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Advances in the pathogenesis and possible treatments for multiple hereditary exostoses from the 2016 international MHE conference

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Abstract

Multiple Hereditary Exostoses (MHE) is an autosomal dominant disorder that affects about 1 in 50,000 children worldwide. MHE - also known as Hereditary Multiple Exostoses (HME) or Multiple Osteochondromas (MO) - is characterized by cartilage-capped outgrowths called osteochondromas that develop adjacent to the growth plates of skeletal elements in young patients. These benign tumors can affect functioning of the growth plates, leading to skeletal growth retardation or deformations. They can also encroach on nerves, tendons, muscles and other surrounding tissues and cause motion impairment, chronic pain and early onset osteoarthritis. In about 2–5% of patients, the osteochondromas can become malignant and life-threatening. Current treatments mainly consist of surgical removal of most symptomatic tumors and correction of major skeletal defects, but physical difficulties and chronic pain usually continue and patients may undergo multiple surgeries throughout life. Thus, there is an urgent need to find new treatments to prevent or reverse osteochondroma formation. Given its complexity, this goal would require: a much better understanding of MHE cellular pathogenesis; new insights from skeletal genetics, development, glycobiology, stem cell biology, drug discovery, cancer biology and orthopedics; and possible multidisciplinary efforts. To promote such key objectives, the 2016 International MHE Research Conference was convened to assess the field and to define future directions. The Conference included physicians, physician-scientists and scientists expert in MHE and related areas and had three major aims: to provide a forum for the presentation of most up-to-date and advanced clinical and basic science data and insights in MHE and related fields; to stimulate the forging of new perspectives, collaborations and venues of research; and to publicize key scientific findings within the biomedical research community and share insights and relevant information with MHE patients and their families. This report provides a description, review and assessment of all the exciting and promising studies presented at the Conference and delineates a general roadmap for future MHE research targets and goals.

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Keywords

Multiple Hereditary Exostoses; Hereditary Multiple Exostoses; Heparan sulfate; EXT1; EXT2; Multiple Osteochondromas; Exostoses; Growth plate; Skeletal development; Perichondrium; BMP signaling

Introduction

Multiple Hereditary Exostoses (MHE) is a rare, pediatric, autosomal dominant musculoskeletal disorder that affects about 1 in 50,000 children worldwide (1, 2). MHE, also known as Hereditary Multiple Exostoses (HME) or Multiple Osteochondromas (MO), is amongst the most common disorders within the NIH Office of Rare Diseases classification and one of the most frequent causes of bone tumors. As its name implies, MHE is characterized by osteochondromas that are benign cartilage-capped outgrowths with a bony stem developing adjacent to the growth plates of long bones, vertebrae, ribs and pelvis in juvenile patients (3, 4). The tumors can affect the normal functioning of growth plates, leading to growth retardation and skeletal deformations. They can also impinge on nerves, tendons, blood vessels, muscles and other surrounding tissues, causing motion impairment, chronic pain and even early onset osteoarthritis. In about 2–5% of patients, the osteochondromas transform into malignant chondrosarcoma that, because of their common resistance to chemo- or radiation therapy, can be life threatening (5, 6). No new osteochondromas form after the end puberty when all the growth plates close and the skeleton reaches maturity.

Current treatments for MHE mainly consist of surgical removal of most symptomatic tumors and correction of major skeletal defects such as deformations, limb length discrepancies and joint ankylosis, and patients often undergo more than 40–50 surgeries by age 18 (7). However, because the osteochondromas are so numerous and often difficult to reach and resect, many are left in place; consequently, a considerable number of patients struggle with pain and physical difficulties through life (8, 9). As recent attentive and systematic studies indicate, patients can suffer from additional non-skeletal health problems that include social and learning difficulties, sleep disorders and neuropathies (10–12). MHE may also involve alterations in postprandial lipid clearance and pancreatic beta-cell reserve due to smaller pancreas volume (13, 14), making MHE a syndrome rather than a pure skeletal condition.

MHE is linked to heterozygous loss-of-function mutations in *EXT1* (8q24.1) or *EXT2* (11p11–12) (15–17) that encode Golgi-associated glycosyltransferases responsible for the biosynthesis of heparan sulfate (HS) (18). HS is a complex glycosaminoglycan polymerized from alternating N-acetylglucosamine and glucuronic acid residues, and both EXT1 (the enzyme) and EXT2 (the chaperone-like and non-enzymatically active partner of EXT1) are needed for its synthesis (19, 20). The HS chains are polymerized and covalently linked to small subset of HS-rich proteoglycans (HSPGs) and undergo extensive and concurrent chain modifications including sulfation at various positions and glucuronic acid epimerization, resulting in chains with a high degree of structural complexity and biological specificity (21, 22). The HSPG family includes cell surface proteoglycans (4 syndecans, 6 glypicans, CD44V3, betaglycan and CD47), pericellular/extracellular matrix proteoglycans (perlecan,

agrin and Type XVIII collagen), and serglycin (21, 23, 24). The HSPGs are expressed in a tissue-specific manner and have a number of important developmental and physiologic functions (24). One of their main roles is to interact with growth factors, morphogens, and receptors, all characterized by possessing a specific HS-binding domain (25, 26). The HSPGs regulate protein availability, distribution, turnover, and signaling, thus affecting cell adhesion and migration, cell-cell interactions and communication, cell differentiation and morphogenesis, lipoprotein metabolism and proteases involved in hemostasis and development (21, 23, 24). The *EXT* deficiency in MHE results in partial truncation of the HS chains, which leads to lower levels of HS in multiple tissues and plasma (27–29), potentially explaining the impact of the mutations on multiple organ systems.

