Multiple Hereditary Exostoses (MHE):

Elucidating the Pathogenesis of a Rare Skeletal Disorder Through Interdisciplinary Research

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Summary

An interdisciplinary and international group of clinicians and scientists gathered in Philadelphia, PA several months ago to attend the 4th International Research Conference on Multiple Hereditary Exostoses (MHE), a rare and potentially severe skeletal disorder also known as Multiple Osteochondromas (MO) or Hereditary Multiple Exostoses (HME). The large majority of MHE cases are caused by autosomal dominant mutations in *EXT1* or *EXT2*, genes that encode Golgi-associated glycosyltransferases responsible for heparan sulfate (HS) synthesis. The HS chains are key constituents of cell surface- and extracellular matrix-associated proteoglycans that include syndecans, glypicans and perlecan. Affected individuals are thus HS-deficient, can display skeletal growth retardation and deformities, and consistently develop benign, cartilage-capped bony outgrowths (termed exostoses or osteochondromas) near the growth plate of skeletal elements such as long bones, ribs and vertebrae. In up to 2% of the patients, the exostoses progress to malignancy, becoming peripheral chondrosarcomas. Current treatments are limited to surgical removal of symptomatic exostoses. There are no definitive treatments to inhibit further formation and growth of exostoses, prevent transition to malignancy, or address other medical problems often experienced by MHE patients, including chronic pain. Thus, the goals of the Conference were to assess the current understanding of MHE pathogenesis, identify key gaps in information, envision possible future therapeutic targets and strategies and discuss ways to test and implement them. To stimulate and advance MHE research, the Conference also brought together scientists and clinicians from the closely related biomedical research fields of glycobiology, skeletal developmental biology, cell signaling, invertebrate development, and pediatric skeletal dysplasias. This report provides a description and assessment of the exciting and promising findings in MHE and related fields presented at the Conference and the directions in which research on MHE will move in the future to uncover more effective treatments. The Conference underlined the critical usefulness of gathering experts in several research fields to forge new alliances, identify cross-fertilization areas and in doing so, to benefit both basic and translational biomedical research on the skeleton.

Cartilage and Bone Development in the Limbs

The long bones are a common site for the formation of exostoses in MHE patients. Thus, there has long been an interest to decipher this biology and how it relates to normal limb skeletal development. The limb skeleton develops when mesenchymal progenitor cells originating from the lateral plate mesoderm form condensations within the developing limb bud. The progenitors within condensations give rise to chondrocytes during the process of chondrogenesis or cartilage formation, while the surrounding cells remain mesenchymal in nature and give rise to a primitive perichondrium (1, 2). With further development, the chondrocytes proliferate rapidly until cells nearest the center of the anlagen exit the cell cycle, begin the process of hyperthrophic differentiation, and establish the architecture of the growth plate. Chondrocytes within the developing growth plate transition from round, slowly proliferating cells near the epiphyseal ends to flat, rapidly proliferating cells that form columns to drive longitudinal growth. The proliferating chondrocytes ultimately exit the cell cycle and undergo further differentiation from pre-hypertrophic to hypertrophic and finally terminally hypertrophic chondrocytes. Concurrently, progenitor cells in the initial perichondrium become more organized into distinct layers and those adjacent to prehypertrophic and hypertrophic chondrocytes in the diaphyseal region undergo osteoblastic differentiation and produce the bone collar. The development of cartilage and perichondrium are tightly linked, as signals from both populations of cells affect the growth and maturation of the other in a coordinated manner (3, 4). As skeletal rudiments expand and develop, vasculature from the surrounding tissues infiltrates the diaphyseal center of each element, leading to the formation of a marrow cavity that requires apoptosis of terminally hypertrophic chondrocytes and the subsequent ossification of the cartilaginous matrix by invading osteoblasts. It is this process of chondrocyte proliferation, hypertrophy, and apoptosis along with communication between cartilage and perichondrium that drives normal longitudinal growth and development of long bones (1, 2).

Multiple Hereditary Exostoses, EXT proteins, and Heparan Sulfate

Multiple Hereditary Exostoses (MHE), also known as Multiple Osteochondromas (MO) and Hereditary Multiple Exostoses (HME), is an autosomal dominant disorder effecting 1 in 50,000 individuals and is

characterized by slightly short stature, growth deformities of bones, and multiple cartilage-capped bony exostoses (osteochondromas) that develop on the metaphyses of long bones and other sites including ribs and vertebrae (5). MHE typically presents early during postnatal life with most affected individuals (>80%) being diagnosed by the age of 10 (5, 6). Individuals affected with MHE often undergo multiple surgeries (sometimes greater than 20) to remove symptomatic osteochondromas or address deformities, most during childhood and adolescence (7).

