate chains result in specific growth factor signaling defects as well as impaired fibroblast-matrix interactions.

New Advances in Heparan Sulfate/Heparin Analysis and Biosynthesis

Robert J. Linhardt

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The heparan sulfate/heparin family of glycosaminoglycans and proteoglycans has critical importance in physiology and pathophysiology as well as being a critical therapeutic agent. Disaccharide analysis is a first step in the analysis of glycosaminoglycans. Following tissue recovery of glycosaminoglycans or proteoglycans disaccharide composition can now be definitively determined on as little as 10^4 cells using liquid chromatography (LC)-mass spectrometry (MS) analysis. Oligosaccharide mapping affords critical structural information on sequence motifs the size of protein binding sites within glycosaminoglycans. Sequencing of these oligosaccharides is now possible using Fourier transform ion cyclotron resonance (FTICR) MS with electron detachment dissociation. MS analysis of full glycosaminoglycan chains, particularly ones with low sulfo group substitution levels, is now possible. Using preparative gel electrophoresis, the recovery of a single intact glycosaminoglycan for sequence analysis may now be possible.

Improved analytical technology and a better understanding of glycosaminoglycan and proteoglycan structure should help us establish structure-activity relationships (SAR). Analysis of the glycosaminoglycan-ome of embryonic stem cells as they differentiate down different lineages is resulting in important insights into heparan sulfate/heparin SAR. While signaling through cell surface heparan sulfate is clearly tied its primary structure or sequence, the precise relationship still remains unclear.

An alternative approach to understanding the structure of heparan sulfate/heparin is to better understand heparin biosynthesis in the Golgi. Two approaches currently underway in our laboratory to explore biosynthesis include the metabolic engineering of CHO cells to control heparan sulfate/heparin structure and the construction of an artificial Golgi on a digital microfluidic platform. These approaches should also provide an insight into the control of the outcome of biosynthesis in the Golgi.

Finally, research is underway to develop large-scale enzyme-assisted synthesis of heparan sulfate/heparin. This worked is directly aimed at replacing animal sourced pharmaceutical heparin with a bioengineered product. This work also offers an inexpensive approach to the synthesis of heparan sulfate/heparin oligosaccharides. These can also be made with the incorporation of isotopes, unnatural functional groups or reactive functional groups. These modified heparan sulfate/heparin oligosaccharides should greatly assist the study of the biology of this important family of molecules.

Elucidating the Functions of Heparan Sulfates: Towards Glycomics Strategies

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The structural diversity of heparan sulphates (HS) remains an enigmatic molecular puzzle, and the potential role of specific sequences is a complex and controversial issue. To address the central question of the extent of biological specificity of HS sequences, we have established a number of new experimental strategies aimed at developing glycomics strategies for studying HS. These include:

- Rapid isolation and purification of HS from tissues;
- Improved tags for high sensitivity disaccharide composition analysis using fluorescence detection;
- Generation of oligosaccharide libraries which can be exploited for binding studies and bioassay screening;
- Array methodologies for high throughput analyses including glycoarrays for probing binding specificities and "glycobioarrays" for screening biological responses;
- Improved methods for separation of HS oligosaccharides;
- Accurate sequencing methods using electrospray-mass spectrometry.

The application of these methods has yielded a variety of data that provide novel insights into the biological selectivity of HS structures, and demonstrate that chemical information encoded in the complex sulfation sequences of HS chains conveys functional specificity. Further application and extension of these strategies will permit development of glycomics scale approaches to decode the molecular basis of HS function in specific biological contexts.

Restoring Function to Heparan-Sulphate Deficient Cells

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Embryonic stem (ES) cell differentiation is dependent on the presence of heparan sulphate (HS). We have demonstrated that during differentiation, the evolution of specific cell lineages is associated with particular patterns of glycosaminoglycan (GAG) expression with, for example, different HS epitopes synthesised during neural or mesodermal lineage formation. Cells deficient in HS production (EXT1-/- and EXT1+/- ES cells) are able to be maintained as a stem cell population but are unable to differentiate normally to various lineages. We have been using these cells to understand how cells lacking cell surface HS coordinate multiple signalling pathways and have found that it is often the ability to switch off specific intracellular signals that is at fault in these cells. We have also observed that the addition of soluble GAG saccharides to cells with or without cell surface HS can influence the pace and outcome of differentiation, often correcting the deficiencies in intracellular signalling, again highlighting specific pattern requirements for particular lineages. We are combining this work with ongoing studies into the design of artificial cell environments where we have optimised 3D scaffolds, generated by electrospining or by the formation of hydrogels. By permeating these scaffolds with defined GAG oligosaccharides we can to control the mechanical environment of the cells (via the scaffold architecture) as well as their biological signalling environment (using the oligosaccharides). Initially, these scaffolds have been used to demonstrate that they can restore function to HS-deficient cells in vitro however; in the near future we hope to use these scaffolds in vivo to present defined GAGs to HS deficient cells with the aim of influencing cell behaviour. A focus of such studies will be the application of GAG-bearing scaffolds to the sites of exostosis removal with the aim of reducing the reoccurrence of exostosis formation and the need for further surgery.

Session 4

Studies Using Non-Mammalian Model Animals

Friday, October 30, 2009

15:50 – 17:30

Zebrafish as a Model for Multiple Hereditary Exostoses

Aurelie Clement, Malgorzata Wiweger and Henry H. Roehl

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As part of a large scale forward genetic screen for zebrafish developmental mutants, more then twenty alleles (representing >6 genes) were isolated that confer a "cartilage-tumour" phenotype. In collaboration with the Chein Laboratory (University of Utah, Salt Lake City), we have positionally cloned alleles for three of these genes and shown that they encode null mutations in Ext2, Extl3 and Papt1. We have shown the all three genes are required for synthesis of heparin sulphate (HS). Surprisingly, homozygous mutant embryos develop normally and have only been shown to have defects in four processes: pectoral fin development, axon sorting, osteoblast differentiation and cartilage morphogenesis. This is explained in part by the presence of maternally provided transcripts for these three genes in the early embryo.

In order to elucidate the roles of HS during skeletal development, we have analysed the skeletal defects of ext2-/- fish. During early development, nascent chondrocytes flatten and intercalate to give rise to a morphology that resembles a 'stack of pennies'. In ext2-/- embryos this process does not occur and chondrocytes remain round and disorganized. Early chondrogenesis proceeds normally indicating that HS is required during morphogenesis and not differentiation of the cartilage skeleton. Mutations in zebrafish wnt5a confer a similar phenotype, suggesting that HS acts with the non-cannonical Wnt pathway to establish cell polarity within pre-cartilage condensations. Both perichondral bone formation as well as chondrocyte hypertrophy are lost in ext2-/- mutants indicating that chondral ossification also requires HS. Finally, dermal bone ossification and expression of markers of osteoblast differentiation are reduced in ext2-/- larvae.