The haploinsufficiency in *EXT1* or *EXT2* in MHE patients can in itself cause certain pathologies such as lipid metabolism defects and wound healing delays (14, 30), but is not sufficient to cause osteochondroma formation (31, 32). In line with Knudson's hypothesis of tumorigenesis (33), osteochondroma formation requires a "second hit" such as loss of heterozygosity (LOH), aneuploidy or other genetic changes that would further lower the HS levels in affected cells and tissues (34). Murine models for MHE have provided strong and direct evidence for this thesis. Single heterozygous $Ext1^{+/-}$ or $Ext2^{+/-}$ mutant mice were found to be largely normal and less than 10% of them developed solitary osteochondromas only in ribs and certain genetic backgrounds (20, 29). In sharp contrast, numerous osteochondromas at typical anatomical locations developed in double heterozygous $Ext1^{+/-}$;$Ext2^{+/-}$ mice (29) and in mice in which both *Ext1* alleles were conditionally and stochastically ablated in growth plate chondrocytes and perichondrium (35–37). Other studies showed that chondrocytes in zebrafish mutants lacking *dackel* (corresponding to mammalian *Ext2*) grew aberrantly and lost polarity, invading surrounding tissues, thus mimicking traits of MHE chondrocytes (38). Human MHE growth plate chondrocytes display disorganized primary cilia and loss of cellular polarity, suggesting that the cells were unable to perceive cilia-mediated signals and maintain cell alignment and location, thus contributing to osteochondromas (39). Huegel et al. showed that *Ext1* and HS regulate perichondrial phenotype and border function in developing long bones and that *Ext1* loss in perichondrial progenitor cells led to osteochondroma formation (40). Boundary defects may be reinforced by changes in microRNAs demonstrated in MHE chondrocytes (41). Studies also indicated that the HS deficiency in MHE cartilage and perichondrium could be exacerbated by abnormally high expression of heparanase (42, 43). In sum, there has been significant progress in uncovering aspects of the genetics and cellular pathogenesis of MHE using both animal models and human MHE specimens. However, much remains to be understood mechanistically, and relatively little has so far been achieved towards the development of treatments to prevent osteochondroma formation and/or growth (44).

As pointed out above, MHE is not simply a skeletal disease, but encompasses non-skeletal pathologies (9). Unlike skeletal abnormalities, these additional pathologies occur with varying frequency and have been discounted or unappreciated in the past. However, recent human and mouse studies have provided evidence that HS deficiency can in fact cause physiological defects in kidney organization and podocyte structure (13), neuronal cell signaling and mouse behavior (45, 46), lipid metabolism (47, 48), intestinal epithelial barrier function (49), pancreatic beta-cell reserve (50), and inflammation and infection (51, 52).

Thus, there is little doubt that HS has encompassing roles in many tissues and organs (21) and that the HS deficiency in MHE can provoke a variety of pathologies or deficiencies in patients.

The 5th International Research Conference on MHE

The 5th International MHE Research Conference was organized to assess the status of most recent MHE research and provide a springboard for the future of the field. Its key and specific goals were to: (i) maintain research momentum; (ii) provide a forum for sharing the most recent and unpublished findings from major laboratories working on MHE; (iii) include experts from related fields to share in this knowledge, provide critical input and fresh perspectives, and be inspired to work directly on this debilitating pediatric disorder individually or in collaboration; (iv) improve our understanding of the medical and clinical complexities of MHE; and (v) use all the biological and biomedical insights gained in animal model systems and from patients and donated specimens to begin to envision therapeutic approaches for treating MHE by pharmacological, cellular or genetic means together or without surgery. To accomplish these objectives, the Conference was organized around major themes including HS biochemistry and metabolism, skeletogenesis and protein signaling, cancer biology, stem cell biology, and therapeutic approaches. It also included a clinical session for patients and families. As in past MHE Conferences, the 5th Conference provided many opportunities for brainstorming and promoting collaborations and synergy amongst laboratories studying this and related diseases. The program united an eclectic mixture of investigators from several clinical and biological disciplines, enabling the participants to formulate comprehensive and multi-disciplinary concepts in MHE and envision strategies and goals. Expected outcomes included: a refined and more comprehensive understanding of MHE as a syndrome; dissemination of most current data and insights; sharing of reagents, methods and technologies across the research community; and identification of possible and plausible means for preventive or therapeutic interventions to counter osteochondroma formation and possibly, rectify other MHE-associated pathologies.

The Conference was held in May 2016 at St. Mary’s Medical Center and the Paley Advanced Limb Lengthening Institute in West Palm Beach and consisted of 6 biomedical and clinical research sessions, followed by a special session for patients and orthopedic specialists. Each session had 5–6 speakers and followed an interactive workshop format. A total of 85 individuals –including 35 senior speakers; 13 trainees (PhD, MD, and BS); representatives from the MHE research foundations; staff members from the Paley Institute and the University of California; and 26 MHE family members - had an opportunity to present. The format, roster and size of the meeting were designed to maximize interactions among participants in an informal setting. In addition to the scientific sessions, a final session for families focused on direct interactions with MHE medical specialists to discuss individual MHE prognosis. Sarah Ziegler, Director of the MHE Research Foundation, chaired this session. Speakers and attendees hailed from 11 countries on 4 continents: United States, Argentina, Canada, China, France, Germany, Israel, Japan, Netherlands, Norway, and the United Kingdom

Major themes and research areas covered at the conference are the following.