The majority of MHE cases are caused by loss-of-function mutations in *EXT1* or *EXT2* (8, 9). These genes encode Golgi-associated glycosyltransferases that are responsible for the synthesis of heparan sulfate (HS), a key component of cell surface-associated HS-rich proteoglycans (HSPGs) such as syndecan and glypican and matrix-associated proteoglycans including perlecan (10, 11). Both EXT1 and EXT2 are needed for HS synthesis since the proteins form multimeric complexes in the Golgi (12, 13). The HS chains of HSPGs impact a number of signaling proteins critical to skeletal development, such as Indian hedgehog (Ihh) (14-16), Fibroblast growth factors (FGFs) (17-19), Bone morphogenetic proteins (BMPs)(20, 21), and Wnts (22-24). The HSPGs can function as co-receptors for some of these proteins, but can also influence their distribution, range of action, stability, and action on target cells (13). In general, the MHE-associated phenotypes are widely believed to arise from impaired HS synthesis and accompanying HS deficiency in the skeleton and other tissues and organs. However, it remains unclear how the HS deficiency alters cell signaling within the cartilage and/or surrounding perichondrium in developing and growing skeletal elements, as well as, whether it may also affect other processes and events leading to the clinical MHE phenotype.

The 4th International Research Conference on MHE

For more than a decade, the patient advocacy MHE Research Foundation has been a driving force in directing research interest and attention to this skeletal disorder, providing a resource for patients and families, and organizing an International Research Conference every 3 years. On November 1, 2012, nearly fifty musculoskeletal and other research scientists, clinicians, MHE affected individuals, and MHE Research Foundation supporters gathered over four days for the 4th International MHE Research Conference in

Philadelphia, Pennsylvania organized by one of us (MP). The Conference was designed to 1) describe and discuss recent advances in our cellular and molecular understanding of cartilage and bone development, 2) debate the pathogenic mechanisms that may underlie osteochondroma formation and the development of biomedical and clinical phenotypes associated with MHE, 3) identify opportunities to form meaningful and novel collaborations to advance MHE-related research, and 4) formulate ideas about how MHE may be more appropriately treated and/or prevented. The meeting began with stirring remarks from Sarah Ziegler and Craig Eaton, National Research Director and President of the MHE Research Foundation, respectively. Both gave personal accounts of how MHE has affected their children and families and how the disease continues to represent a major medical and personal burden for thousands of patients in this country alone. They stressed the importance of the Conference format with multiple disciplines coming together to forge a cohesive and mutually beneficial research agenda on MHE. They also underlined the impact prior Conferences have had and will continue to have to move the research agenda ahead and provide continuous hope to patients and families alike that a cure may one day be found.

The Clinical Care and Genetics of MHE

MHE presents a number of clinical challenges to orthopaedic surgeons who provide the bulk of care to affected individuals. **John P. Dormans** (Children's Hospital of Philadelphia, USA) described and discussed the several types of clinical problems that MHE patients experience and suffer from throughout skeletal growth and thereafter. Because osteochondromas continue to grow in size prior to skeletal maturity, most also become symptomatic during those years, if they are going to. Both patients with sporadic, isolated osteochondromas and those with MHE undergo surgical excision of symptomatic osteochondromas that irritate neighboring tendons, nerves, or vessels. Some osteochondromas can become so large that excision is pursued to avoid obstruction or damage to another skeletal element. Bigger challenges arise from the forearms and lower legs (below the knee), where the impact on linear growth of bones can differentially affect one bone more than the other, leading to tethering, bowing, and angular growth deformities. While surgical interventions have been described for these scenarios, each is fraught with complications. **Dror Paley** (Paley Advanced Limb

5

Lengthening Institute, USA) reviewed the challenges that remain after skeletal maturity is reached. Bulky osteochondromas can impede the range of motion in joints, leading to painful degeneration. Therefore, aggressive surgical techniques to re-shape the bones are often pursued to correct these impingement scenarios. Other clinicians who interface with MHE affected individuals are medical geneticists. Inactivating mutations in either *EXT1* or *EXT2* are identified in over 90% of MHE patients, with *EXT1* mutations approximately twice as common compared to *EXT2* mutations. Both **Wim Wuyts** (University of Antwerp, Belgium) and **Struan Grant** (Children's Hospital of Philadelphia, USA) discussed large-scale efforts to improve our understanding of genetic modifiers that may result in the variable clinical phenotype often observed in MHE patients, even among members of the same family who carry identical *EXT* mutations. Dr. Grant described the single nucleotide polymorphism genomic profiling underway at the Children's Hospital of Philadelphia and Dr. Wuyts described the European collaborative effort to collect a growing cohort of patients with detailed clinical data regarding genotype-phenotype correlations. Dr. Wuyts also reviewed his team's recent work to delineate the genetics underlying the approximately 10% of MHE patients lacking germline *EXT* gene disruptions. Speculation with regard to this patient subgroup has ranged as far as a search for a third MHE causative gene, but none has been identified so far. Dr. Wuyts and colleagues have taken another approach, deep sequencing of the lymphocytes of these individuals, who as a rule also lack a family history of the disease and generally have a less severe phenotype. His team has identified mosaicism for loss-of-function mutations in either *EXT1* or *EXT2* in all individuals sequenced thus far.