There are currently two models for the genetic mechanism that gives rise to osteochondromas in MHE patients: reduced gene dosage and loss-of-heterozygosity (LOH). One caveat in the LOH model is that HS is secreted and thus a homozygous mutant cells that arises may be rescued by neighbouring cells. To test this directly, we transplanted ext2-/- cells into wildtype fish and assayed chondrocyte polarity. In most cases, ext2-/- cells were rescued by neighbouring wildtype chondrocytes and stacked normally. However, in several cases the mutant chondrocytes behaved autonomously and formed an apolar cluster of cells on the edge of the cartilage. This data argues that mutant cells can loose polarity and that cells that lie on the edge of the cartilage may become autonomous because of the lack of contact with neighbouring wildtype cells.

Developmental Regulation of Heparan Sulfate Proteoglycan Synthesis

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In the fruitfly Drosophila, the genes *toutvelou* (*ttv*) and *sister of toutvelou* (*sotv*) encode GAG polymerase subunits orthologous to human EXT1 and EXT2 that are affected in Hereditary Multiple Exostoses. We isolated mutations in *sotv*, and showed that both Sotv and its partner co-polymerase Ttv, are essential for enzymatic activity. Importantly we found that mutations in *sotv* and *ttv* result in wide ranging developmental defects and impair the activity of 3 major signaling pathways – Hedgehog (Hh), Wnt and Bone Morphogenetic Protein (BMP) - in larval imaginal discs that give rise to adult tissues (Bornemann et al., 2004, *Development* 131, 1927-1938). This work highlighted the fact that consequences of mutations in the EXT genes are likely to reflect dysregulation of multiple growth factor signaling pathways, in contrast to previous studies that argued for a more restricted effect on Hedgehog signaling alone.

More recently we investigated how loss of *sotv* and *ttv* activity affects early embryonic patterning. Surprisingly, in the embryo the absence of GAG chains perturbs only Wnt/Wg and Hh but not BMP signaling. We found that GAG chain synthesis is under tight temporal regulation and HSPG core proteins are not glycosylated in the early embryo during the first 3 hours post-fertilization, when BMP activity specifies cell fates along the dorsal ventral axis. GAG chain synthesis is initiated an hour later when Wg and Hh signaling play critical roles. The absence of GAG modifications in the early embryo is unexpected since HSPGs are required for BMP signaling at other stages and argues that the mechanism by which a BMP activity gradient is established in the embryo is significantly different from that utilized in imaginal discs. The absence of detectable GAG chain polymerase activity in the early embryo was surprising because high levels of *ttv* and *sotv* transcripts are present at these stages. Examination of the genomic organization, expression data and cDNA clones revealed that these maternally provided transcripts have complex structures typically associated with mRNAs that are translationally regulated. Our data show that the mRNA structure of *ttv* is critical for regulating protein levels, suggesting a novel mechanism for regulating GAG chain synthetic activity. Importantly, this mechanism is likely to be evolutionarily conserved since we have identified similar complex 5' UTR in several human enzymes that play critical roles in GAG synthesis and modification (Bornemann et al., 2008, *Development* 135, 1039-1047).

Current studies indicate that the 5' UTR from human Ext1 can function as an IRES and direct regulated translation in Drosophila. This exciting finding argues that these regulatory mechanisms may be evolutionarily conserved, and that *cis*-elements and *trans*-acting factors identified in Drosophila could be relevant to understanding the basis of the human disease.

HS Fine Structure and FGF Signaling Pathways Converge at Cilia: Does Cilia Function Have a Role in HME?

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Several peptide growth factors, including members of the FGF, TGFb, BMP and Wnt families, have been implicated in the regulation of cartilage and bone growth. The functions of these factors are regulated in part by complex Heparan Sulfate Proteoglycans (HSPGs) at the cell surface. Alterations in genes involved in synthesis of Heparan Sulfate (HS) chains, as exemplified by mutations in EXT1 and EXT2, lead to Hereditary Multiple Exostoses (HME), perhaps by misregulating complex cell-cell signaling pathways. Heparan sulfate (HS) is an unbranched chain of repetitive disaccharides, typically attached to the core proteins of Heparan sulfate proteoglycans (HSPGs), which specifically bind peptide growth factors at the cell surface or secreted extracellularly. HS chains contain sulfated domains termed the HS "fine structure" which are catalyzed by HS O-sulfotransferases (OSTs).

Our working hypothesis is that there is a "Glycocode" embedded in the fine structure of HS, and that distinct members of the HS 3-OST family contribute to distinct signatures within this code, which then regulate specific cell-cell signaling pathways. Using gene knock-down screens in zebrafish, we have uncovered distinct roles for three members, 3-OST-5, 3-OST-6 and 3-OST-3Z expressed in the same cell lineages. Strikingly, both 3-OST-5 and 3-OST-6 are required for distinct functions of cilia, which are cell surface organelles found on most epithelia in vertebrates. Recently, cilia have been implicated as recipients of some cell-cell signaling pathways, for example, Hedgehog signaling. However, little is known about cell-cell signaling pathways that control the formation or function of cilia. We found that 3-OST-5 is required for normal cilia length, whereas 3-OST-6 controls cilia motility. Knockdown of a third 3-OST family member has normal cilia function. In collaboration with Jeff Esko's lab, we find that knockdowns of each of these three 3-OST family members cause a similar reduction of a 3-O-sulfated disaccharide subunit in the HS chains, so the differences in cellular phenotypes are not simply due to bulk changes in sulfation. Further analyses indicate that 3-OST-5 and 3-OST-6 modulate distinct cell-cell signaling pathways in cilia formation or function, presumably by distinct glycocodes.

Using several genetic, morpholino and pharmacological approaches, we have recently shown that fibroblast growth factor (FGF) signaling, via FGF8, FGF24 and FGF receptor1 (FGFR1), regulates cilia length and function in diverse epithelia during zebrafish and *Xenopus* development (Neugebauer et al., 2009 *Nature*). Strikingly, both FGFR1- and 3-OST-5-dependent pathways converge on the same Intraflagellar Transport (IFT) regulatory pathway to control cilia length. These results suggest a fundamental and highly conserved role for FGF signaling and 3-OST-5 in the regulation of cilia length in multiple tissues.

Given that cell signaling pathways and 3-OST-dependent fine structures converge on cilia formation and function, we propose that a subset of developmental disorders ascribed HSPG

misregulation, such as Hereditary Multiple Exostoses, might be due in part to altered cilia function.