HS in MHE and related pathologies

Over 90% of MHE patients bear heterozygous mutations in *EXT1* or *EXT2* (53), and direct measurements of blood and tissue HS levels have shown that the patients have a systemic HS decrease of about 50% (27). As indicated above, such partial loss of HS may in itself be sufficient to cause physiologic abnormalities in tissues and organs such as the liver (14), but it is not sufficient to alter the behavior and functioning of growth plate and perichondrial cells and provoke osteochondroma formation (34). The latter appears to require a much greater loss of HS, and genetic studies with human osteochondroma specimens and in mouse models have solidified this tenet as pointed out above. Genomic analyses have indicated that LOH or other chromosomal rearrangements are detectable in at least a portion of human osteochondroma samples (31, 32, 54, 55), thus likely leading to steep decrease in HS production within the tumors. In mice, compound heterozygous loss of *Ext1* and *Ext2* or conditional ablation of both *Ext1* alleles are needed to trigger a stereotypic MHE phenotype in which the mice develop multiple tumors at multiple and typical locations, including long bones, vertebrae and ribs (29, 35, 36). The conclusions stemming from these studies are that a partial loss of HS seems to be tolerated by growth plate cartilage and perichondrium and does not alter their behaviors and function, but a steeper HS loss would render the cells tumorigenic and prone to produce osteochondromas.

Matthew Hilton (Duke University) engineered two new mouse models in which loss of both *Ext1* alleles could be induced by mating “floxed” *Ext1* mice ($Ext1^{f/f}$) with *Matrilin1-Cre* or *Col2a1-Cre*™. The former model inactivated *Ext1* in all chondrocytes, but the animals did not develop osteochondromas; instead, they exhibited skeletal developmental defects similar to those in other models in which *Ext1* is compromised systemically by hypomorphic expression (e.g., $Ext1^{Gt/Gt}$) (56). The second genetic model - $Col2a1Cre™;Ext1^{f/f}$ - results in a stochastic deletion of both *Ext1* alleles within chondrocytes and perichondrial cells both targeted by *Col2a1Cre* (57). The mutant mice contained islands of chondrocytes lacking HS and exhibited a high frequency of osteochondroma formation. One interpretation of the data was that osteochondromas arise from uneven expression of HS across cartilage. Interestingly, transgenic systemic overexpression of heparanase, the endo- -D-glucuronidase that cleaves heparan sulfate at specific subsites in the chain, was found to reduce HS across all tissues and to reduce osteochondroma incidence in $Col2a1Cre™;Ext1^{f/f}$ mice, perhaps by pervasive and pleiotropic effects and collapsing HS gradients. Though intriguing, the data would need to be reconciled with the strong endogenous heparanase expression observed in human osteochondromas from MHE patients (43), possibly suggesting that gene dosage and/or restricted heparanase expression are important.

Marion Kusche-Gullberg, PhD (University of Bergen, Norway) presented work examining the influence of HS chains on bi-directional communication and interactions that occur between stromal fibroblasts and tumor cells. An in vitro model was described based on mixed multicellular three-dimensional spheroids composed of human tumor cells and wild type or *Ext1*-deficient fibroblasts. Differential effects in expression profiles were noted in genes involved in tumor cell proliferation and invasion, whereas several genes encoding

extracellular matrix proteins or those regulating cell motility were suppressed (58). The data indicated how HS can have non-cell autonomous effects, possibly relevant to models of MHE in which only small cohorts of cells undergo stochastic loss of *Ext* function or to certain anatomical sites such as tissue boundaries where the levels of HS can vary and be modulated.

Andrea Vortkamp (University of Duisburg, Germany) presented studies on HS in osteoarthritis and articular cartilage maintenance. Osteoarthritis (OA) is characterized by progressive loss of articular cartilage due to slow and chronic degradation of cartilage matrix and loss of phenotypic expression in chondrocytes (59). Clonal deletion of *Ext1* in type II collagen-expressing chondrocytes in *Col2*^{rtTA}*Cre*; *Ext1*$^{e2fl/e2fl}$ mice was found to induce formation of enlarged, atypical cells within articular cartilage, as seen in previous studies (37). The abnormal cells were largely devoid of HS, produced an altered extracellular matrix, and appeared to separate themselves from surrounding wild type chondrocytes. Surprisingly, the clonal loss of HS did not lead to OA during ageing or in a surgical OA model, but actually seemed to have a protective effect on cartilage maintenance. It remains unclear whether this surprising effect is due to protective release of beneficial growth factors by the HS-deficient cell clones, increased turn-over and remodeling of the matrix, or other mechanisms operating within articular cartilage over time.

Two studies presented at the Conference suggested that the immune system might affect osteochondroma formation. Maureen A. McGargill (St. Jude Children's Research Hospital) showed that mice with a germline deletion of *Erk1* and a conditional deletion of *Erk2* mediated by *CD4-cre* (*DKOCD4* mice) spontaneously developed multiple osteochondroma-like lesions over time. Histological analyses revealed excessive accumulation of hypertrophic chondrocytes possibly originating from growth plates, but no mononuclear infiltrates indicative of inflammation. Osteochondroma-like lesions developed faster and were more severe in *DKOCD4* mice that lacked T cells, indicating that T cells play an important role in regulating cartilage formation and homeostasis. Development of osteochondroma-like lesions was also influenced by changes in microbiota and inflammatory stimuli, demonstrating previously unappreciated roles of the immune system in cartilage homeostasis (60). Joseph Yost (University of Utah) reported studies indicating that defects in immunological cell lineages and wound responses occurred in zebrafish with mutations in genes encoding *Syndecan 2* core protein, *Ext2* or HS chain modification enzymes. A surprising number of certain homozygous recessive mutants appeared to have normal development and morphology and were viable through adulthood. Live imaging distinguishing specific immune lineages revealed that the more penetrant mutants had underlying defects in specification and propagation of immune cell lineages and substandard immune system responses to wounding. These findings suggested that defects in immune system and wound healing could be possible cofactors in non-skeletal symptoms and osteochondroma formation in MHE patients.