Advances in Animal Models of MHE

For several years many attempts have been made to develop an appropriate animal model of MHE. Because most MHE patients bear heterozygous mutations in *EXT1* or *EXT2*, mice were created to mimic them and possibly serve as a model. However, heterozygous $Ext1^{+/-}$ or $Ext2^{+/-}$ mice did not mimic the MHE skeletal phenotype and were largely normal. Less than 10% of them exhibited solitary osteochondromas and in the ribs only (25). Homozygous $Ext1^{-/-}$ and $Ext2^{-/-}$ mutants were early embryonic lethal prior to establishing the skeleton, reaffirming the fundamental nature of the EXT genes for life (26, 27). An additional mouse model was

6

created bearing hypomorphic *Ext1* alleles (also embryonic lethal during later developmental stages); these mutants displayed a significant reduction in HS throughout the cartilage that resulted in delayed growth plate chondrocyte differentiation and bone formation. Osteochondroma development was not observed in these mice, possibly because of their early lethality (16). Because a few MHE patients bear compound heterozygous mutations in both *EXT1* and *EXT2*, double heterozygous $Ext1^{+/-};Ext2^{+/-}$ mice were created and examined. These mice were born at expected mendelian ratios and, interestingly, did display stereotypic osteochondromas along their long bones and ribs by 2 months of age (15, 27). In other respects, however, even these mice did not mimic clinical MHE and, for example, lacked skeletal deformation or growth retardation. The failure of these animal models to reproduce clinical MHE could have multiple explanations and was highly debated at the Conference. One view expressed was that the "gene dosage" model may not be correct. This model hypothesizes that proper cartilage development and long bone growth would require normal levels of HS synthesis and when levels dropped below a certain "threshold" due to EXT haploinsufficiency, an environment would be generated that is conducive to osteochondroma formation. Alternatively, a loss-of-heterozygosity (LOH) model has been proposed such that MHE affected individuals inheriting a single dysfunctional copy of either *EXT1 or EXT2* would undergo a second somatic mutation within a normal *EXT* allele, resulting in localized osteochondroma formation (28, 29). Consistent with this model, two groups developed mice with clonal, sporadic deletions of *Ext1* floxed alleles using *Cre* promoters that target cells of both chondrocyte and perichondrial/osteogenic lineages and presented their data at the Conference (30, 31). These groups utilized inducible *Col2Cre* transgenes to recombine *Ext1* floxed alleles that ultimately resulted in sporadic or mosaic gene deletions primarily in chondrocytes and some perichondrial cells. All mice with these chimeric growth plates and/or perichondria (homozygous mutant *Ext1* cells surrounded by wild-type cells) developed multiple osteochodromas on their long bones and other MHE related phenotypes, indicating that the sporadic loss of HS synthesis may represent the underlying cause for the MHE pathology (30, 31). While these studies have been critical in establishing the importance of a mosaic *Ext1* loss-of-function to the MHE pathology, the precise role for HS, the exact cellular origin of osteochondromas, and whether disruptions in specific signaling molecules contribute to this pathology remain a mystery. In addition, since MHE patients can display over 100

7

independent osteochondromas at distinct anatomical sites, it remains unclear how LOH could account for such a high frequency of independent events.

Matthew Hilton (University of Rochester Medical Center, USA) presented unpublished data comparing multiple new genetic models that would lend further support to the hypothesis that mosaic disruptions in HS within the cartilage and perichondrium underlie the MHE pathology. Work from the Hilton lab demonstrated that use of the *Matrilin1Cre (32)* to efficiently remove *Ext1* floxed alleles from chondrocytes resulted in delayed chondrocyte hypertrophy and altered long bone growth, without the development of osteochondromas during embryogenesis. He also showed that another inducible *Col2Cre* transgene (33) could be used to delete *Ext1* floxed alleles sporadically in greater than 50% of chondrocytes and perichondrial cells. This resulted in similar alterations to long bone growth and delayed chondrocyte differentiation. These mice also developed multiple clusters of chondrocytes within and protruding from the perichondrium near the hypertrophic zone that resembled early osteochondromas. Further, these mice displayed persistent cartilage within endocortical regions metaphyseal to their growth plates. Since these mice die just after birth for unknown reasons, it is not possible to assess the potential for definitive postnatal osteochondroma development. Interestingly, when the sporadic *Ext1* deficient mice also expressed a heparanase (*Hpa*) transgene (34), which drives a global reduction of HS on the surface of cells, the cartilage growth defects were ameliorated including failure to form the early osteochondroma-like perichondrial clusters. These preliminary studies provide further support to the concept that localized and sporadic disruptions in normal HS and HSPGs may play a critical role in the pathogenesis of MHE and provide some clues to the treatment of MHE.