New Aspects of Multiple Osteochondromas – A Lesson from *dackel (dak/ext2)* Zebrafish Mutant

M. Wiweger[1], Z. Zhao[1], R. van Merkesteyn[2], H. Roehl[3] and P.C.W. Hogendoorn[1]

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Multiple Osteochondromas (MO; previously known as HME/MHE) is characterized by the formation of cartilaginous bone tumors (osteochondromas) at multiple sites in the skeleton, bone curving, short stature, bursa formation and impingement of nerves, tendons and vessels. MO is also known to be associated with arthritis, general pain, scarring, increased risk of bone fracture and malignant transformation. MO patients present additional complains but the relevance of those needs validation. We use a zebrafish mutant called *dackel (dak)* as a model for MO. *dak* is mutated in a homologue of the human *EXT2* gene, and is the only Heparan Sulfate-deficient vertebrate being available that can complete embryo development when homozygous for a mutation in an *ext* gene. The *dak (-/-)* mutant displays such severe phenotypes that the identification of more settled defects is thus possible. Histological analysis revealed that the *dak (-/-)* mutant has very severe defects associated with the formation and the morphology of teeth. At 5 days post fertilization 100% of homozygote *dak/ext2* mutants had a single tooth formed at the end of the 5th pharyngeal arch, whereas wild-type fish had developed three teeth, located in the middle of the pharyngeal arch. *dak (-/-)* teeth had abnormal morphology (they were shorter and thicker than in the WT) and scattered ossification at the tooth base. Deformities such as spitted crowns and enamel lesions were found in 20% of the heterozygote *dak/ext2* adults. The *dak*-tooth morphology was partially rescued by a treatment with either FGFs or TGF that was added into fish water. The number of teeth was not affected unless a higher dosage of FGF8 was admitted locally. In order to validate our findings in zebrafish, a dental questionnaire was designed and sent out to the HME Research Foundation for further distribution among MO patients and their families. Until now, we have received 23 replies from MO patients, half of whom stated that they have malformed and/or displaced teeth with abnormal enamel; further indicating that MO might indeed be also associated with dental problems. The analysis of *dak* skeleton also revealed severe bone defects in all homozygote larvae and in 20% of heterozygote adult *dak/ext2* mutants. Bone malformations in *dak/ext2* mutants coincided with reduced expression of osteoblast markers and enhanced TRAP staining. This indicates that the remodeling of the skeleton might be altered in MO, and so patients might be at higher risk of developing osteoporosis. Skeletal and dental defects, similar to those present in *dak/ext2* mutant, were also observed in the *acerebellar (ace/fgf8)* zebrafish mutants. This observation suggests that FGF signaling might be a potential target for prophylactics in MO.

Session 5

Developmental Biology of Bone and Cartilage

Saturday, October 31, 2009

9:00 – 11:50

PTH/PTHrP Receptor Signaling is Required for Maintenance of the Growth Plate in Postnatal Life

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PTHrP (PTHrP), regulated by Indian hedgehog (Ihh) and acting through the PTH/PTHrP receptor (PPR) is crucial for normal cartilage development. These observations suggest a possible role for PPR signaling in the postnatal growth plate; however, the role of PPR signaling in postnatal chondrocytes is unknown, because embryonic lethality of PPR null mice does not allow examination of the physiological role of PPR signaling in postnatal chondrocytes. To overcome this issue, we have generated tamoxifen-inducible and conditional Coll2-CreERt:floxed PPR ($PPR^{fl/fl}$)-knockout mice to allow such postnatal analysis.

To generate *Coll2-CreERt:*$PPR^{fl/fl}$ mice, we crossed *Coll2-CreERt:*$PPR^{fl/fl}$ mice to $PPR^{fl/fl}$ mice*Coll2-CreERt:*$PPR^{fl/fl}$ and $PPR^{fl/fl}$ control mice were treated with single dose of 0.5 mg tamoxifen per body at postnatal day (P)3, and the effects on bone development were analyzed at P6, P7 and P10. Three days after treatment of tamoxifen, the columns of chondrocytes in the growth plate were disrupted, and the region of hypertrophic chondrocytes expressing collagen X was greatly expanded into the middle of the growth plate. Furthermore, histological analysis of tibia of P10 showed that the premature closure of the growth plate had already occurred in *Coll2-CreERt:*$PPR^{fl/fl}$ mice. To test the possibility that the deletion of PPR signaling could affect cell death of chondrocytes, we performed the TUNEL assay and immunohistochemistry for cleaved caspase-3 in growth plates. We observed ectopic TUNEL and activated caspase 3 positive cells in the columnar region of growth plate chondrocytes, along with an increase in apoptotic cells at the end of the hypertrophic layer of *Coll2-CreERt:*$PPR^{fl/fl}$ at 4 days after tamoxifen administration. Simultaneous administration of a low phosphate diet, that prevents apoptosis of late hypertrophic chondrocytes in normal growth plates, prevented the disappearance of the growth plate after tamoxifen administration.

These results suggest that activation of the PPR is required for continued survival of chondrocytes in the postnatal growth plate. Postnatal ablation of PPR in chondrocytes results in premature closure of the growth plates and permanent deformity of bone epiphyses. Moreover, chondrocyte apoptosis through the activation of a caspase 3 pathway may be involved in the process of growth plate closure by postnatal deletion of PPR in chondrocytes.

FGF Signaling in Skeletal Development and Repair

Kai Yu, Gregory Schmid, Mario Martinez, Jennifer McKenzie, Chikashi Kobayashi, Linda Sandell, Matthew Silva, David Ornitz

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Mutations in Fibroblast Growth Factor Receptors result in chondrodysplasia and craniosynostosis syndromes that affect both long bone and cranial bone development. These skeletal phenotypes underscore the essential role for FGF signaling in skeletal development. To investigate specific functions of FGFs, mouse models have been used to conditionally inactivate FGF receptors in different limb bud and skeletal compartments. These studies have identified unique roles for FGF signaling at almost every stage of skeletal development, beginning at the earliest stages of limb bud formation and continuing throughout embryonic skeletal development. More recent studies have revealed expression of FGF signaling molecules during postnatal bone growth and during the repair of skeletal fractures, suggesting additional roles for FGF signaling during adult skeletal growth, homeostasis and repair.

To address function of FGF receptors, conditional gene disruption is being used to target *Fgfr1* and *Fgfr2*. *Prx1-Cre* and *Dermo1-Cre* effectively target limb bud mesenchyme. Lineage tracing experiments show that these *Cre* genes target an osteo-chondro progenitor cell that will give rise to all chondrocytes and osteoblasts in long bones. Although use of these Cre genes to target FGF receptors suggest roles for FGF signaling during bone development, earlier phenotypes resulting from defects in the limb bud complicate this analysis. To identify specific roles for FGF receptors in at later stages of development, the *Osx-Cre* gene is being used to target *Fgfr1* and *Fgfr2,* in all osteoblasts and a subset of chondrocytes. *Osx-Cre* conditional knockout mice display, at birth, severe loss of intramembranous bone but surprisingly normal long bones. However, postnatally, long bone growth is significantly slowed. These studies show that FGF signaling is essential for normal osteoblast mediated postnatal bone growth.

To address functions of FGF signaling during fracture repair, we have developed two mouse models. Endochondral repair is induced with a stabilized tibial fracture model and woven bone repair is induced through an ulnar stress fracture model. Preliminary studies suggest that induction of FGF signaling during endochondral bone repair decreases chondrogenesis and leads to increased osteoid formation. Ongoing studies will evaluate the effects induced FGF signaling on bone strength following complete fracture healing.

