

**CONFERENCE
ON
MULTIPLE HEREDITARY EXOSTOSES
ABSTRACT BOOK**

OCTOBER 25-27, 2002

**ARIZONA CANCER CENTER, UNIVERSITY OF ARIZONA
1515 North Campbell Avenue
Tucson, Arizona**

and the

**HILTON TUCSON EAST HOTEL
7600 East Broadway, Tucson, Arizona**

The Molecular Genetics Program gratefully acknowledges support from our sponsors:

**THE MIZUTANI FOUNDATION for GLYCOSCIENCE
THE ORTHOPAEDIC RESEARCH SOCIETY
MHE COALITION
THE NATIONAL INSTITUTES of HEALTH
THE ARIZONA CANCER CENTER**

CONFERENCE
ON
MULTIPLE HEREDITARY EXOSTOSES ABSTRACT BOOK
OCTOBER 25-27, 2002

ARIZONA CANCER CENTER, UNIVERSITY OF ARIZONA
1515 North Campbell Avenue, Tucson, Arizona
and the
HILTON TUCSON EAST HOTEL
7600 East Broadway, Tucson, Arizona

The Molecular Genetics Program gratefully acknowledges support from our sponsors:

THE MIZUTANI FOUNDATION for GLYCOSCIENCE
THE ORTHOPAEDIC RESEARCH SOCIETY
MHE COALITION
THE NATIONAL INSTITUTES of HEALTH
THE ARIZONA CANCER CENTER

TABLE OF CONTENTS

Session I: Clinical Presentation and Treatment of MHE

Michael J. Goldberg, Tufts University School of Medicine, Tufts-New England Medical Center, Boston, MA

George Thompson, Case Western Reserve University, University Hospitals of Cleveland, Rainbow Babies & Children's Hospital, Cleveland, OH

"Clinical Presentation and Treatment of MHE" ----- p. 4

William Cole, University of Toronto Research Institute, The Hospital for Sick Children, Toronto, Canada

"Surgical Considerations in the Excision of Exostoses in Growing Individuals" p. 5

Session II: Medical Genetics

Wim Wuyts, University of Antwerp, Department of Medical Genetics, Antwerp, Belgium

"Hereditary Multiple Exostoses: a genetic overview" ----- p. 6

Gregory Schmale, University of Washington, Seattle, WA

Wendy H. Raskind, University of Washington, Seattle, WA

"Mutations in the EXT1 and EXT2 genes" ----- p. 7

Christine Alvarez, University of British Columbia, British Columbia's Children's Hospital, Vancouver, British Columbia, Canada

"Genotype Phenotype correlation in Hereditary Multiple Exostoses" ----- p. 8

Session III: Biochemistry of EXTs

Marion Kusche Gullberg, University of Uppsala, Department of Medical Biochemistry and Microbiology, Uppsala, Sweden

"EXT1 and 2 proteins and heparan sulfate biosynthesis" ----- p. 9

**Afternoon
Workshop:**

An Introduction to Therapeutic Strategies

Jeffery Mai, University of Pittsburgh School of Medicine, Pittsburgh, PA

"Gene transfer & protein transduction approaches for therapy" ----- p. 10

Evening

Workshop:

Issues in Living with MHE

Jacqueline Hecht, University of Texas Medical School at Houston, Houston, TX

Sarah Ziegler, MHE Coalition, Whitestone, NY

Chele Zelina, MHE Coalition, Olmsted Fall, OH

“Living with Multiple Hereditary Exostoses” ----- p. 11

Session IV:

Bone Development, Repair and Related Disorders I

Henry Kronenberg, Endocrine Unit, Massachusetts General Hospital and Harvard Medical School, Boston, MA

“Actions of parathyroid hormone-related protein on the growth plate” - p. 12

Benjamin Alman, University of Toronto, The Hospital for Sick Children, Toronto, Ontario, Canada
“Hedgehog and Parathyroid hormone related protein signaling in enchondromatosis” p. 13

Jacqueline Hecht, University of Texas Medical School at Houston, Houston, TX

“EXT1 and EXT2 Germline Mutations are the Most Common Cause of Diminished Heparan Sulfate in Exostosis Growth Plates” ----- p. 14

Session V:

Bone Development, Repair and Related Disorders II

T. Michael Underhill, University of Western Ontario, London, Ontario, Canada

“Importance of RAR-Mediated Gene Repression in Skeletal Development” --- p. 15

Yoshihiko Yamada, National Institute of Dental and Craniofacial Research, NIH, Bethesda, MD

“Perlecan mutations in mice and humans: critical role of perlecan in skeletal development and diseases” ----- p. 16

Fred Kaplan, University of Pennsylvania School of Medicine, Philadelphia, PA

“MHS: Multiple Hereditary Skeletons - An Endochondral Conundrum” ----- p. 17

Session VI:

EXT Function in Morphogenesis I

Dan Wells, University of Houston, Houston, TX

“Non-exostoses related alterations in the EXT1 deficient mice” ----- p. 18

David Ornitz, Washington University School of Medicine, St Louis, Missouri

“FGF Signaling in Skeletal Development” ----- p. 19

Jeffery Esko, University of California – San Diego, San Diego, CA

“A Murine Model For Hereditary Multiple Exostoses (HME)” ----- p. 20

Rahul Warrior, University of California – Irvine, Irvine, CA

“Modulation of growth factor signaling by the ext2 tumor suppressor gene” p. 21

Session VII:

EXT Function in Morphogenesis II

Scott Selleck, University of Minnesota, Minneapolis, MN

“Proteoglycans as regulators of growth factor/morphogen distributions in tissues” p. 22

Gyeong-Hun Baeg, Howard Hughs Medical Institute, Harvard Medical School, Boston, MA

“The role of HSPGS in the formation of the Wingless gradient in Drosophila” p. 23

Siu Ing (Inge) The, University of Massachusetts Medical School, Boston, MA

“The role of Drosophila EXT1 homolog tout velu in Hedgehog distribution” -- p. 24

Session I: Clinical Presentation and Treatment of MHE

Michael J. Goldberg, MD, Professor and Chairman, Department of Orthopaedics, Tufts University School of Medicine, Tufts-New England Medical Center

George Thompson, MD, Professor of Orthopaedic Surgery and Pediatrics, Case Western Reserve University, University Hospitals of Cleveland, Director, Pediatric Orthopaedics, Rainbow Babies Children's Hospitals

“Clinical Presentation and Treatment of MHE”-----

The goals for this session are (1) to detail the differential diagnosis of children with multiple osteochondromas, and related conditions, (2) outline the patterns of growth of those with multiple hereditary exostosis, (3) present the management of the common deformities resulting from MHE; and (4) specifically discuss the difficult management of osteochondroma that involve the hip and spine.

From the perspective of the pediatric orthopaedic surgeon, the child with multiple bumps may have one or several different conditions. A similar molecular defect may be found in disorders that are phenotypically different; and a similar clinical picture may result from different gene defects. For the clinician however, diagnosis and syndrome identification are important inasmuch as management and natural history may be different.

Syndrome identification and clinical features of multiple hereditary exostosis (Type 1 and Type 2); metachondromatosis; proteus syndrome and Langer Gideon and trichorhinophalangeal syndromes (Type 1 and 2) will be presented.

Children with multiple hereditary exostosis have a disturbed growth pattern. Final height for many falls at or below the 3rd percentile and is further complicated by disproportionate short limbs, mainly in the mesomelic segment (radius/ulna; tibia/fibula). Growth hormone to increase final height is theoretically contraindicated. Although each osteochondroma has the potential of producing an impairment, common deformities are encountered. These include bilateral genu valgum; progressive ulnar deviation of the hand and wrist; limitation of the forearm pronation/supination; and valgus of the foot and ankle. Osteochondroma in certain vulnerable areas (eg: hip and spine) can produce substantial functional disability.

There is not universal agreement as to how best to manage each of the common deformities. Indeed, here is controversy regarding when to remove a single specific osteochondroma.

Session I: Clinical Presentation and Treatment of MHE

William G. Cole, MD, FRACS, FRCSC, Head, Division of Orthopaedics and Senior Scientist, Research Institute, Hospital for Sick Children, Professor of Surgery and Genetics, University of Toronto

“Surgical Considerations in the Excision of Exostoses in Growing Individuals” -----

Before skeletal maturity, exostoses may be excised if they produce functional impairment and/or pain. These problems become more evident as the child and the exostoses grow. Chondrosarcomas rarely occur in the skeletally immature child. Care evaluation of symptomatic exostoses is required before a final decision regarding surgery is made with families. Computerized tomograms with 3-dimensional reconstruction of the affected bones are helpful in planning excision of one or more exostoses. The proximity of the exostosis to the adjoining growth plate, neurovascular structures, bones, joints and viscera need to be determined.

The cap of the exostosis may be large and overhang neurovascular structures. Under these circumstances, it is often safer to plan a surgical approach to the base rather than to the cap of the exostosis. The neurovascular structures are identified and traced towards the lesion. In this way, the base of the exostosis can be defined while the adjoining neurovascular structures are retracted. Removal of the trabecular bone of the exostosis enables the lesion to be collapsed. The later procedure takes the tension off adjoining neurovascular and other structures which can then be more safely dissected from the exostosis. The cartilage cap is completely removed in order to prevent regrowth of the lesion.

The periosteum (perichondrium) on the growth plate side of the base of exostoses is usually white and thick in contrast to the thinner normal periosteum elsewhere around the lesion. Distal-tibial exostoses may grow laterally resulting in significant bowing of the fibula. Three-dimensional imaging reveals that the fibula is thin, scalloped and sclerotic at the site of impingement of the tibial exostosis. Removal of the coarse trabecular bone of the lesion enables the cap to be separated from the fibula. The fibular deformity does not have to be corrected. Over the following 6-12 months, the fibula spontaneously thickens and remodels with loss of the clinical deformity.

At skeletal maturity, it is generally impractical to remove more than a few symptomatic lesions from the bones of individuals with MHE. Consequently, affected adults need advise concerning the circumstances in which they need to subsequently consult with a musculoskeletal tumor service. Protocols are needed for the monitoring of exostoses of the spine, chest and pelvis as chondrosarcomas occurring in these sites often only become clinically apparent once they have grown to a huge size.