HS, protein signaling and skeletogenesis

The mechanisms by which osteochondromas exclusively form next to the growth plates remain unclear and somewhat controversial. Several studies have suggested that HS loss in

MHE causes aberrant distribution and action by HS-binding signaling proteins and growth factors, including members of the hedgehog, bone morphogenetic protein (BMP)/ transforming growth factor β (TGFβ), fibroblast growth factor (FGF) and Wnt families (29, 40). Alterations in growth factor distribution and action could potentially provoke abnormal behaviors in chondrocytes and/or neighboring perichondrial cells, leading to osteochondroma initiation and growth (34). Hence, one of the most pressing issues in current MHE research is to uncover the mechanisms by which HS regulates the normal function of signaling proteins and factors (25, 26) and how alterations in such key homeostatic mechanisms affect skeletal cell behavior and induce tumor formation. It is interesting to point out that the osteochondromas and their growth plate-like cartilage caps are usually oriented perpendicularly to the axis of the endogenous growth plates, and it is wholly unknown how this occurs and what it may mean pathologically.

Yingzi Yang (Harvard School of Dental Medicine) reported that FGF and Wnt5a signaling coordinates directional proximo-distal elongation and patterning in developing limbs through regulation of planar cell polarity (PCP), a mechanism by which cells within developing fields such as the limb bud become polarized in a specific direction (61). Genetic evidence was provided suggesting that altering the Wnt5a gradient direction affected the spatial orientation of PCP as revealed by Vangl2 phosphorylation. The data raised the possibility that the directionality of osteochondroma formation may reflect re-orientation of such mechanisms and growth factors/morphogen gradients orthogonally with respect to endogenous growth plate and perichondrium.

Elazar Zelzer (Weizmann University, Israel) discussed proprioceptive mechanosensors and regulation of spinal alignment. A series of genetic studies in which *Runx3* was inactivated selectively in peripheral sensory neurons induced peripubertal scoliosis without skeletal dysplasia, which led to the conclusion that these sensors coordinate the alignment of the vertebral column. These findings uncover a central role for the mechanosensory system in maintaining spinal alignment and provide a mechanistic explanation for adolescent idiopathic scoliosis. The spine is not usually included in general physical assessment of MHE patients (62), but a recent study from Japan strongly indicates that this may need to change (63). The authors clinically assessed 50 MHE patients (average age 28), used a disease severity classification system that considered skeletal deformities and osteochondroma number, and closely inspected the spine of each patient. They found that mild to moderate scoliosis was actually common amongst the patients. Scoliosis spanned King type I to type IV in severity, but not type V. Though the data need to be verified in other and larger patient cohorts, they point to the possibility that defects in skeletal development and/or mechanosensory mechanisms may affect aspects of spine physiology during the course and progression of MHE. If confirmed and further assessed, the data could possible lead to changes in standard of care of MHE patients in the future.

Chondrocytes and osteoblasts originate from a common mesenchymal precursor, the osteochondroprogenitor (OCP), and help build the vertebrate skeleton. The signaling pathways that control lineage decisions in OCPs are incompletely understood. Two studies demonstrated the importance of FGF signaling in fate decision and differentiation in the chondrogenic and osteogenic lineages. David M. Ornitz (Washington University) showed

that FGFs regulate the balance between osteogenesis and chondrogenesis and overall skeletal growth through FGF receptors 1 and 2 (Fgfr1 and Fgfr2), both of which are expressed in the osteoprogenitor lineage. Conditional knockout mice lacking both receptors were created using an osteoprogenitor-specific *Osx-Cre* driver. The mutants showed a ~50% reduction in body weight and bone mass, and impaired longitudinal skeletal growth via cell autonomous functions of FGF signaling. The double knockout mice also showed growth plate defects that appeared to be due to a non-cell autonomous feedback pathway regulating Fgfr3 in growth plate chondrocytes, leading to suppression of chondrocyte proliferation (64). Wentian Yang (Brown University) and collaborators conditionally deleted *Ptpn11* in mouse limb and head mesenchyme that encodes the protein tyrosine phosphatase Shp2. They found that the *Shp2*-deficient mice exhibited increased cartilage mass and deficient intramembranous and endochondral ossification, suggesting that *Shp2*-deficient OCPs become more frequently chondrocytes than osteoblasts. Interestingly, mosaic *Shp2* deletion at E13.5 in *Prrx1-Cre*^{ERt2}-expressing OCPs led to osteochondromas and enchondromas. Mechanistic studies suggested that Shp2 regulates fate determination of OCPs via post-translational phosphorylation and SUMOylation of Sox9, at least in part via the PKA signaling pathway.

Condyles are present at the epiphyseal ends of long bones where they participate in articulation between skeletal elements. Karen Lyons (University of California, Los Angeles) reported that mice lacking the type I TGFβ receptor ALK5 in the growth plate (*ALK5CKO*) exhibited loss of specific condyles and cartilaginous protrusions in their limbs and also a severe and lethal chondrodysplasia. Preliminary data indicated that the loss of ALK5 prevented isotropic expansion of epiphyseal/resting zone cartilage and instead promoted growth plate column formation. Surprisingly, *Alk5* ablation led to only a modest reduction in Smad2/3 activation, which is downstream from ALK5; instead, there was a massive up-regulation of BMP Smad1/5/8 activation. The data, along with the considerably milder and viable phenotype of mice lacking Smad2/3, suggested the very interesting conclusion that a major role for ALK5 in cartilage might not be to activate the TGFβ pathway, but rather to prevent excessive BMP signaling. As described below, Yu Yamaguchi and one of us (MP) have reported evidence recently that inhibitors of BMP signaling can reduce osteochondroma formation, signifying that this signaling pathway and/or modulation of TGFβ signaling may serve as novel therapeutic strategies for MHE.

MHE and related cancers

The hallmark of MHE is the formation of benign osteochondromas next to the growth plates. *EXT* mutations occur in other types of cancer as well, and *EXT1* and *EXT2* are classified as tumor suppressors (18, 65). In about 2–5% of MHE patients, osteochondromas progress to malignant chondrosarcomas, but the mechanisms underlying such transformation are unknown (54). Thus, one of the sessions was devoted to discussion of bone and cartilage tumors and the roles of HS function and metabolism in tumor formation.