Understanding the role of Ihh and proteoglycans interaction during bone development

Allard, P., Tabin, C.

Patrick Allard[1], Maria Pazyra[2], Rosalind Segal[2], Monia Zuntini[3], Luca Sangiorgi[3], Clifford Tabin[1] 1- Genetics, Harvard Medical School. Boston, MA. 2- Dana Farber Cancer Institute. Harvard. Boston, MA. 3- Rizzoli Orthopedic Institute**,** Bologna, Italy. e-mail: pallard@genetics.med.harvard.edu.

Heparan-sulfate proteoglycans (HSPGs) have recently been recognized as mediators of intercellular signaling during development (for review see Lin, 2004). During bone development specifically, loss of function of genes implicated in the synthesis of HSPGs, namely Ext1 and Ext2, show a clear defect in the patterning of the growth plate. Ext1 heterozygous or hypomorphic embryos display an increased chondrocyte proliferation and a delay in chondrocyte differentiation (Koziel et al, 2004; Hilton et al, 2005). At the molecular level, Ext1 mutant embryos show a reduced Ihh mRNA expression but an increased Ihh protein distribution (Koziel et al, 2004; Hilton et al, 2005). Consistent with that result, PTHrP mRNA levels, which is downstream of Ihh signaling, is up-regulated (Koziel et al, 2004). While these studies address the role of HSPGs during growth plate development, they do not specifically address the function of HSPGs in mediating Ihh signaling in that context. Our objective is to investigate the role of sulfated proteoglygans (SPGs) in mediating Ihh signaling during bone development. To this aim, we will specifically abolish the interaction of Ihh with SPGs.

The interaction of proteins with SPGs is mediated by a domain, termed the Cardin-Weintraub domain, consisting of a short stretch of basic amino-acid (Cardin and Weintraub, 1989). Examination of Ihh amino-acid sequence revealed the presence of a stretch of basic amino-acid highly conserved in identity and location with the described functional Cardin-Weintraub (CW) domain of Shh. Ihh CW domain is also completely evolutionary conserved from zebrafish to human. Next, we mutated Ihh CW domain *in vitro* and observed that although wild-type Ihh can bind heparin*,* mutation of Ihh CW domain strongly diminishes Ihh affinity for heparin sulfate and chondroitin sulfate proteoglycans. We also showed that Ihh requires its CW domain to bind efficiently to bone sections. Furthermore, Ihh binding to bone sections is also dependent on the presence of heparan-sulfate chains. We are currently generating a mouse knock-in carrying a mutation of the CW domain in Ihh. Finally, in collaboration with Dr Luca Sangiorgi, we are also screening human patients that present chondrodysplasia phenotypes consistent with a mutation in Ihh CW domain.

Altered Matrix at the Chondro-Osseous Junction Leads to Defects in the Hematopoietic Stem Cell Niche

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During endochondral ossification (EO), hypertrophic cartilage blueprints define all subsequent skeletal elements where a hematopoietic marrow forms. A link between altered endochondral skeletogenesis and aberrant hematopoiesis in the marrow was first established by the skeleto-hematopoietic disease phenotype of mice transgenic (Tg; AJP 160:2019, 2002) or null (KO; JCB 149:983, 2000) for collagen X, a hypertrophic cartilage-specific matrix protein. In the collagen X mice, skeletal disease manifestations involve both hypertrophic cartilage and trabecular bone, comprising the chondro-osseous junction (coj); hematopoietic aberrations include marrow hypoplasia, altered B lymphocyte profile throughout life and both inflammatory and hematopoietic cytokine mis-expression (Dev Dyn 237:2693, 2008). Of note, many of these altered cytokines bind heparan sulfate proteoglycans (HSPGs), which are reduced or lacking in the coj of the collagen X mice. In vitro ELISAs and in vivo parasite challenge confirmed an impaired immune response in all subsets of collagen X Tg and KO mice. To assess if these hematopoietic changes are due to defects in the hematopoietic stem cell (HSC) population or in the HSC niche environment, neonatal bone marrow transplantations and in vitro co-culture assays were used. These assays confirmed that the hematopoietic defects in the collagen X mice result from an altered EO-derived coj environment. We present a hypothesis depicting an EO-derived HSC niche where we propose that the collagen X/HSPG network sequesters hematopoietic cytokines and growth factors at the coj; disruption of collagen X function and the collagen X/HSPG network causes an imbalance in cytokine metabolism, leading to impaired hematopoiesis and immunity.

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Metachondromatosis: Expanding the Clinicopathologic Spectrum

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Metachondromatosis is a rare autosomal-dominant disorder in which patients develop multiple benign cartilaginous tumors in childhood. The primary tumor is an osteochondroma-like exostosis that, unlike an osteochondroma, usually occurs in the digits, points towards the affected joints, and may regress spontaneously. The enchondromas, in distinction from those in enchondromatoses, most often occur in the iliac crests and metaphyses of long bones. Patients may also develop periarticular calcifications as well as avascular necrosis of the femoral head. Fewer than 50 cases of metachondromatosis have been reported and the disorder has not been mapped in the human genome. Reported cases have not provided sufficient detail to define the pathology of these unique tumors. Metachondromatosis shares features with the autosomal dominant disorder, Multiple Hereditary Exostoses (MHE), in which patients develop multiple osteochondromas in the long bones. Loss of function mutations in two genes, *EXT1* and *EXT2*, involved in heparin sulfate polymerization, have been associated with HME, but not in the only published metachondromatosis patient examined. We studied additional cases of metachondromatosis in order to expand our understanding of the clinical syndrome and, in particular, to define the histopathology of this rare syndrome. The lesions were compared with normal growth plates as well as with 50 osteochondromas, both sporadic and syndromic, from age-matched controls.

We identified and reviewed 16 osteochondroma-like exostoses, which we have called metachondromas, excised from three metachondromatosis patients followed at Children's Hospital Boston in the last 20 years. Two patients were related as second cousins and the third patient was from an unrelated family. All three lacked mutations in *EXT1* and *EXT2*. The majority of tumors were removed from the digits (11/16), six from the hands and five from the feet, and most often affected the proximal phalanx. Five were removed from the long bones, two from the distal femur and three from the distal tibia. All excised lesions were symptomatic, most obstructing joint function. Unlike the metaphyseal-based osteochondromas, excised metachondromas extended from the metaphysis to the epiphysis, spanning the physis (11/16), or were exclusively epiphyseal (5/16). At least two lesions were found to resolve with age, while others, including adjacent lesions, grew as the patients aged. All patients had enchondromatous lesions on the digits and distal long bones, as well as multiple new exostoses that continued to develop into adolescence that were not resected. One patient developed avascular necrosis of the femoral head in childhood.