Session II: Medical Genetics

Wim Wuyts, PhD University of Antwerp, Department of Medical Genetics,
Universiteitsplein 1-2610 Wilrijk (Belgium)

“Hereditary Multiple Exostoses: a genetic overview” -----

Hereditary multiple exostoses (MHE/EXT) is an autosomal dominant bone disorder characterized by the presence of bony outgrowths (exostoses) which are mainly located at the juxta-epiphyseal regions of the long bones. The exostoses develop shortly after birth and increase in number and size throughout childhood, causing pain by pressuring neighboring tissues. Several complications have been described with the most life threatening being the malignant transformation of an exostosis into a chondrosarcoma.

In the past ten years two EXT causing genes, EXT1 on chromosome 8q24 and EXT2 on chromosome 11p11-p12, have been identified by positional cloning methods and presence of a third locus (EXT3) on 19p has been suggested. Sequence analysis of both EXT genes revealed significant homology throughout the entire protein, but most striking in the carboxy terminal region. This observation leads to the identification of 3 additional homologous EXT-like (EXTL) proteins defining a protein family currently comprising 5 members. Recently, functional analysis has shown that the members of the EXT/EXTL family are glycosyltransferases with EXT1 and EXT2 forming a functional complex involved in heparan sulfate biosynthesis.

Mutation analysis in multiple EXT patients has revealed that loss of function mutations in EXT1 and EXT2 are responsible for the majority of EXT Cases. In addition to isolated EXT, multiple exostoses can also be found in syndromes such as Langer-Giedion and P11pDS, which are caused by larger chromosomal rearrangements in the EXT1 and EXT2 chromosomal regions.

Session II: Medical Genetics

Gregory Schmale, MD, Assistant Professor, University of Washington, Seattle, Children's Hospital & Regional Medical Center

Wendy H. Raskind, MD, PhD, Associate Professor, University of Washington, Seattle

“Mutations in the EXT 1 and EXT 2 genes” -----

In the past seven years, since EXT1 (8q24.1) and EXT2 (11p11-13) genes responsible for HME were identified, multiple EXT mutations have been described. A review of the literature documented 82 and 39 different mutations in the EXT1 and EXT2 genes respectively. Twenty of the EXT1 mutations and 4 of the EXT2 mutations were identified in more than one family. The EXT1 gene mutations include 45 frameshift, 14 missense, 16 nonsense and five splice site mutations, as well as two mutations with one or more amino acid deletions. The EXT2 gene mutations included 16 frameshift, 6 missense, 11 nonsense and six splice site mutations. In each gene there were proportionally more mutations in the first exons, with fewer than expected mutations in the carboxy terminal regions. There are clustered deletions following a polycytosine tract in exon 6 of EXT1 resulting in a frameshift mutation and protein termination. No similar strings have been noted in EXT2. Such polypyrimidine tracts have been proposed as deletions hot spots. Numerous missense mutations affecting residue R340 in EXT1 have been observed, suggesting that this region of the gene serves a crucial function. No similar region was seen in EXT2.

Although there has been some suggestion that a third locus for Hereditary Multiple Exostoses (HME) exists on chromosome 19, no gene has been identified by the group who reported that linkage, and no other group has yet confirmed this location in another population. No mutations in the EXT-like genes (EXTL-1-2-3) have been identified to date in any patient with MHE.

Session II: Medical Genetics

Christine Alvarez, MD, FRCSC, Assistant Professor, Department of Orthopaedics, University of British Columbia, British Columbia's Children's Hospital

“Genotype Phenotype correlation in Hereditary Multiple Exostoses” -----

C.M. Alvarez, S.J. Tredwell, M.R. Hayden, British Columbia Children's Hospital

Introduction – Hereditary Multiple Exostoses is an autosomal dominant condition caused by a mutation in one of the EXT genes. The gene product of EXT1 is felt to be involved in the tumor suppressor system. Mutations in EXT1 and EXT2 result in complete loss of function of the gene resulting in exostoses development. HME has a wide spectrum of clinical presentation. The question addressed is whether the variability is based on the genetic make-up of these patients with respect to the EXT genes or whether there are secondary, non-genetic forces affecting the expression of the disease.

Objective – To explore the variation of the disease expression in HME to determine if a genotype-phenotype correlation exists.

Method – DNA was extracted from blood. Linkage analysis using polymorphic satellite markers was utilized and based on this the appropriate EXT gene was sequenced. Mutation identification and verification was completed. Phenotypic data, 78 parameters in total, were drawn from clinical and radiographic examinations. These were divided into 4 major categories, limb alignment, lesion quality, limb segment lengths and stature and range of motion. Phenotypic variables were compared between patients with mutations in EXT1 and EXT2, and analyzing males and females separately. Care was taken to ensure that other factors such as mutation type, severity and location were not influencing phenotype.

Results – This is a pilot study consisting of 10 families with 75 participating members and 32 affected individuals. Mutations were identified and verified for 8 of the 10 families, leaving 26 subjects with known genotypes and complete phenotypic information.