Matthew L. Warman (Children’s Hospital, Boston) gave a keynote presentation entitled “Why don’t all cancer causing mutations cause cancer?” A high percentage of mutations in oncogenes or tumor suppressors often cause tissue malformation, but may not result in

cancer. Explanations for this observation include phenotypic suppression by surrounding wild type cells or the need for mutations or epigenetic changes in modifier genes. Some tumors also undergo “reversibility”, i.e. the initial tumor growth is subsequently suppressed leading to spontaneous regression, possibly due to secondary mutations or epigenetic alterations. Another complicating factor is that cells/mutations responsible for development of the tumor may be gone by the time the tumor is biopsied. To gain insight into mosaicism and genetic alterations taking place in tumor cells, modern techniques for sequencing DNA and RNA from single cells from tumors using Nanopore technology are being developed. Their application to osteochondromas might provide clues about variable penetrance of osteochondromas and the overall MHE severity phenotype in patients bearing identical mutations, including family members (66).

Isocitrate dehydrogenases are soluble enzymes encoded by *IDH1* and *IDH2*. Somatic mutations in either gene are present in the majority of enchondromas, which are benign cartilage tumors forming inside (but not next to) the bone and can be precursors to malignant chondrosarcomas. Benjamin A. Alman (Duke University) showed that mice expressing *Idh1-R132Q* mutation in one allele in chondrocytes exhibited disordered growth plates, with persistence and scattered distribution of type X-collagen expressing hypertrophic chondrocytes. Homozygous mutants did not survive after neonatal stages, but induction of *Idh1-R132* mutant after weaning resulted in multiple enchondroma-like lesions. Interestingly, this mutation alters enzymatic activity from conversion of isocitrate to α-ketoglutarate to production of D-2-hydroxyglutarate, which can alter DNA methylation. The data raise the interesting possibility that “oncometabolites” could contribute to bone tumor formation (67).

Ernest (Chappie) Conrad (Seattle Children’s Hospital) discussed the clinical difficulties in diagnosing patients with malignant chondrosarcoma and the need for biomarkers. Equally perplexing is the difference in malignant transformation in MHE patients (2–5%) versus patients with Ollier Disease/Multiple Endochondromas (10–46%) (68). Although association of risk varies with the size of the primary tumors, other contributing factors remain unknown. Judith V.M.G. Bovée (Leiden University Medical Center, Netherlands) discussed other potential molecular culprits in cartilage tumors. Secondary mutations causing malignant transformation of osteochondromas or enchondromas may include alterations in the pRb and p53 pathways, Hedgehog signaling, metabolic pathways (e.g. mTOR), apoptosis and survival mechanisms (e.g. Bcl family members, survivin), and several tyrosine kinases (e.g. Src) (69, 70). These genes represent potential targets for chemotherapy that would be especially relevant and beneficial to patients with un-resectable chondrosarcomas.

Veronique M. Lefebvre (Cleveland Clinic) presented work on cell fate specification in skeletogenesis and chondrosarcoma. CHIP-seq was used to study enhancer elements in cartilage specific genes. “Super-enhancers,” genomic regions composed of multiple enhancers bound to multiple transcription factors, were found in several genes involved in cartilage differentiation, including *Sox6* and *Sox9* and genes involved in proteoglycan production and glycosaminoglycan modification (*Acan*, *Chst11*, *Syndecan 4* and *Ndst1*). Interestingly, Sox 4,11 and 12 (termed SoxC genes) are expressed in perichondrium. Inactivation of all three genes by *Prrx1-Cre* (that targets the early limb bud mesenchymal

population) resulted in defects in growth plate, lack of synovial joints, poor definition of perichondrium-growth plate boundary and interestingly, formation of cartilaginous outgrowths. Clearly, the SoxC genes are needed to maintain the normal phenotype of perichondrium and this may be in association with strong Wnt/β-catenin signaling. Decreased SoxC expression would lead to decreased Wnt/β-catenin signaling, which is a well known anti-chondrogenic mechanism (71), causing ectopic chondrogenesis and cartilage formation in perichondrium. The data point to the interesting possibility that the SoxC genes may be part of the mechanisms derailed by *Ext* and HS deficiencies that would cause osteochondroma formation in MHE and/or other cancers.

Joanna Phillips (University of California, San Francisco) presented studies on HS modifications and the impact on platelet-derived growth factor (PDGF) signaling in glioblastoma (GBM). Of particular interest was the observation that expression of *Sulf2*, an endosulfatase that modifies the sulfation levels and patterns in HS chains at the cell surface, was elevated in GBM subtypes that exhibit amplification of PDGF receptor expression. Sulfs have not yet been analyzed in osteochondromas, but as indicated above, other studies suggested higher heparanase expression that could amplify the impact of HS reduction due to *Ext* deficiency (42, 43). Overall, the studies raise the possibility that extracellular alterations in HS structure and levels could contribute to tumorigenicity. Ralph Sanderson (University of Alabama at Birmingham) discussed how trimming of HS chains by heparanase could impact cell signaling and promote tissue remodeling, driving tumor growth, invasion, metastasis, osteolysis and angiogenesis in multiple myeloma. His group discovered that treatment of myeloma cells with anti-myeloma drugs such as bortezomib and melphalan significantly up-regulated expression and secretion of heparanase and resulted in chemoresistance (72, 73). These findings indicate that heparanase is a viable target for anti-cancer therapy. Roneparstat (previously known as SST0001) is a rationally developed heparanase inhibitor produced by modification of porcine mucosal heparin to eliminate its anticoagulant activity. Preclinical studies of Roneparstat in animal models of multiple myeloma indicated anti-tumor efficacy and pharmacodynamic effects consistent with its anti-heparanase activity in vivo (74). A recently completed Phase I study in advanced refractory multiple myeloma patients revealed excellent tolerability of this drug. These exciting findings might be adapted for the treatment of MHE. Roneparstat was shown to inhibit chondrogenesis in vitro; since this step is the first to occur in osteochondroma development, Roneparstat may indeed be a possible therapeutic for MHE as well (42).