The histologic features of metachondromas are unique. All were covered by an outer fibrous capsule and most (14/16) contained only a partial hyaline cartilage cap with underlying bone. Unlike in osteochondromas, the predominant cartilage mass was within a central core. The cap of both lesions was reminiscent of the growth plate, but more disorganized with prominent proliferative and hypertrophic chondrocytes and incomplete zonation forming bone by endochondral ossification. The cartilaginous core of metachondromas was even more

hypertrophic and less organized with irregular or absent columns. Entrapped growth plate was observed in some cases. Mild hypercellularity and binucleate chondrocytes were common, but clustering was not. The cells were mildly pleomorphic with a large nucleus and a single prominent nucleolus. Excessive myxoid degeneration of the matrix was common and islands of necrotic cartilage were present in several tumors. Unlike osteochondromas, metachondromas primarily form bone on the outer surface of the central cartilage rather than beneath a cartilage cap, although the latter process also occurs. In summary, we found that the exostoses of metachondromatosis shared some features with osteochondromas, but have distinct clinical, radiographic, gross and histologic findings to warrant separate a classification, which we have called the metachondroma.

Session 6

Role of Heparan Sulfate in Bone Development / Novel MHE Mouse Models

Saturday, October 31, 2009

13:00 – 15:20

Mechanisms of Exostosis Formation in Mouse Models of HME

Maurizio Pacifici[1] and Jeffrey D. Esko[2]

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The mechanisms by which exostoses form along the growth plates of long bones and other skeletal elements remain largely unclear. Since the majority of HME patients carry loss-of-function mutations in *Ext1* or *Ext2*, other groups previously created heterozygous null *Ext1* (*Ext1*$^{+/-}$) mice that were expected to display traits of HME patients. Surprisingly, the mutant mice did not completely mimic the human phenotype. Exostosis-like masses were observed in 10-20% of the mice only, and the ectopic masses were rather small and atypical in organization and were limited to the ribs. Based on immunohistochemical evidence that human exostoses contain far less heparan sulfate (HS) than would be expected of a heterozygous *Ext* mutation (about 50% of control levels), we reasoned that mice producing lower amounts of HS chains may be able to mimic the human condition more closely. Thus, we created and examined double heterozygous *Ext1*$^{+/-}$/ *Ext2*$^{+/-}$ mice. Indeed, the double hets mice did display stereotypic exostoses along their long bones that were characterized by a distal cartilaginous cap followed by a pseudo growth plate and were oriented at a 90° angle with respect to the long axis of the long bones. We even observed osteochondromas masses at other locations. The data strongly indicate that exostosis formation and organization are intimately sensitive to, and dependent on, HS production and/or content and that frequency of exostosis formation can be increased by progressive decreases in *Ext* expression. At the Conference, we will present data from an additional mouse model of HME and data from mesenchymal cell cultures that provide important insights into the mechanisms of exostosis induction and formation.

Osteochondromagenesis: Somatic Loss of Heterozygosity Modeled via *Cre*-Mediated Inversion of the Second Exon of *Ext1* in Chondrocytes

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Multiple hereditary exostoses (MHE) is caused by heterozygosity for mutations in *EXT1* or *EXT2*. Osteochondroma pathogenesis in this disorder remains elusive. Mice homozygous null for homologous *Ext1* or *Ext2* die prenatally; only rarely will a heterozygote form a rib osteochondroma-like excrescence. Origin of osteochondromas in the physeal chondrocytes or adjacent perichondrium is also unclear.

Gene-targeting generated a unique conditional allele of *Ext1* with *trans*-orientation *loxP* sites flanking exon 2. Mice bearing this *Ext1*^{e2neofl} were crossed with mice bearing transgenic *Cre*-recombinase driven by a doxycycline-inducible collagen IIa1 promoter (C*ol2-rtTA-Cre*). Doxycyline was administered during week 2 of life. Mice were sacrificed for phenotypic analysis at 4, 6, and 10 weeks. Phenotypic analysis included histology of ribs and knees and microCT. Crossing to mice bearing *Cre*-recombinase driven by *Osterix* expression and activated by tamoxifen (*Osx-CreERT*) at P8 tested competing cells of origin.

PCR from cartilage containing tissues demonstrated both forward- and reverse-orientation exon 2 in *Ext1*$^{e2neofl/e2neofl}$;C*ol2-rtTA-Cre* mice after receiving doxycycline. Homozygotes lacking *Cre* and heterozygotes with *Cre* had no demonstrable phenotype. Homozygotes with induced *Cre* consistently developed numerous osteochondromas. *Ext1*$^{e2neofl/e2neofl}$;*Osx-CreERT* mice, in contrast, formed no osteochondromas.

Reversible *Cre*-mediated inversion of a *trans*-floxed genomic fragment results in a 50:50 distribution of forward and reverse orientation alleles and a small fraction of cells with homozygous disruption. This genetic recapitulation of somatic loss of heterozygosity of *Ext1* in physeal chondrocytes generated numerous osteochondromas, when heterozygosity for a null-allele failed to. This argues that loss of heterozygosity is critical to the phenotypic expression of MHE. That osteochondromas do not form when *Ext1* disruption is induced in pre-osteoblasts argues for a proliferating chondrocytes as the cell of origin.

Molecular Characterization of Osteochondroma Development in Mice

Virginia Piombo, Kevin B. Jones, Charles Searby, Gail Kurriger, Florian Grabellus, Peter Roughley, Jose A. Morcuende, Joseph A. Buckwalter, Mario R. Capecchi, Val C. Sheffield, Andrea Vortkamp

Hereditary multiple exostoses syndrome (HME) is a dominant inherited human disorder characterized by short stature and exostoses (osteochondromas) of the growth plat. HME results from mutations in EXT1 or EXT2, which encode glycosyltransferases necessary for the synthesis of heparansulfate (HS). Investigation of hypomorphic Ext1 ($Ext1^{gt/gt}$) mice demonstrated that reduced HS levels lead to an increased range of Ihh signaling and consequently to a delay in hypertrophic differentiation of chondrocytes. Here we have analyzed chimeric mice, in which clones of Ext1 mutant cells have been induced by Doxycycline-dependent exon inversions. We show that, in contrast to heterozygous deletion of Ext1, these mice develop exostoses of the axial skeleton. On morphological level later stages of exostoses resemble that of human patients with a bony shaft connected to the bone of the affected skeletal element. On molecular level the en of chondrocyte markers indicated that the cell organization of the cartilaginous cap of the osteochondroma mimics that of the of the growth plate. Analysis of early exostoses stages revealed that the origin of the osteochondroma is an early chondrocyte. We have investigated the genotype of the osteochondroma cells by genomic PCR of small clones of cells. We found the majority of cells to be homozygous for the mutant allele. These are also negative for the production of HS indicaing that loss of heterozygosity is required for osteochondroma development. Nevertheless we found a subset of wildtype cells in early exostoses stages indicating that these cells might be incorporated into the osteochondroma tissue.