Lesion number revealed individuals with mutations in EXT1 had more lesions than in EXT2 and males had more lesions than females, EXT1 had more lesions located on the flat bones and males had more metaphyseal flaring than females. EXT1 patients were shorter than EXT2 but no gender difference was noted. In terms of segment lengths all HME patients had more shortening of the upper extremities than lower extremity. For limb alignment and bony deformity EXT1 had more abnormal features than EXT2 and males were worse than females. Range of motion of all tested joints showed no clinically significant restrictions.

Conclusions – Mutations in EXT1 appear to be associated with a worse phenotype. Males are worse than Females. These results show a trend towards a phenotype-genotype correlation in HME in that EXT1 and males are the most severely affected.

Table 1. Mutation Identification

Family	Gene	Mutation	Novel	Type	Severity
1	EXT1	G1019A R to H	No	MS	Mild
2	EXT2	G730T E to X	Yes	NS	Severe
3	EXT2	X751T Q to X	Yes	NS	Severe
4	EXT1	?		?	?
5	EXT2	G679A D to N	No	MS	Mild
6	?	?		?	?
8	EXT1	G1174A	Yes	SS	Severe
16	EXT1	G1723C	Yes	SS	Severe
17	EXT2	451 del4 + T458C Stop 1293	Yes	FS	Severe
18	EXT1	C357G	Yes	NS	Severe

Session III: Biochemistry of EXTs

Marion Kusche-Gullberg, Docent, University of Uppsala, Department of Medical Biochemistry and Microbiology

“EXT 1 and 2 proteins and heparan sulfate biosynthesis” -----

Heparan sulfate, a highly negative charged polysaccharide present at the cell surface and in the extracellular matrix, is an important mediator of complex biological processes. The polysaccharide is elongated by the alternating transfer of glucuronic acid and N-acetylglucosamine units, from the corresponding UDP-sugars, to the non-reducing end of the growing chain. Concomitant with elongation, the polymer is modified through a series of reactions that requires the action of several different enzymes. The extent of these reactions varies, giving rise to heparan sulfate chains with different structural properties. However, the precise roles of these enzymes in determining the degree of chain elongation and modification are poorly understood. Recent studies have shown that EXT1 and EXT2 encode proteins involved in heparan sulfate chain elongation and that the EXT proteins like most of the enzymes involved in heparan sulfate biosynthesis are type II membrane proteins which reside in the Golgi.

Analysis of the catalytic activities of the EXT1 and EXT2 indicate that the biological function heparan sulfate polymerization unit is a complex of EXT1 and EXT2 and that complex formation induces a relocalization from the endoplasmic reticulum to the Golgi compartment. To understand the individual roles of EXT1 and EXT2, the proteins have been expressed in different mammalian cell systems either alone or together with the first heparan sulfate modifying enzyme the glucosaminyl N-deacetylase/N-sulfotransferase (collaboration with L. Kjellen and coworkers). The effect of reduced EXT 1 expression on heparan sulfate structure has been studied in fibroblasts isolated from mice with a gene trap mutation in EXT1 (collaboration with K. Sugahara and coworkers).

Afternoon

Workshop: An Introduction to Therapeutic Strategies

Jeffery Ching-Kwei Mai, University of Pittsburgh School of Medicine,
Graduate Student Researcher/Registered Student, Biochemistry & Molecular Genetics

“Gene transfer & protein transduction approaches for therapy” -----

Our laboratory has focused on gene transfer approaches for rheumatoid arthritis, as well as cancer gene therapy. Our studies have included adenovirus-mediated gene therapy in murine and rabbit models of arthritis and a phase I clinical trial for the treatment of rheumatoid arthritis. A general overview will be provided on current gene transfer approaches which may have relevance for the therapy of MHE. We have also recently characterized a number of arginine and lysine-rich protein transduction domains (PTDs), which are able to efficiently enter cells in the absence of heparan sulfate and other glycosaminoglycans. Given the highly efficient level of delivery of both small and large cargos by PTDs, these peptides are attractive as agents for use in protein therapy, as well as vectors for delivering therapeutic peptides and nucleic acids. These PTDs and their potential applications will be discussed.

**Evening
Workshop: Issues in Living with MHE**

Sarah Ziegler, MHE Coalition, National Director and Coordinator of Research,
Executive Director, The National MHE Registry

Chele Zelina, MHE Coalition, President

Susan Wynn, MHE Coalition, Vice President/MHE and Me, Director

Jacqueline Hecht, PhD, Professor of Pediatrics, University of Texas Medical School at Houston,
University of Texas Houston Medical Center

“Living with Multiple Hereditary Exostoses” -----

The MHE Coalition is a support group that has a unique perspective as our large collection of over 800 families offers the medical and research community a glimpse into the lives of individuals and families living with MHE. Many of the people who contact our organization for support have moderate to severe cases of MHE and as such face a number of challenges, some of which have not been appreciated as part of the natural history of this condition. These issues will be discussed in an interactive format. While many families have multiple affected members and some can trace the disease back several generations, we are also contacted by those who have no family history and seek us out when their child is newly diagnosed in order to obtain information that is not readily available from their health care providers. Living with MHE can be a fine balancing act for most families. The physical and emotional impacts of this disease on daily living will be discussed and are based on our own experiences and those of hundreds of families we represent. Topics to be discussed include: emotional, psychological, educational, financial, medical treatment, genetic testing and family planning issues. All of these topics are interwoven into a large patchwork quilt, entitled “Living with MHE,” which is compiled in the MHE Coalition Information Packet that will be available to workshop participants. Opportunities for research studies will also be discussed.