Stem cell biology

Original studies of specimens from MHE patients suggested the presence of early microchondromas within perichondrium along the physis (growth plate) and the groove of Ranvier, suggesting that precursor cells in perichondrium give rise to neo-chondrocytes clonally expanding and developing into osteochondromas (32). Conditional *Ext1* ablation in perichondrium flanking the epiphyseal long bone region, which encompasses the groove of Ranvier, does indeed cause ectopic cartilage formation in mice (40). Like perichondrium, the groove of Ranvier is thought to house progenitor cells (75), suggesting the possibility that osteochondroma formation in MHE involves defects in resident stem cell behavior and niches.

Conceptually, MHE might also share elements with regenerative processes in which a stem cell undergoes activation. HS regulates many of the cell signaling pathways important for wound repair and regeneration (21). To examine the impact of *Ext1* on wound repair and regeneration, one of us (Anne Phan, University of California, San Diego) examined the response to cutaneous skin lesions and digit amputations in *Ext* deficient mice. Surprisingly, double $Ext1^{+/};Ext2^{+/-}$ heterozygous mice showed faster repair of full-thickness dorsal skin excisional wounds. This phenotype was recapitulated in wild type animals treated with glycosides that modulate glycosaminoglycan formation, suggesting a pharmacological approach to stimulate wound repair. Additionally, digit regeneration studies showed that wild type mice, like children (76), can regenerate the digit tip if amputations occur in the 3rd phalangeal element above the nail root and the wound is allowed to close and form a blastema. More proximal amputations below the nail root and in the 2nd phalangeal element do not. No effect on 3rd digit regeneration was noted in $Ext1^{+/-}Ext2^{+/-}$ mice, but amazingly, mutant mice regenerated digit-tips after an amputation through the more proximal 2nd phalangeal element. We note however, digit regeneration was highly variable and poorly penetrant amongst mice. These findings raise the possibility that the formation of osteochondromas may involve recruitment of processes and progenitor cells normally involved in regenerative and repair responses.

Yang Chai (University of Southern California) discussed craniofacial mesenchymal stem cells in bone tissue homeostasis and repair. Craniofacial bones are derivatives of the neural crest and thus have a different origin as compared to those producing much of the skeleton. *Gli1+* progenitors within the suture mesenchyme were found to serve as the major mesenchymal cell population for craniofacial bones. Ablation of *Gli1+* cells led to craniosynostosis and arrest of skull growth. Interestingly, the calvarial sutures possessed much stronger regeneration capacity than non-suture areas during experimental calvarial bone repair, and the healing rate of calvarial bone was inversely related to suture-injury distance. Some of these very exciting data have been published (77).They strongly suggest that the potential for bone healing is not evenly distributed within the calvaria bones but is greatly enriched within the sutures and their mesenchymal stem cells and niche. This is analogous to the preferential distribution of progenitors in perichondrium and groove of Ranvier and their likely roles in osteochondroma formation (40), a process invoking skeletal repair processes as postulated above

T. Michael Underhill, PhD (University of British Columbia, Canada) also discussed the role of mesenchymal progenitors in tissue renewal and regeneration. Mesenchymal progenitors, one type of adult progenitor cells, can remain in a quiescent state, but become activated in response to various signals. Mouse models were presented that involve knock-in of a reporter into the *Hic1* gene (hypermethylated in cancer), which is largely restricted to quiescent mesenchymal progenitor cells. Lineage tracing and marker analysis showed that the *Hic1+* cells generally resided in a perivascular location and significantly contributed to multiple mesenchymal linages, including beige and white adipocytes, osteocytes, marrow stromal cells, and myofibroblasts. RNA-seq analyses of isolated mesenchymal progenitors revealed that “activated” cells had multiple roles in regeneration, in particular in muscle regeneration. Mouse models of this type might prove useful for tracing the origin of cells in osteochondromas.

April Craft (Boston Children's Hospital) discussed how pluripotent stem cells (PSCs), including both embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), can be used to reproducibly and efficiently generate articular chondrocytes and growth plate-like chondrocytes from mouse and human PSCs (78). Human PSCs expressed lineage specific mRNAs in appropriate sequence during in vitro differentiation, and retained their lineage-specific phenotypes when implanted in vivo. Cartilage tissues generated from hPSCs resisted ossification when implanted in vivo, whereas hypertrophic chondrocytes derived from hPSCs underwent endochondral ossification, akin to endogenous growth plate maturation. Additional studies compared the articular and growth plate cartilage structures created in vitro and in vivo from hPSCs to respective endogenous tissues. These techniques might prove useful for studying diseased tissues from MHE patients and could elicit expandable and renewable populations of mutant cells to study their basic pathogenic mechanisms.

Hiroshi Nakato (University of Minnesota) reported on studies on the control of stem cell activity by HS in Drosophila. One area of research was to understand how changes in HS sulfation regulate intestinal stem cell division during normal midgut homeostasis and regeneration. Deficiency in Sulf1, the extracellular endosulfatase that reduces HS sulfation, resulted in increased intestinal stem cell division. Using a regeneration model, his group found that in *Sulf1* mutants intestinal stem cells failed to properly halt division at the termination stage. Conversely, overexpression of *Sulf1* during early regeneration suppressed intestinal stem cell division. Taken together, these findings indicate that Sulf1 is required for terminating intestinal stem cell division at the end of regeneration (79). As pointed out above, it remains to be explored whether Sulf1 has roles in progenitor cell function and osteochondroma formation.