Yu Yamaguchi (Sanford-Burnham Medical Research Institute, USA) also presented unpublished data from mouse genetic experiments designed to determine whether the osteoblastic lineage is important in MHE and osteochondroma formation. His laboratory utilized both the *Fsp1-Cre (35)* and *Oc-Cre (36)* mice to delete *Ext1* floxed alleles within cells of the perichondrium/periosteum that give rise to osteoblasts, as well as other cell populations. Both of these mouse genetic models developed osteochondroma-like lesions in various bones and therefore implicate this cell lineage as a contributor to osteochondroma development in MHE. Furthermore,

8

the *Oc-Cre; Ext1^{f/f}* mice also developed a low bone mass phenotype caused by enhanced osteoclastogenesis, and therefore suggest that osteoblast derived HS is important for osteoblast-osteoclast crosstalk.

In an effort to decipher which signaling pathway alterations are important to the MHE pathology affecting osteochondroma size and frequency, **Andrea Vortkamp** (University of Duisburg-Essen, Germany) presented unpublished data from experiments where she combined FGF and IHH pathway gain- and loss-of-function alleles in the background of one of the *Col2Cre* driven mosaic *Ext1* homozygous mutant mice (30). These data suggested that FGF signaling may influence the severity of the MHE pathology, and that IHH signaling may induce the transformation of osteochondroma to chondrosarcoma. This work further established that MHE-related osteochondromas are likely caused by a LOH, not simply via *Ext* gene haploinsufficiency, and that multiple signaling pathways may be affected by aberrant HS synthesis within the cartilage and surrounding extracellular matrix.

Julianne Huegel, a graduate student in the laboratory of **Maurizio Pacifici** (Children's Hospital of Pennsylvania, USA), demonstrated that *Ext1^{f/f}* murine neonatal limb explants cultured with an Adeno-Cre virus developed ectopic cartilage near the perichondrium that resembled osteochondroma-like structures, and the cells within and around these structures exhibited reduced HS and enhanced BMP signaling. Additionally, she presented data using the small molecule, Surfen, to inhibit HS function in limb-bud micromass and limb explant cultures. These experiments suggested that HS inhibition increased BMP diffusion and signaling, promoted cartilage formation from progenitors, and even enhanced endogenous heparanase expression. Higher heparanase expression has been reported to occur in the osteochondromas of MHE patients.(37) Intriguingly then, as the HS levels decrease or HS function is inhibited, the cells respond by increasing heparanase expression, further lowering HS levels/function. All these mechanisms could converge to markedly decrease the bioactivity and bioavailability of HS, likely leading to osteochondroma formation.

Henry Roehl (University of Sheffield, UK) presented an analysis of *Ext* mutant zebrafish and demonstrated the involvement of HS in chondrocyte organization and polarity during cartilage development via regulation of the Wnt/planar cell polarity (PCP) pathway. A novel multilox technology for zebrafish, an adaptation of the brainbow technology, was also introduced as a means to analyzing PCP signaling in mutant

and wild-type cartilage at the cellular level. This technological advance will serve as a powerful tool for PCP and cartilage analyses via the mosaic expression of various fluorescently tagged signaling molecules.

Xinhua Lin (Cincinnati Children's Hospital, USA) demonstrated the utility of the Drosophila genetic model system when analyzing *brother of tout-velu* (*botv*; homologue of *EXTL3*) mutations during wing and eye development. In addition to previously identified roles of *botv* in Wnt, Hh, BMP, and FGF signaling, Dr. Lin presented data indicating that *botv* and HS regulate cell surface expression of *unpaired*, a ligand for the Jak/Stat signaling pathway, and that *dally*, an HSPG of the glypican family, was also crucial for normal Jak/Stat signaling.

Collectively, these studies suggest that localized and/or tissue specific disruptions in HS-mediated signaling may be important for contributing to the various aspects of the MHE pathology. Specifically, defects in multiple pathways likely lead to disruptions in chondrocyte polarity, proliferation, and differentiation and altered perichondrial/osteoblastic proliferation and differentiation that may elicit osteochondroma initiation and frequency, as well as, altered long bone growth and morphology. As suggested, specific signaling impairments may also result in the cellular transformation to a malignant phenotype leading to chondrosarcoma.