Session IV: Bone Development, Repair and Related Disorders I

Henry Kronenberg, MD, Professor of Medicine, Chief, Endocrine Unit, Massachusetts General Hospital and Harvard Medical School

“Actions of parathyroid hormone-related protein on the growth plate” -----

Parathyroid hormone-related protein (PTHrP) acts to modestly increase chondrocyte proliferation and to dramatically halt the exit of chondrocytes from the proliferative pool, thus delaying chondrocytes differentiation into post-mitotic, hypertrophic chondrocytes. The mediators of PTHrP's actions are largely unknown. We have used two approaches to identify the roles of G proteins thought to act downstream of the PTH/PTHrP receptor in the growth plate. Chimeric growth plates containing a mixture of normal chondrocytes and chondrocytes descended from embryonic stem cells homozygous for ablation of the *Gs cx* gene were examined. As with chondrocytes missing the PTH/PTHrP receptor, the *Gs Cx (-/-)* chondrocytes left the proliferative pool very early and became hypertrophic prematurely. Surprisingly, however, the normal chondrocytes did not undergo extra rounds of proliferation, despite the expected increase in PTHrP production at the top of the growth plate. Excess Gq activation of the PTH/PTHrP receptor in this setting may, in some way, account for this result. To explore further the role of Gq signaling by the PTH/PTHrP receptor in the growth plate, mice were generated with “knock-in” of a mutant PTH/PTHrP receptor that signals through *Gs cx* normally but cannot activate Gq/phospholipase C because of mutations introduced into the receptor's second intracellular loop. The resultant mice have growth plates in vitro confirm that phospholipase C signaling acts to oppose the actions of adenylate cyclase on the growth plate. In the wild-type growth plate, the actions of adenylate cyclase dominate the response to PTHrP.

Targets downstream of *Gs* are still uncertain. One likely target is the cdk inhibitor, p57. Knockout of p57 rescues much of p57 mRNA and protein. Other cell cycle proteins and transcription factors are plausible mediators of PTHrP action, as well.

Session IV: Bone Development, Repair and Related Disorders I

Benjamin Alman, MD, FRCSC, Assistant Professor, Canadian Research Chair, Program in Developmental Biology and Division of Orthopaedic Surgery, University of Toronto, The Hospital for Sick Children

“Hedgehog and Parathyroid hormone related protein signaling in enchondromatosis” -----

Enchondromas are common benign cartilage tumors of bone. They can occur as solitary lesions, or as multiple lesions in enchondromatosis (Ollier’s and Maffucci’s diseases). Clinical problems caused by enchondromas include skeletal deformity and the potential for malignant change to chondrosarcoma. The extent of skeletal involvement is variable in enchondromatosis, and may include dysplasia that is not directly attributable to enchondromas. Enchondromas are usually in close proximity to, or in continuity with, growth plate cartilage. Consequently, they may result from abnormal regulation of proliferation and terminal differentiation of chondrocytes in the adjoining growth plate. In normal growth plates, differentiation of proliferative chondrocytes to post-mitotic hypertrophic chondrocytes is regulated in part by a tightly coupled signalling relay involving Parathyroid hormone related protein (PTHrP) and Indian hedgehog (IHH). PTHrP delays the hypertrophic differentiation of proliferating chondrocytes, while IHH promotes chondrocyte proliferation. We identified a mutant PTH/PTHrP type I receptor (PTHR1) in human enchondromatosis that signals abnormally *in vitro* and causes enchondroma-like lesions in transgenic mice. The mutant receptor constitutively activates Hedgehog signaling. We demonstrated that excessive Hedgehog signaling is sufficient to cause formation of enchondroma-like lesions, by expressing a transcription factor activated by hedgehog signaling (Gli-2) driven by type II collagen regulatory elements in mice. This data shows that dysregulation of IHH-PTHrP signaling in the growth plate can cause cartilage neoplasia, and suggest an important role for hedgehog signaling in the etiology of enchondromatosis.