Stochastic Conditional Knockout of *Ext1* Reveals an Unexpected Relationship between Biallelic Inactivation of the Gene and the Development of Multiple Exostoses

Kazu Matsumoto[1], Fumitoshi Irie[1], Susan Mackem[2], and Yu Yamaguchi[1]

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Individuals with MHE carry heterozygous loss-of-function mutations of *Ext1* or *Ext2*, which together encode an enzyme essential for heparan sulfate synthesis. Despite the unambiguous identification of causative genes, there are a number of enigmatic issues and unanswered questions surrounding MHE. Among them, three questions are of particular interest: (i) whether osteochondroma in MHE is a true neoplasm or a developmental defect; (ii) whether loss of heterozygosity is the underlying genetic mechanism of MHE; and (iii) why $Ext1^{+/-}$ mutant mice, which faithfully mimic the genotype of human MHE, are resistant to osteochondroma formation, especially in long bones.

To test the hypothesis that biallelic inactivation of *Ext1* occurring in a small fraction of chondrocytes is the pathogenic mechanism of MHE, we employed a method of stochastic inactivation of loxP-flanked *Ext1* alleles ($Ext1^{flox}$) using a tamoxifen-dependent Cre transgene driven by the *Col2a1* promoter ($Col2\text{-}Cre^{ERT}$). We originally intended to control the level of recombination using different doses of tamoxifen. Unexpectedly, $Col2\text{-}Cre^{ERT};Ext1^{flox/flox}$ mice developed multiple osteochondromas and other MHE-like bone deformities without tamoxifen treatment. We found that the non-induced $Col2\text{-}Cre^{ERT}$ transgene drives stochastic recombination in a small fraction of chondrocytes (~5% in long bones). ($Col2\text{-}Cre^{ERT};Ext1^{flox/flox}$ mice that are raised without tamoxifen treatment are designated as *Ext1*-SKO [stochastic knockout] mice.) The penetrance of the long bone exostosis phenotype in *Ext1*-SKO mice was 100%, whereas bowing deformity and subluxation of the radius and scoliosis were observed in 92% and 58% of *Ext1*-SKO mice, respectively. In contrast, neither heterozygous *Ext1*-SKO mice (i.e., $Col2\text{-}Cre^{ERT};Ext1^{flox/+}$) or $Prx1\text{-}Cre;Ext1^{flox/+}$ mice developed these phenotypes at all, supporting the requirement for biallelic inactivation. Surprisingly, osteochondromas (cartilage cap region) developed in *Ext1*-SKO mice are not clonal growths of *Ext1*-null chondrocytes, but mixtures of *Ext1*-null and wild-type chondrocytes at highly variable ratios. This heterogeneous nature of osteochondroma might be a part of the reason why previous studies on loss of heterozygosity have not generated an unequivocal conclusion. Our results indicate that, although biallelic inactivation of *Ext1* is required for its *initiation*, chondrocytes comprising osteochondroma are not clonal, and therefore osteochondroma is not considered to be a neoplasm in its strictest sense. Our results also suggest that *Ext1*-null chondrocytes exert unexpectedly potent cell non-autonomous effects on the behavior of wild-type chondrocytes. This mouse model provides novel insight not only into the genetic mechanism of MHE but also how heparan sulfate controls tissue development.

Session 7

Role of Heparan Sulfate in Non-Skeletal Tissues

Saturday, October 31, 2009

15:40 – 17:10

Do Mutations in EXT1 or EXT2 Affect Non-Skeletal Tissues?

Jeffrey D. Esko

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Heparan sulfate proteoglycans reside on the plasma membrane of virtually all animal cells studied to date and represent major components of extracellular matrices. Studies of model organisms and human diseases demonstrate their importance in development and normal physiology. A recurrent theme is the electrostatic interaction of the heparan sulfate chains with protein ligands, which affects metabolism, transport, information transfer, support and regulation in all organ systems studied to date (Bishop et al., 2007). The importance of these interactions is exemplified by phenotypic studies of mice and humans bearing mutations in the core proteins or the biosynthetic enzymes responsible for assembly of the heparan sulfate chains. Most of these conclusions have been based on homozygous mutations, which lead to profound alterations in heparan sulfate structure. Hereditary Multiple Exostoses (HME) is caused by autosomal dominant mutations in genes that code for subunits of the heparan sulfate copolymerase, EXT1 and EXT2. In most tissues studied to date, heterozygous mutations in either gene results in truncated chains. However, the phenotype associated with these mutations appears to be restricted to the cartilage growth plate, which is somewhat surprising given the biological importance of heparan sulfate in other tissues. Recent studies of heparan sulfate in several systems will be discussed, including studies of vascular permeability, lipoprotein metabolism mediated by vascular and hepatic proteoglycans, and microbial infection. Further phenotypic analyses of patients and model organisms are needed to determine if truncation of the chains caused by etiological mutations in EXT1 or EXT2 result in changes in physiology and metabolism.

Deficient Heparan Sulfate and N-Glycosylation Contribute to Protein-Losing Enteropathy in Humans and Mice

Hudson Freeze

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Protein losing enteropathy (PLE), the loss of plasma proteins through the intestine, occurs in Congenital Disorders of Glycosylation (CDG) and long after corrective heart surgery (Fontan procedure) in some children. We believe that environmental insults (inflammatory challenge and increased venous pressure) precipitate PLE in genetically at-risk patients. Curiously, these PLE patients lose heparan sulfate (HS) from the intestinal epithelial cells, but it returns when PLE subsides. We wanted to model PLE in epithelial cells, intestinal explants, and mice to understand how N-glycosylation and HS loss contribute to PLE. Results from cell monolayers, mucosal explants, and from mice were fully consistent. Mice lacking HS on their intestinal epithelial cells increase enteric protein loss, which is greatly compounded when venous pressure and/or TNFa/IFNg challenge are also imposed. HS normally blunts cytokine effects by preventing cytokine binding and/or signaling that destabilizes tight junctions allowing protein leakage. Non-anticoagulant heparin reverses protein loss in HS-deficient cells and mice. Heparin injections also reverse PLE in post-Fontan and CDG patients. To determine effects of reduced N-glycosylation, we knocked down phosphomannose isomerase (PMI/*Mpi*, Fru-6-P <--> Man-6-P) in epithelial cells. Protein leakage increased and cytokine challenge further exacerbated it. However, HS loss and PMI deficiency were additive, suggesting that impaired N-glycosylation does not cause HS loss. We have now made a PMI hypomorphic mouse containing a patient mutation. This line also shows increased enteric protein leakage in feces and in through mucosal explants. A combination of HS loss and N-glycosylation deficiency increases intestinal permeability leading to enteric protein leakage. Non-anticoagulant heparin may be therapeutic for PLE. Clinical trials are in early stages.

Supported by NHLBI, the Children's Hearts Fund, and the March of Dimes Foundation.