Osteochondromas in Other Conditions

Another approach toward understanding the biology of osteochondromagenesis has been to study it in relation to other skeletal disorders. **Fred Kaplan** (University of Pennsylvania, USA) described recent investigations into solitary osteochondromas observed in patients with fibrodysplasia ossificans progressiva (FOP). FOP patients bear a consistent activating mutation in ALK2, the BMP type I receptor. Ninety percent of FOP patients develop osteochondromas on the proximal tibia and 100 percent of them develop one somewhere in the skeleton. How ALK2 constitutive activation leads to osteochondromagenesis remains to be deciphered.

Wentian Yang (Brown University, USA) and **Margot Bowen** (a graduate student in the laboratory of **Matt Warman**, Harvard Children's Hospital, Boston) discussed their research surrounding a very rare disorder called Metachondromatosis. Recent discoveries attribute this condition, characterized by development of both enchondromas (cartilage growths within the metaphyses of bones) and osteochondromas, to mutations in the

PTPN11 gene and loss of its product, the SHP2 protein. Work with mice bearing a conditional allele of the gene have identified the cathepsin K-expressing cells in the ossification groove of Ranvier as a source of metachondromatosis, osteochondromas and enchondromas in mice. Explant cultures have honed in on a role for SHP2 in promoting chondrocyte differentiation.

Chondrosarcoma

One of the most feared manifestations of MHE is the development of a chondrosarcoma or cartilage malignancy in the cap of an osteochondroma. This is a rare event, occurring in up to 2% of MHE patients. **Carlos de Andrea** (Leiden University Medical Center, Netherlands), whose flight was prevented by the aftermath of super-storm Sandy, intended to present data explaining at least in part the biology of peripheral chondrosarcomagenesis. In their investigations into the cartilaginous caps of osteochondromas, they have identified multiple areas in each characterized by clonal loss of an *EXT* gene or retention of a wild-type allele. The peripheral chondrosarcomas that arise from osteochondromas do not maintain the same chimerism for the functional allele. Most peripheral chondrosarcomas consistently retain the wild-type allele, while a few (12 percent) are homozygous null for an *EXT* gene. It is not known how these relate to the peripheral chondrosarcomas that arise in MHE specifically, as most of the patient cohorts were sporadic in character. Nonetheless, even most sporadic osteochondromas have regions of heterozygosity and homozygosity for loss of an *EXT* gene, suggesting that the former expands in the development of a peripheral chondrosarcoma.

Kevin Jones (University of Utah, USA) described efforts with a new mouse model of peripheral chondrosarcoma to understand this biology better. The model was developed by conditional disruption of cell cycle regulators in chondrocytes that also bear mosaic clonal disruption of *Ext1*. His results confirmed the findings of the Leiden group prospectively in the mouse, wherein peripheral chondrosarcomas can arise from either the *Ext1*-null or *Ext1*-wildtype-bearing portions of an osteochondroma.

Marion Kusche-Gullberg (University of Bergen, Norway) explored a model that may elucidate the outgrowth of wild-type *Ext1* cells impacted by neighboring $Ext1^{-/-}$ chondrocytes. Working with stromal

11

fibroblasts lacking *Ext1*, cultured in spheroids with tumor cells, she showed that the tumor cells increased in migration and proliferation in the presence of *Ext1*$^{-/-}$ neighboring cells.

Benjamin Alman (Duke University School of Medicine, USA) whose research focuses on the development and malignant transformation of enchondromas, described his recent work with *IDH*, a gene noted to be mutated in the majority of human enchondromas. The primary metabolite that accumulates in the absence of IDH is alpha-hydroxyglutarate. Dr. Alman's group was disappointed by the lack of a growth plate phenotype in *Idh1* knock-out mice, but found instead that mouse fetal limb explants exposed to excess exogenous alpha-hydroxyglutarate increased Hedgehog signaling and impaired chondrocyte differentiation, effects that could be inhibited by cyclopamine. In other work, his team found that xenografted chondrosarcoma cells were dramatically inhibited by antagonism of Wnt signaling. Thus, Hedgehog and Wnt signaling remain central foci for investigation into both benign neoplasia and malignant transformation of chondrocytes.