Session IV: Bone Development, Repair and Related Disorders I

Jacqueline Hecht, PhD, Professor of Pediatrics, University of Texas Medical School at Houston, University of Texas Houston Medical Center

“EXT1 and EXT2 Germline Mutations are the Most Common Cause of Diminished Heparan Sulfate in Exostoses Growth Plates” -----

Jacqueline T Hecht (1,2*), Elizabeth Hayes (1), Catherine R. Hall (1), Hong Li (1), Richard Haynes (2), William Cole (3), Robert Long (4), Mary C. Farach-Carson (4). And Daniel D. Carson (4)

(1)Department of Pediatrics, University of Texas Medical School at Houston, Shriners Hospital for Children in Houston, TX; (2) Department of Surgery, Hospital for Sick Children, Toronto, Canada; (3) Department of Biological Sciences, University of Delaware, Newark, DE

Hereditary multiple exostoses (HME) is a condition associated with development and growth of bony exostoses juxtaposed to the growth plate at the ends of long bones. HME is caused by germline mutations in EXT1 and EXT2 genes. The EXT1 and EXT2 genes are glycosyltransferases that play pivotal roles in the biosynthesis of heparan sulfate on proteoglycans. Heparan Sulfate is an important constituent of cell surface and extracellular matrix proteoglycans and influences growth factor bioavailability and activity. We, and others, have previously suggested that a two hit mutational model applies to the development of an exostosis where a germline mutation coupled with a somatic mutation results in the loss of EXT1 or EXT2 function and subsequent tumor formation. We report the results of direct sequencing and LOH analysis of twenty-one exostoses from nineteen HME families and twelve solitary exostoses. Of the twenty-one exostoses screened, we found one HME case with a mutation in the EXT1 and EXT2 genes and one solitary case in which two somatic mutations, a deletion and LOH was identified. This provides limited support for the two hit hypothesis involving the EXT1 and EXT2 genes for the development of an exostosis.

To evaluate the effect of EXT1 and EXT2 mutations on growth plate chondrocytes, we evaluated four growth plates from two HME and two solitary exostoses. Mutational events were correlated with the presence/absence and distribution of heparan sulfate and the normally abundant proteoglycan, perlecan (PLN). DNA from HME Patients demonstrated heterozygous germline EXT1 or EXT2 mutations, and DNA from one solitary exostosis demonstrated a somatic EXT1 mutation. No LOH was observed on any of these samples. The chondrocyte zones of four exostosis growth plates showed the absence of heparan sulfate, as well as, diminished and abnormal distribution of PLN. Furthermore, *in vitro* metabolic labeling of HME chondrocytes derived from several patients demonstrates >90% reduction in heparan sulfate and, unexpectedly, chondroitin/dermatan sulfate synthesis. These results indicate that while multiple mutational events do not occur in the EXT1 or EXT2 genes, there is diminished amount of heparan sulfate in these exostosis growth plates. Taken together, these findings suggest that heterozygous mutations lead to a functional knock-out of the exostosis chondrocytes ability to synthesize heparan sulfate chains and imply a link between heparan sulfate assembly and that of other proteoglycans in cartilage.

(This work is supported by Shriners Hospital grant and NIH grant)

Session V: Bone Development, Repair and Related Disorders II

T. Michael Underhill, B.Sc., PhD, Assistant Professor, University of Western Ontario, School of Dentistry, Division of Oral Biology

“Importance of RAR-Mediated Gene Repression in Skeletal Development” -----

T. Michael Underhill, Lisa M. Hoffman and Andrea D Weston, Department of Physiology and School of Dentistry, Faculty of Medicine and Dentistry, The University of Western Ontario, London, Ontario, Canada.

Development of the appendicular skeleton relies on a complex interplay between multiple signaling pathways to coordinate condensation and differentiation of chondroprogenitors. Although the phenotypic changes associated with chondroblast differentiation have been well characterized, much less is known about the mechanisms underlying these changes. Retinoid excess has been associated with congenital malformations of the skeleton, premature closure of the growth plate and reduced bone mass, indicating that many of the stages within the skeletogenic program appear to be affected by retinoids. To better understand the role of retinoids in chondrogenesis we have used a variety of approaches to assess retinoic acid receptor function in chondrogenesis. Overexpression of a weak constitutively form of the retinoic acid receptor, RSR, in the limbs of transgenic mice causes appendicular skeletal defects due to an interference of chondrogenesis (J. Cell Biol. 136:445-457). Analysis of these animals shows that transgene expression prevents chondroblast differentiation, maintaining a prechondrogenic phenotype, even in response to bone morphogenetic proteins (BMPs) (Weston et al., J. Cell Biol. 148:679-690). Subsequently, we have established a close association between RAR activity and the transcriptional activity of SOX9, a transcription factor required for cartilage formation. Specifically, inhibition of RAR-mediated signaling in primary cultures of mouse limb mesenchyme, results Sox9 expression and activity. This induction is attenuated by the histone deacetylase inhibitor, TSA and by co-expression of a dominant-negative N-CoR-1, indicating an unexpected requirement for RAR-mediated repression in skeletal progenitor differentiation. These results will be presented along with additional findings that provide a molecular framework for understanding the processes underlying the commitment and differentiation of chondrocytes.

This research was supported by grants to T.NM.U. from the Canadian Institutes of Health Research and the Canadian Arthritis Network.