Noriaki Ono (University of Michigan) presented data suggesting that cells located in the resting zone of postnatal growth plate are a novel type of skeletal stem cells. These cells displayed distinct characteristics compared to adult bone marrow mesenchymal stromal stem cells. To study if resting growth plate cells are important in bone growth as well, the group utilized a genetic label-retention strategy based on a Tet-Off system regulating expression of a histone-linked GFP. Exploiting the fact that the resting cells express *PTHrP* (80), the cells were irreversibly labeled using *PTHrP-creER* BAC transgenic mice, and they and their progenies were traced over time. The cells subsequently formed columnar chondrocytes, hypertrophic chondrocytes and subchondral bone in the primary spongiosa. Reporter-positive cells also formed large colonies in vitro, indicating their replicative capacity. Thus, the data suggested that cells in the resting zone of growth plate represent a novel type of skeletal stem cells. Whether these cells contribute to bony tumors such as osteochondromas or endochondromas remains unknown.

Therapeutic approaches

Surgery is currently the major treatment option for MHE patients by which the most symptomatic osteochondromas are resected and major skeletal defects can be corrected. However, as pointed out above, patients often have numerous osteochondromas that cannot be resected or would require multiple surgeries. These lingering tumors can cause life-long

problems in addition to increasing the probability of malignant transformation. Currently, there are no pharmacological treatments for MHE other than palliative care for pain. Thus, there is an urgent need to identify potential druggable targets and drug-like agents that might prove of therapeutic value. One of the major goals of the Conference was the identification of potential pharmacological approaches for treating the disease, taking advantage of various model organisms, high-throughput screening technologies, and studies of other bone and cartilage disorders where drug development efforts have succeeded or have made progress.

One of us (Jeffrey Esko, University of California, San Diego) discussed several novel approaches to develop drugs for treating MHE. Given that the primary defect in MHE is HS assembly, methods to restore HS were discussed. Ideas included use of subclinical doses of low molecular weight heparin as a method of “substrate replacement therapy”. Initial studies in murine model of MHE did not reveal significant reduction in the number, size or time of onset of disease, but additional studies were suggested using non-anticoagulant heparin (e.g. Roneparastat). Jian Liu (University of North Carolina) described current methods for chemoenzymatic synthesis of HS and heparin. Synthesis of heparin and HS oligosaccharides using a purely chemical approach remains challenging. However, a chemoenzymatic method was developed to prepare synthetic heparin and HS, using 15 different enzymes including sulfotransferases, an epimerase and glycosyltransferases (81). The availability of HS oligosaccharide libraries offers a unique tool to study the function and activity relationship of HS in biological systems. Applications include binding studies of signaling proteins, competition experiments performed in cultured cells and substrate replacement therapy. Jian Liu’s methods could yield large quantities of defined, non-anticoagulant heparinoids for treating MHE.

Esko also described a high-throughput screening assay to identify compounds that augment HS on the plasma membrane. One compound was identified and is currently under development. In addition, genome wide screens using CRISPR/Cas9 identified novel genes that modulate HS formation. These genes represent other potential targets for drug development efforts.

Laurence Legeai-Mallet (INSERM U1163, Imagine Institute, Paris Descartes University, Paris, France) discussed novel strategies to correct skeletal development. Innovative therapies to treat skeletal genetic disorders included replacement of defective/missing proteins or genes or normalization of aberrant signaling pathways. Potential therapeutic strategies emerged from studies on achondroplasia, the most frequent form of dwarfism. Several preclinical studies are being carried out that include treatment with: C-type natriuretic peptide analog (BMN111), intermittent PTH injections; soluble FGFR3, Meclozine, Statin and a pan-FGFR tyrosine kinase inhibitor (NVP-BGJ398). The success of any therapies targeting pediatric disorders, such as MHE, lies on early diagnosis and effective targeted correction of the affected pathway, while maintaining normal development and homeostasis of the remainder of the skeleton.

Chondrodysplasias and other genetic diseases that affect bone and cartilage formation are often studied in animal models, but sometimes there are differences in animal models versus humans. Because human tissues are difficult to obtain on a routine basis, the advent of

induced pluripotent cells (iPSCs) is making it possible to create human chondrocytes and cartilage from skin fibroblasts from patients with chondrodysplasias. Noriyuki Tsumaki (Kyoto University, Japan) described the application of iPSC technology to disease modeling for chondrodysplasias. His group found that cartilaginous tissues derived from hiPSCs generated from patients with FGFR3 chondrodysplasia mutations reproduced the disease pathology and thus offer a novel model to develop therapeutic agents. Catherine Merry (University of Nottingham, United Kingdom) developed a similar approach to derive chondrocytes from hiPSCs obtained from MHE patients. She reported the identification of a plasma-derived protein (inter-α-inhibitor) as a culture additive that negates the traditional requirement for surface coating of tissue culture plastic with extracellular matrix for human pluripotent stem cell culture. Her group also developed 3D culture environments to better mimic the in vivo conditions, using hydrogel-based or fibrous scaffold-based culture conditions.

José Luis Millán (Sanford Burnham Prebys Medical Discovery Institute) pioneered methods for treating hypophosphatasia caused by mutations in tissue-nonspecific alkaline phosphatase. These methods involve enzyme replacement targeted to bone (82). Skeletal mineralization involves matrix vesicles that initiate and propagate mineralization from intralumenal to extravesicular space, followed by deposition of mineral onto the collagenous matrix (83). The phosphatases are involved in mineralization in various ways, including the hydrolysis of pyrophosphate (a strong anti-mineralization agent) and local release of phosphate. Thus, enzyme replacement therapy compensated for low alkaline phosphatase activity, reversed pyrophosphate accumulation and allowed resumption of mineralization. Targeting these mechanisms could be useful to prevent and treat diverse forms of ectopic calcification, including arterial calcification. Whether these methods might prove useful for treating MHE remains speculative at the moment.