Deficiency of Heparan Sulfate in Excitatory Neurons Causes Autism-like Behaviors in Mice

Fumitoshi Irie and Yu Yamaguchi

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The role of heparan sulfate (HS) in mammalian brain development is well established. The function of HS in the brain, however, is not limited to development. HS is highly concentrated in synapses in the adult brain and the HS proteoglycan syndecan-2 controls the formation of dendritic spines, the key postsynaptic structure of excitatory neurons. Electrophysiologically, enzymatic elimination of HS has been shown to impair the expression of hippocampal synaptic plasticity. AMPA-class glutamate receptors have been shown to bind heparin. Considering these biological observations suggesting a synaptic function for HS, it is of interest that there are reports, both scientific and anecdotal, that MHE is sometimes associated with neurological (such as generalized pain) and mental (such as autistic traits) conditions.

To study the role of HS in adult brain function and behavior, we generated conditional *Ext1* knockout mice specifically targeted to postnatal excitatory neurons using a CaMKII-Cre transgene. This particular CaMKII-Cre transgene drives recombination specifically in forebrain excitatory neurons starting only after P14. This property allows us to essentially rule out developmental defects of the brain as the cause of possible physiological or behavioral phenotypes. Indeed, extensive histological analyses revealed no detectable abnormalities in the cytoarchitecture of the brain of *CaMKII-Cre;Ext1*$^{flox/flox}$ mice. Moreover, *CaMKII-Cre;Ext1*$^{flox/flox}$ mice have normal visual, olfactory, and motor functions.

Intriguingly, *CaMKII-Cre;Ext1*$^{flox/flox}$ mice displayed an array of behavioral deficits that are relevant to human autism, namely: (i) impairment in social activities, such as reduced social interaction with littermates of the same genotype and the avoidance of unfamiliar wild-type mice; (ii) reduced fear of physical danger; (iii) stereotyped behavior; (iv) hyperlocomotion; and (v) hypersensitivity to certain types of sensory stimuli. Neuronal activation following social or fear stimulation, as assayed by immunodetection of rapid induction of c-Fos, was attenuated in the amygdala of *CaMKII-Cre;Ext1*$^{flox/flox}$ mice. Electrophysiological analysis revealed that AMPA glutamate receptor-mediated excitatory postsynaptic activity is reduced in the basal amygdala pyramidal neurons of *CaMKII-Cre;Ext1*$^{flox/flox}$ mice. Surface expression of AMPA receptors is decreased in the *Ext1*-null primary neurons, which is restored by reintroduction of *Ext1* by transfection. Mice carrying heterozygous inactivation of *Ext1* in excitatory neurons (*CaMKII-Cre;Ext1*$^{flox/+}$) or in the entire brain (*Nestin-Cre;Ext1*$^{flox/+}$) displayed a partial behavioral phenotype. Our results demonstrate that HS plays a physiological role in the regulation of synaptic transmission, and that its elimination from synapses results in electrophysiological and behavioral deficits. These results also suggest that the anecdotal information known among families with MHE patients regarding the frequent association of autistic and Asperger-like traits may be true, and that the mental aspect of MHE would require a systematic scientific study.

Session 8

Biology of MHE and Related Bone Disorders

Sunday, November 1, 2009

9:00 – 12:15

Gli2 and p53 Cooperate to Regulate IGFBP-3–Mediated Chondrocyte Apoptosis in the Progression from Benign to Malignant Cartilage Tumors

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Clinical evidence suggests that benign cartilage lesions can progress to malignant chondrosarcoma, but the molecular events in this progression are unknown. Mice that develop benign cartilage lesions due to overexpression of Gli2 in chondrocytes developed lesions similar to chondrosarcomas when also deficient in p53. Gli2 overexpression and p53 deficiency had opposing effects on chondrocyte differentiation, but had additive effects negatively regulating apoptosis. Regulation of *Igfbp3* expression and IGF signaling by Gli and p53 integrated their effect on apoptosis. Treatment of human chondrosarcomas or fetal mouse limbs explants with IGFBP3 or by blocking IGF increased the apoptosis rate, and mice expressing Gli2 developed substantially fewer tumors when also deficient for *Igf2*. IGF signaling meditated apoptosis regulates the progression to malignant chondrosarcoma.

Although molecular mechanisms responsible for the progression of benign to malignant tumors of epithelial origin have been identified, they have not been demonstrated in mesenchymal tumors. Here we used a mouse model of enchondromatosis to show that p53 deficiency can cause chondrosarcomas to arise from benign lesions. An unexpected role for IGFBP3 in this progression was found. Human cartilage tumors have low levels of *IGFBP3* expression compared to normal chondrocytes, with chondrosarcomas having lower levels than benign lesions, suggesting *IGFBP3* level as a prognostic factor in cartilage tumors. Furthermore, IGFBP3 treatment or IGF signaling blockade increased chondrosarcoma apoptosis, suggesting a therapeutic approach to chondrosarcomas, a tumor for which there is no universally effective chemotherapy.

Primary Cilia Organization Orchestrating Cell Polarity in the Growth Plate and its Loss in Osteochondroma

C. de Andrea, M. Wiweger, F. Prins, J.V.M.G. Bovee, S. Romeo, P.C.W. Hogendoorn

The growth plate (GP) is a cartilaginous template for the elongation of long bones. In its regulation heparan sulphate proteoglycan (HSPG) and primary cilia (PC) play a role. Impaired HSPG biosynthesis is associated with osteochondroma (OC) formation. PC function as cell's antennas that receive and transduce mechanical and chemical signals from the surrounding cells and the extracellular matrix. We evaluated the organization of PC in GP and OC and its relation with cell polarity. The constituting cells of the human GPs (n=5) and OCs (n=5) had PC as documented by confocal microscopy using anti-acetylated α-tubulin antibody. The PC assembly was similar in both as judged by electron microscopy and the immunolocalization of KIF3A motor protein and γ-tubulin. The PC organization in the GP reflected that chondrocytes non-polarized (resting chondrocytes) became polarized (proliferating and hypertrophic chondrocytes) orienting the cilium parallel to the longitudinal axis of the bone. The PC alignment formed one virtual axis which crossed the center of column of chondrocytes. The ciliary axes showed the polarity axis of the GP. In OC, PC were randomly located in the central or in the lateral-medial region of the cells related to the growth axis of the tumor. The PC organization in OC reflected loss of cell polarity that seemed to be a key event and might indicate impaired cell movement or problems in cell-matrix interaction. We also demonstrated the dynamicity of PC whose presence/absence was correlated with the cell cycle.

Osteochondromas & the FOP Metamorphogene

Kaplan FS, Deirmengian GK, Chakkalakal S, Shore EM

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Sudden developmental novelty in highly conserved tissue repair mechanisms can lead to catastrophic medical consequences. A recurrent missense mutation in the gene encoding activin receptor A type I/Activin-like kinase 2 (ACVR1/ALK2), a bone morphogenetic protein type I receptor, creates a novel metamorphogene (ACVR1 c.617G>A;R206H) that causes fibrodysplasia ossificans progressiva (FOP) (1,2). FOP is a rare and disabling autosomal dominant disorder that causes a plethora of pathologic processes including dysregulated morphogenesis, abnormal tissue repair, skeletal metamorphosis, degenerative joint disease, and osteochondromas (3).