Cell Signaling in Skeletal Development

To further our understanding of cell signaling in skeletal biology and how multiple signaling pathways may influence the MHE pathology, the Conference brought together numerous experts in the field of skeletal developmental biology. **Ernestina Schipani** (Indiana University School of Medicine, USA) presented her recent studies on understanding the role of the E3 ubiquitin ligase that targets hypoxia inducible factors (HIFs) via the von Hippel Lindau gene (VHL), during cartilage and bone development. Dr. Schipani demonstrated that removal of *Vhl* floxed alleles within chondrocytes led to a severe shortening and thinning of cartilage elements. *Vhl*-deficient cartilage elements were deformed and hypocellular, then eventually collapsed as a result of cellular growth arrest and cell death. Interestingly, removal of *Vhl* floxed alleles within all of the developing limb skeletogenic mesenchyme resulted in synovial fibrosis, foci of ectopic cartilage, and perisoteal osteochondroma-like and fibrosarcoma-like masses that eventually invaded and replaced the collapsed cartilage structures.

Michael Underhill (University of British Columbia, Canada) presented work on the role of retinoic acid (RA) signaling and the RA-metabolizing enzyme, CYP26B1, during the commitment, expansion, and

differentiation of skeletal progenitor cells. Dr. Underhill analyzed *Cyp26b1* mutant mice and reported their severe limb defects that were largely attributable to alterations in skeletogenic cell fate to that of a tendonous lineage. Limb-bud mesenchymal progenitor cultures from these mice and wild-type cells treated with a CYP inhibitor confirmed that RA signaling is critical for inhibiting chondrogenesis and maintaining the progenitor status of these cells.

Karen Lyons (University of California – Los Angeles, USA) first reviewed mouse genetic experiments that prove the importance of BMP signaling during cartilage development via SMAD 1/5/8 signaling, and then presented the unexpected finding that the co-SMAD, SMAD4, has a much more limited role in cartilage development according to *in vivo* analyses of *Smad4* conditional mutant mice. Furthermore, Dr. Lyons demonstrated that BMP-mediated SMAD 1/5/8 signaling is up-regulated in the absence of SMAD4 and that TGFβ-mediated signaling in cartilage may function primarily via non-canonical pathways.

Hank Kronenberg (Harvard Medical School, USA) presented data focused on the mechanisms by which Ihh and parathyroid hormone-related protein (PTHrP) signaling delays chondrocyte hypertrophy during cartilage growth plate development. Functional PTHrP/PTHrP-R signaling during chondrocyte differentiation is mediated via the heteromeric G protein, Gs, to activate adenylate cyclase and suppress RUNX2, a critical inducer of chondrocyte hypertrophy. Dr. Kronenberg presented mouse genetic studies using *Hdac4* mutant mice combined with *Pthrp* over-expressing mice to demonstrate the requirement for HDAC4 in mediating PTHrP signaling and the inhibition of chondrocyte hypertrophy, an effect caused by the ability of nuclear HDAC4 in blocking the stimulatory actions of MEF2c on RUNX2.

David Ornitz (Washington University School of Medicine, USA) presented data from *Fgfr1* and *Fgfr2* double conditional (DKO) mutant mice using the *OsterixCre* transgene that targets prehypertrophic chondrocytes and the osteogenic lineage. These data identified a critical role for FGF signaling in regulating multiple aspects of cartilage and bone development. Specifically, the limb skeleton of DKO mutant mice exhibited delayed chondrocyte terminal maturation and turnover of the cartilage matrix, as well as, reduced longitudinal bone growth and decreased trabecular bone mass.

Bjorn Olsen (Harvard Medical School, USA) presented provocative data concerning the role of paracrine versus intracrine VEGF signaling mechanisms. VEGF signaling is important during skeletal development in regulating chondrocyte survival, angiogenesis, osteoblast and osteoclast differentiation, and cell fate decisions of mesenchymal progenitors toward the osteoblastic and adipocytic fates, as well as, BMP-induced conversion of vascular endothelial cells to mesenchymal stem-like cells (EndMT). Utilizing conditional mutant mice for *Vegfr1* and *Vegfr2*, Dr. Olsen reported that MSC differentiation toward the osteogenic lineage requires intracellular VEGF signaling that is distinct from the normal role of secreted VEGF signals. Furthermore, he noted that adipogenic differentiation of MSCs is regulated independently from these VEGF receptors.

Linda Sandell (Washington University, USA) described her work on site-1 protease (S1P), a proprotein convertase that processes ER-bound latent proteins into free and active forms. Conditional ablation of this gene in cartilage caused a severe skeletal phenotype in mice. The phenotype included a marked reduction in collagen II, an essential component of cartilage matrix, and ER stress. This combination of phenotypes points to regulatory cross talk between matrix production/secretion, tissue homeostasis and ER stress in chondrocytes. Dr. Sandell presented additional work on their quest to identify genes involved in articular cartilage tissue maintenance and repair, functions that often fail after cartilage injury, leading to osteoarthritis. To this end, the group has created several distinct inbred lines of mice that display differential capacity for tissue repair. Each was subjected to experimental osteoarthritis-induction by mechanical destabilization of the knee. The mice of one line resisted osteoarthritis development and maintained relatively intact articular cartilage structure, suggesting strong homeostatic/healing capacity. Another line was more susceptible to the surgical insult, exhibiting rapid loss of articular cartilage and osteophyte formation. These approaches promise to elicit a wealth of information on cartilage phenotypic regulation, healing and homeostasis. Insights could also be used to design ways to mitigate skeletal tissue abnormalities in various conditions, including MHE.