Session V: Bone Development, Repair and Related Disorders II

Yoshihiko Yamada, BS, MS, PhD, Chief, Molecular Biology Section, Craniofacial Developmental Biology and Regeneration Branch, National Institute of Dental Research, National Institutes of Health

“Perlecan mutations in mice and humans: critical role of perlecan in skeletal development and diseases”

Perlecan has been identified as a major heparan sulfate proteoglycan in basement membrane and in some other extracellular matrices. The perlecan gene (*HSPG*) is more than 100 kb in size with at least 94 exons and encodes a ~400-kDa protein core with three glycosaminoglycan chains covalently attached. Perlecan has various biological activities, such as influencing cell growth and differentiation by modulating cellular signaling and by interacting with matrix and with growth factors. We and other groups previously created gene knockout mice for the perlecan gene in order to determine the role of perlecan in development. The perlecan-null mice showed chondrodysplasia, with micromelia, a narrow thorax, dyssegmental ossification of the spine, craniofacial abnormalities, and, in 6% of mice exencephaly. The radiographic, clinical, and chondro-osseous morphology of the mice are remarkably similar to a lethal autosomal recessive disorder in humans, dyssegmental dysplasia, Silverman-Handmaker type (DDSH). Individuals have a flat face, micrognathia, cleft palate, reduced joint mobility, and frequently have a encephalocoele. We identified a homozygous 89-bp duplication in exon 34 of *HSPG2* in a pair of siblings with DDSH born to consanguineous parents and heterozygous point mutations in the 5' donor site of intron 52 and in the middle of exon 73 in the third unrelated patient, causing skipping of the entire exons 52 and 73 of the *HSPG2* transcript, respectively. These mutations are predicted to cause a frameshift, resulting in a truncated protein core. The cartilage matrix from the patients stained poorly with antibody perlecan. Truncated perlecan was not secreted by the patient fibroblasts. Thus DDSH is caused by a functional null mutation of perlecan. These findings demonstrate the critical role of perlecan in cartilage development.

Through linkage mapping and a positional candidate approach, Nichole et al. identified homozygous mutations in *HSPG2* of two SJS (Schwartz-Jampel syndrome) patients. SJS is a rare autosomal recessive skeletal dysplasia associated with myotonia. Patients with SJS survive and show much milder phenotypes compared to DDSH. However, the mechanisms causing the phenotype are unknown. We identified five different mutations that resulted in various forms of perlecan in three unrelated SJS patients. Heterozygous mutations in two SJS patients produced either truncated perlecan that lacked domain V or significantly reduced levels of wild type perlecan. The third patient has a homozygous 7kb-deletion that resulted in producing reduced amounts of nearly full-length perlecan. Thus partial functional mutations of *HSPG2* cause SJS.

Perlecan is present in the muscle BM and enriched at the neuromuscular junction (NMJ). To define the role of perlecan in NMJ function in vivo, we examined expression of molecules clustering at the NMJ of perlecan-null mice, which we previously created. We observed that most of molecules localized at normal NMJ, such as acetylcholine receptor (AChR), α - and β -dystroglycans, utrophin, rapsin, and agrin, were concentrated at the NMJ of perlecan-null mice. However, acetylcholinesterase (AChE) was completely absent at the perlecan-null NMJ, whereas it co-localized with perlecan and other clustered molecules at normal NMJ. Thus, it is likely perlecan is the unique acceptor molecule for AChE at the NMJ and that the enrichment of AChE at the synapse depends entirely on the presence of its binding partner, perlecan. Our results confirm that perlecan binds AChE and may explain the poor movement of newborn perlecan-null mice and the myotonia phenotype of SJS.

Session V: Bone Development, Repair and Related Disorders II

Fred S. Kaplan, MD, Professor of Orthopaedic Molecular Medicine, Chief, Division of Metabolic Bone Diseases and Molecular Orthopaedics, Director of the Center for Research in FOP and Related Diseases, University of Pennsylvania School of Medicine, University of Pennsylvania Medical Center, Hospital of the University of Pennsylvania,

“MHS: Multiple Hereditary Skeletons - An Endochondral Conundrum” -----

Frederick S. Kaplan, MD; Lourdes Serrano de la Pena, PhD; Jaimo Ahn, PhD; Paul C. Bilings, PhD; and Eileen M. Shore, PhD Departments of Orthopaedic Surgery, Medicine and Genetics, The University of Pennsylvania School of Medicine, Philadelphia, PA

Fibrodysplasia ossificans progressiva (FOP) is an autosomal dominant disorder that is characterized by congenital malformation of the great toes and by post-natal episodes of heterotopic endochondral ossification that lead to the formation of a heterotopic skeleton. In FOP patients, the process of heterotopic endochondral ossification and the resulting ectopic bone appear normal; the timing and location of induction of the bone formation are abnormal.

Given the role of BMP signaling in the induction of bone formation, alterations in the BMP signaling pathway were hypothesized to be involved in the pathogenesis of FOP. The first molecular insights into FOP arose from observations of increased BMP4 mRNA and protein expression in cells obtained from FOP patients, suggesting the presence of altered BMP4 regulation and/or signal transduction.