One of us (MP) in collaboration with Kevin Jones at the University of Utah, re-examined craniofacial X-ray scans from MHE patients and over half of them exhibited moderate defects or osteochondroma-like outgrowths in the endochondral cranial base, specifically in the clivus. Similar phenotypes were observed in the cranial base of mutant *Col2-Cre*^{ER};*Ext1*$^{f/f}$ or *Aggrecan-Cre*^{ER};*Ext1*$^{f/f}$ mouse models of MHE (84). These findings indicate that not only the cervical vertebrae (3) but also the cranial base may be affected by MHE, expanding the potential anatomical sites and structures that a pharmacologic therapy would need to reach in patients.

Yu Yamaguchi (Sanford Burnham Prebys Medical Discovery Institute) presented data that Ext1 knockout specifically targeted to the progenitor cells in the perichondrium (*Fsp1-Cre;Ext1*$^{F/F}$mice) leads to osteochondroma formation (85). This confirms data first reported by one of us (MP) and collaborators using mouse models of MHE (40). Osteochondromas were found to develop from mesenchymal stem cell-like progenitor cells residing along the boundary between growth plate and perichondrium. Both groups showed that *Ext1*-deficient progenitor cells display enhanced BMP signaling and chondrogenic differentiation in vitro. Most importantly, Yamaguchi reported that systemic administration of the BMP inhibitor LDN-193189 effectively reduced osteochondroma growth in *Col2-Cre*^{ER};*Ext1*$^{f/f}$ and Fsp1-Cre;Ext1$^{F/F}$ mice (85). A similar osteochondroma-suppressive effect of LDN-193189 was

previously published by the Pacifici's group using *Aggrecan-Cre*^{ER};*Ext1*$^{f/f}$ mouse models of MHE (84). In vitro studies with mouse embryo chondrogenic cells showed that LDN-193189 action resulted in decreased canonical BMP signaling through pSMAD1/5/8. These studies provide a novel therapeutic approach for treating MHE.

Clinical care of MHE patients

A final session was dedicated to patients and was organized by the MHE Research Foundation. Patient advocacy by the MHE Research Foundation has been a driving force in directing research interest and attention to this skeletal disorder. The session was attended by a significant number of MHE patients and their families. The purpose was to provide a layperson summary of major scientific findings, offer an opportunity for researchers, patients and families to mingle and interact, point out issues arising in care and treatment, and facilitate contacts between physicians and MHE patients. One of us (JE) provided an overview of the Conference. His presentation was followed by a MHE Live Patient Clinic Demonstration, led by Drs. Dror Paley, David Friedman and Craig Robbins. Subsequent presentations focused on Best Treatment Strategies in Children and Adults for Upper Extremity Deformities of MHE, Straight Talk on Crooked Legs, MHE of the Hip and How to Restore Hip Motion and MHE of the Spine.

Conclusions and perspectives

The above synopsis of data and insights by participating research groups and from follow-up studies demonstrates that much has been learned over the past 2–3 years about the clinical and biological complexities and pathogenic mechanisms in MHE. It is ever more clear now that MHE is more than a pure musculoskeletal disorder, and can affect patients in additional and insidious manners, including their overall wellbeing and self regard, occupational possibilities, social interactions and independence. Anatomical sites that were regarded to be less affected, such as the thoracic and lumbar spine and the cranial base, have turned out to be affected by mild to moderate scoliosis and osteochondroma-like lesions respectively, making MHE more pervasive than previously realized. In terms of cellular and molecular pathogenesis subtending osteochondroma development, the work presented provides strong evidence that mesenchymal progenitors residing in perichondrium and groove of Ranvier may be major culprits; the cells would shift from a mesenchymal to a chondrogenic lineage, undergo chondrogenesis and initiate tumor formation (40). Transgenic mouse studies have established that compound heterozygous loss of *Ext1* and *Ext2* or conditional loss of both *Ext1* alleles are necessary and sufficient to cause osteochondroma formation. In this regard, it is interesting to note that an appreciable number of MHE patients have been found to bear compound heterozygous mutations in both *EXT1* and *EXT2* (66, 86), possibly indicating that their disease course may not require an additional "second hit" such as LOH and could be more severe given that the double mutation is in every cell. Presented work indicated that osteochondroma formation may directly or indirectly involve additional genes, including *Erk1/2*, *Ptpn11* and *SoxC*, and may also rely on and exploit mechanisms normally involved in tissue repair and regeneration and inflammation. Arguably, a most promising outcome of the Conference was the identification of potential therapeutic means by which osteochondroma formation could be countered. High throughput drug screens conducted by

one of us (JDE) have identified a promising small molecule compound that is undergoing active testing at the moment. Compounds that interfere with canonical BMP signaling have demonstrated effective inhibitory activity against osteochondroma formation in mouse models, in line with the fact that BMP signaling is required for chondrogenesis. These and other proof-of-principle studies have thus provided a strong basis and much impetus and desire to move ahead as readily as possible toward the testing of a therapeutic strategy in a MHE clinical trial.

The Conference was thus a critical and thought-provoking forum for assessing the most recent basic and translational medicine research and creating a platform and springboard for future research. This should include: (1) further studies into the enzymology and regulation of HS synthesis, in particular the possibility of modulating HS assembly through alternate pathways; (2) analysis of how the immune system and physical forces might alter the progression of MHE; (3) identification of the signaling and molecular mechanisms that go awry in MHE due to HS deficiency; (4) further development of current and new therapeutic approaches, such as supplementation therapy, gene therapy, and pharmacological agents to restore normal levels of signaling; and (5) improvement of diagnostic and prognostic tools to assess patients' overall well being and response to treatment. The latter was particularly resonant with the patients and their families, and a multidisciplinary approach may eventually be needed to fulfill that goal.

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