Together, these Investigators described exciting new insights into the critical roles of cell signaling in skeletal development and growth, the complexity of molecular mechanism and targets, the identity of potential new players, and plausible and intriguing targets of therapeutic intervention. As it was expected, studies in

these various areas greatly impacted on the understanding of MHE pathogenesis and osteochondroma formation by pointing to possible new culprits including genes controlling cellular and oxygen metabolism, vascularization, and tissue homeostatic circuits.

Heparan Sulfate Biology

Crucial to our understanding of how signaling pathways and factors regulate chondrocytes and other skeletogenic cells in the context of MHE is an improved understanding of the diverse biological functions of HS. **Robert Linhardt** (Rensselaer Polytechnic Institute, USA) and **Jeremy Turnbull** (University of Liverpool, UK) described their ongoing efforts and progress in synthesis and analysis of GAGs and HS. Specifically, they presented work demonstrating novel procedures for GAG isolation and ultrasensitive structural analysis, sequencing of HS, development of microarray methods, and synthetic chemistry approaches to generate targeted libraries for screening of HS functional diversity.

Cathy Merry (University of Manchester, UK) reported data on the use of *Ext1* null, heterozygous, and wild-type mouse embryonic stem (ES) cells to investigate the role of HS in cell signaling and stem cell differentiation. Interestingly, *Ext1* null ES cells were incapable of differentiation and remained in a "primed state", while *Ext1* heterozygous ES cells adopted an altered morphology and a pro-differentiation phenotype as compared to wild-type ES cells. Directed differentiation assays using these mouse ES cells will allow for better understanding the role of multiple signaling factors and their interactions with HS.

H. Joseph Yost (University of Utah, USA) presented studies using the zebrafish model to demonstrate that each member of the 3-O-sulfotransferase (3-OST) gene family regulates distinct cell signaling pathways, including FGF, BMP, and more. The downstream signaling responses to altered 3-OST function resulted in changes to transcriptional regulation, sarcomere function, cilia formation, and cilia motility. The data presented implicate differential 3-O-sulfation of GAGs as a key regulator of cell signaling from multiple pathways. In related studies, **Rahul Warrior** (University of California at Irvine, USA) described his work on the translational regulation of HS synthesis during Drosophila development. This invertebrate contains three genes involved in HS production, *tout-velu* (*ttv*), *sister of tout-velu* (*sotv*), and *brother of tout-velu* (*botv*)- that

correspond to mammalian *EXT1*, *EXT2* and *EXTL3*, respectively. Gene expression interference studies indicated that loss of HS synthesis does not affect Decapentaplegic (BMP homolog) signaling since the embryos did not ventralize, but did affect Wnt and hedgehog signaling, thus hinting to the complexity of HS regulation of distinct signaling pathways. HS chains were absent in early embryo but, interestingly, all the mRNAs needed for synthesis were present. Analyses revealed that the 5' and 3' UTR contain elements that regulate such translational repression and interestingly, human 5' UTR of *EXT1* can confer developmental regulation when tested in early Drosophila embryos. These interesting data raise the possibility that the EXTs' UTRs and putative gene modifiers affecting their activity could have roles in MHE by modulating *EXT* mRNA translation efficiency and HS production.

Novel Approaches and Possible Therapeutics

Research on a rare disease can obviously benefit from advances in related fields. Also, its pathogenesis can be more complex than it appears and could involve previously unsuspected mechanisms. As importantly, there is an urgent need to conceive, identify and test possible novel therapeutics. Three presentations directly addressed these important issues.

Matthew Warman (Boston Children's Hospital and Harvard Medical School, USA) described his research work to discover the cause of the sporadic and non-hereditary disorder CLOVES (congenital lipomatous overgrowth with vascular, epidermal and skeletal abnormalities) that is characterized by asymmetric somatic hypertrophy and additional defects in multiple organs, including the skeleton. The group used fresh, frozen and fixed archival specimens from six individuals who were the first in their families to have the disease and thus likely to be mosaic. As such, mutations would be absent in peripheral blood but present in tissue lesions. To identity putative mutations, the group resorted to massively parallel sequencing which has facilitated the identification of sporadic causative mutations provided that a sufficient number of individuals with the same phenotype are available as well as affected and non-affected tissues from the each individual. This led to the identification of activating mutations in PIK3CA whose gene product is part of growth factor signaling pathways and regulates phosphoinositide-3-kinase in particular. This innovative and important work is a major

example of the power of this gene discovery approach and could be used as a platform to identify novel causative mutations in MHE patients lacking EXT1 or EXT2 mutations.