Among the least explored functions of the FOP metamorphogene is its ability to stimulate osteochondromas. Osteochondromas are associated with dysregulated BMP signaling and have been considered an atypical feature of FOP, but they may be under-diagnosed because of their often asymptomatic nature (4). A recent study showed that ninety per cent of all FOP patients had osteochondromas of the proximal tibia, and nearly one hundred per cent of all classically affected FOP patients had one or more asymptomatic osteochondromas at other sites (5). Emerging animal models of FOP also confirm these findings (6). Osteochondromas are thus a common phenotypic feature of FOP, a finding that expands the recognized consequences of the FOP metamorphogene to include not only skeletal malformations and skeletal metamorphosis, but also benign osteochondral neoplasms. The FOP metamorphogene dysregulates a highly conserved signaling pathway that has important implications for developmental and regenerative medicine (7).

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On the Clinical Manifestation and the Genetics of Ollier Disease

T.C. Pansuriya, S.H.M. Verdegaal, P.C.W. Hogendoorn, A.H.M. Taminiau, and J.V.M.G. Bovee

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Enchondromatosis is a non-hereditary disease, characterised by the presence of multiple enchondromas with a marked unilateral predominance mainly affecting medulla of the metaphyses and diaphyses of short and long tubular bones of the limbs, especially the hands and feet. The risk of malignant transformation is suggested to be up to 35%. Due to the rarity of these diseases, systematic studies on clinical behaviour providing information how to treat patients are lacking. Also, studies to the genetic cause of Ollier disease are sparse and hampered by low numbers. PTHR1 mutations can be found in only a small proportion (so far 5 of 62) of cases.

We performed a descriptive retrospective EMSOS (European Musculoskeletal oncology Society)-study in which 12 institutes in eight countries participated. 118 patients with Ollier Disease and 15 patients with Maffucci Syndrome were included. Unilateral localization of disease was found in 60% of Ollier patients and 40% of patients with Maffucci Syndrome. One of the predictive factors for developing chondrosarcoma is the location of the enchondromas; the risk increases especially when enchondromas are located in the scapula (33%), humerus (18%), pelvis (26%) or femur (15%). For the phalanges, this risk is 14% in the hand and 16% in the feet. The decision whether or not to perform extensive surgery is difficult, especially in patients who suffer multiple chondrosarcomas. Malignant transformation was found in forty-four patients with Ollier Disease (37%) and eight patients with Maffucci Syndrome (53%). Dedifferentiated chondrosarcoma was not found. Multiple synchronous or metachronous chondrosarcomas were found in 15 patients. Nine patients died (range 21-54 yrs). Seven of them died disease related due to pulmonary metastasis (2 humerus, 2 pelvis, 3 femur). Two patients died from glioma of the brain.

To study the genetic background of Ollier disease we performed SNP analysis using Affymetrix SNP6.0 on 15 enchondromas and 24 chondrosarcomas of different grades from 30 Ollier patients and normal DNA from 12 Ollier patients for paired comparison. We studied tumour tissue since we hypothesized that Ollier disease is a mosaic condition, since it affects multiple bones with an often unilateral predominance. All samples were divided into three groups: normals, enchondromas and chondrosarcomas. The number of numerical genomic changes in the chromosomes were not different for the enchondromas ($p=0.36$) while large genomic aberrations were seen in chondrosarcomas as compared to normals ($p=0.01$). Results are analyzed using R, Partek and Nexus. No common region of Loss of Heterozygosity (LOH) was shared between all enchondromas. LOH at 6p, 9q and 13q is shared by a proportion of chondrosarcomas. A list with candidate genes was generated combining the SNP data with expression profiles generated using Illumina Bead Array and these genes are currently being validated. Enchondromas located in the phalangeal bones of the hands were genetically different from enchondromas located in the long bones. In summary the absence of common copy number variations or loss of heterozygosity suggests that instead point mutations or epigenetic mechanisms seem to play a role in the origin of Olllier disease. Mutation analysis revealed absence of the reported G121E, A122T, R150C and R255H variations in PTHR1 in our series. In addition, mutations in NDST1, the first

modification enzyme of the HS polymer which was recently suggested to compete with EXT1 for binding to EXT2, were also absent.

The MHE Research Foundation

Wings of HOPE as we REACH for the
CURE to Multiple Hereditary Exostoses
www.MHEResearchFoundation.org

The MHE Research Foundation is a nonprofit organization for the support of Researchers, Physicians and Families dealing with, (MHE) Multiple Hereditary Exostoses/(MO) Multiple Osteochondroma, a rare genetic bone disease.

The MHE Research Foundations five point mission is to REACH, advance & support the following.

RESEARCH, to help support researchers in order to one day discover a treatment /cure for MHE. Our foundation works hand in hand with researchers from around the world in this mission.
EDUCATION, to provide vital clinical information/guides benefiting both families and physicians.
ADVOCACY, to bring awareness about this disease throughout the world.
CLINICAL, to help provide resources to families enabling them to locate the medical care they require.
HOPE, the research being conducted on MHE and the informational resources will bring a better quality of life to the families affected by this disease.

Multiple Hereditary Exostoses ("MHE") is a genetic bone disorder in which benign cartilage-capped tumors (exostoses or osteochondromas) grow from the growth plates of long bones or from the surface of flat bones throughout the body. These exostoses can cause numerous problems, such as: compression of peripheral nerves or blood vessels; irritation of tendons and muscles resulting in pain and loss of motion; skeletal deformity; short stature; limb length discrepancy; chronic pain and fatigue; mobility issues; early onset arthritis; and an increased risk of developing chondrosarcoma. MHE is an autosomal dominant genetic disease and patients have a 50% chance of passing this disorder on to their children.

It is not uncommon for MHE patients to undergo numerous surgical procedures throughout their lives to remove painful or deforming exostoses, to correct limb length discrepancies or to improve range of motion. Surgery, physical therapy and pain management are currently the only options available to MHE patients, but their success varies from patient to patient and many struggle with pain, fatigue and mobility problems throughout their lives.

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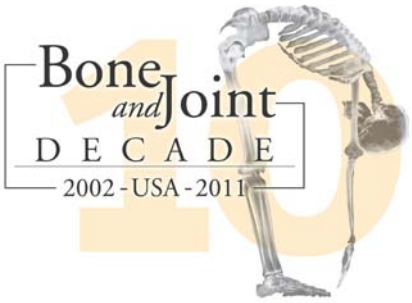
The MHE Research Foundation is proud,
to be a participating member of the
United States Bone and Joint Decade (USBJD) and the
(USBJD) Rare Bone Disease Patient Network



RARE BONE DISEASE PATIENT NETWORK