Luca Sangiorgi (Rizzoli Orthopaedic Institute, Italy) presented data on the identification and potential roles of microRNAs (miRNA) in the MHE/MO pathology. His laboratory isolated osteochondromas from 19 MHE affected individuals and compared their miRNA profiles to that of normal control cartilage (articular and growth plate). His results identified a signature of 8 miRNAs that could distinguish between MHE/MO samples and controls. These miRNAs were found to target the WNT, Hedgehog, TGFβ, NOTCH, and mTOR signaling pathways, as well as, HS and O-glycan biosynthesis, pointing to the complexity of the molecular regulation of MHE at the cellular level.

Jeffrey Esko (University of California – San Diego, USA) presented his plans for high throughput screening of small molecules that may promote HS synthesis. Specifically, his laboratory developed a Chinese hamster ovary (CHO) cell line that expresses about 10-30% of normal HS and intends on using this cell line to find molecules that may augment or restore HS synthesis in defective cells. These small molecules could then be put through secondary and tertiary screens to determine their ability to function in a similar manner within chondrocyte cultures, as well as, in reducing the development and frequency of osteochondromas within various mouse models of MHE.

Conclusions and Perspectives

As the meeting came to a close, it became apparent how useful, constructive, enlightening and far-reaching the Conference format was that brought together world leaders in MHE research and related biomedical fields to open new venues and forge new alliances, identify cross-fertilization areas that will ultimately benefit both basic and translational biomedical research on the skeleton. All investigators participated in a final round of discussions to review the data and insights presented. Their goal was to identify critical areas on which to focus future research efforts. Based on these discussions, a general consensus emerged concerning the basic principles underlying the development of osteochondromas and MHE pathology. Figure 1 illustrates a consensus model whereby sporadic LOH of *EXT* alleles -or other genetic changes resulting in a steep and local

drop in HS- would alter cell signaling and responses, would affect chondrocyte polarity, proliferation and phenotypic stability, and would induce abnormal chondrocyte behavior along the periphery of growth plate and/or ectopic chondrogenic differentiation of perichondrial/groove of Ranvier cells, all processes converging to elicit formation of osteochondromas typical of MHE. From this model and the many additional data and insights presented, the participants generally agreed that future areas of research should include: 1) the formation of a clinical focus group to establish consensus recommendations on assessing MHE clinical severity and appropriate courses of treatment/surgery; 2) further studies into the enzymology and regulation of HS synthesis, structure and function; 3) a search for MHE modifier genes; 4) continued use and development of mammalian and non-mammalian animal models to further identify the underlying signaling and non-signaling mechanisms responsible for MHE; and 5) the development of screens to identify potential HS synthesis altering drugs and/or treatments for MHE. These lofty and hopeful goals were embraced with a renewed sense of determination and commitment for a better understanding of this potentially severe pediatric disorder that continues to affect and afflict thousands of patients and families around the world. Plans were already laid down to reconvene for the 5th International Research Conference on MHE, which will be hosted by Dr. Jeffrey Esko and Sarah Ziegler from October 29 – November 1, 2015 in San Diego, CA.

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Figure Legend:

Figure 1. Model of MHE pathogenesis. (A) Schematic of a growing long bone in MHE patient composed of *EXT*$^{+/-}$ (haploinsufficient) growth plate chondrocytes, surrounding perichondrium, and diaphyseal bone and marrow. (**B**) Sporadic LoH of *EXT* alleles in growth plate chondrocytes or other genetic mechanisms would create mutant chondrocytes or perichondrial cells lacking or nearly lacking HS production (light blue color cells). Similar processes could also occur in progenitors located in the groove of Ranvier. (**C**) Early formation of an osteochondroma initiated by LoH or severe HS loss that would result in altered cell signaling, altered chondrocyte polarity and/or enhanced chondrogenic differentiation of progenitors. D) Later development of the osteochondroma demonstrating a break in the perichondrium and impaired cortical bone formation (yellow), potentially due to disrupted osteoblast differentiation. IC, immature proliferating chondrocytes (blue); PHC, prehypertrophic chondrocytes (purple); HC, hypertrophic chondrocytes (green); THC, terminally hypertrophic chondrocytes (orange); PC, perichondrial osteoblasts; GR, Groove of Ranvier; and BM, bone marrow.