Our recent data indicates that the BMP4 signaling pathway is dysregulated in the cells of patients who have FOP. We have found that FOP cells fail to properly regulate ambient concentrations of BMP4 and fail to appropriately regulate the transcription of BMP pathway genes such as for BMP4 antagonists. Recent preliminary data indicates that the Type IA BMP receptor (BMPRIA) is six to 10 fold more abundant on the surface of FOP cells than control cells and may be constitutively active in FOP cells, while the expression of the Type IB BMP receptor (BMPRIB) is reciprocally downregulated in these cells. These data are consistent with developmental studies that show that postnatal over- expression of BMPRIA can lead to heterotopic ossification and that embryonic under-expression of BMPRIB can lead to digital malformations that closely mimic those seen in patients who have FOP. There are no mutations in the genes encoding BMP4, multiple BMP4 antagonists, or the BMP receptors in FOP patients. Taken together, these data allow us to hypothesize that a primary defect may exist in the BMP4 signaling pathway in FOP cells that leads to the overabundance of BMPRIA on the cell membrane, to constitutive activation of BMPRIA and to the post-natal molecular and cellular pathology of FOP.

Session VI: EXT Function in Morphogenesis I

Dan E. Wells, PhD, Professor and Chairman, University of Houston, Department of Biology and Biochemistry, The Wells Institute for Molecular Pertinacity

“Non-exostoses related alterations in the EXT I deficient mice” -----

Mutations in the EXT1 gene are responsible for human hereditary multiple exostosis (EXT) type 1. Previously, we generated EXT1-deficient mice by gene targeting, EXT1 homozygous mutants fail to gastrulate, are smaller than their littermates, and generally lack organized mesoderm and extraembryonic tissues. RT-PCR analysis of markers for visceral endoderm and mesoderm development indicates the delayed and abnormal development of both these tissues. Immunohistochemical staining revealed a visceral endoderm pattern of Indian hedgehog (IHH) in wild-type E6.5 embryos. However, in both EXT1-deficient embryos and wild-type embryos treated with heparitinase I, IHH failed to associate to the cells. A comparative study using two genetic lines, hybrid and inbred, was done in order to determine if the heterozygous EXT1 deficient mice show phenotypic, morphological, or histological differences. The results indicate significant differences in overall mouse weight between the wild type and heterozygous mice at many time points from 2-14 weeks, with the wild type mice weighing more than the heterozygous mice. However, no significant differences were seen in bone density, bone length, or percent of ossification between the wild type and heterozygous mice of either hybrid or inbred mouse lines. Interestingly, preliminary morphological analysis of the growth plate, indicate increases in the number of cells within the columnar region of the heterozygous mice. This apparently occurs without any associated affect on bone length or density.

Session VI: EXT Function in Morphogenesis I

David Ornitz, MD, PhD, Professor of Molecular Biology and Pharmacology, Washington University School of Medicine,

“FGF Signaling in Skeletal Development” -----

Kai Yu, Zhonghao Liu, Ann Jacob, David Ornitz, Department of Molecular Biology and Pharmacology, Washington University Medical School

Human chondrodysplasia and craniosynostosis syndromes, result from activating or neomorphic mutations in fibroblast growth factor receptors (FGFR) 1-3. These mutations underscore the essential role for FGF signaling in skeletal development. *Fgfrs* 1-3 are differentially expressed throughout skeletal development. *Fgfr1 and 2* are expressed in the perichondrium and periosteum, *Fgfr3* is expressed in proliferating chondrocytes, and *Fgfr1* is expressed in prehypertrophic and hypertrophic chondrocytes. These unique expression patterns suggest unique function in skeletal development. Past experiments have identified FGFR3 as a negative regulator of bone growth.

Embryos harboring homozygous null mutations in *Fgfr1* or *Fgfr2* die prior to skeletogenesis. To address the role of FGFR1 and FGFR2 in normal bone development, a conditional gene deletion approach was adopted. Homologous introduction of *cre* recombinase into the *Dermo1* gene locus allowed robust expression of CRE in mesenchymal condensations giving rise to the osteoblast and chondrocyte lineage. Inactivation of a floxed *Fgfr2* allele with *Dermo1-cre* resulted in mice with skeletal dwarfism and decreased bone density. Although the osteoblast lineage appeared to develop normally, the mature osteoblast was atrophic and produced less bone matrix. These studies demonstrate that FGFR2 signaling is not required for osteoblast differentiation but rather for the normal anabolic function of the osteoblast.

The ligands interacting with FGF receptors (FGFR) in developing bone have remained elusive. We have identified *Fgf18* expression in the perichondrium. Mice homozygous for a targeted disruption of *Fgf18* exhibit a growth plate phenotype similar to that observed in mice lacking *Fgfr3*. These data suggest that FGF18 acts as a physiological ligand for FGFR. In addition, mice lacking *Fgf18* display delayed ossification and decreased expression of osteogenic markers, phenotypes not seen in mice lacking *Fgfr3*. These data demonstrate that FGF18 signals through another FGFR to regulate osteoblast growth. Signaling to multiple FGFRs positions FGF18 to coordinate chondrogenesis in the growth plate with osteogenesis in cortical and trabecular bone.

Session VI: EXT Function in Morphogenesis I

Jeffery Esko, PhD, Professor, Department of Cellular and Molecular Medicine, Associate Director, Glycobiology Research and Training Center, University of California, San Diego

“A Murine Model For Hereditary Multiple Exostoses (HME)” -----

Beverly M. Zak (1), Dominique Strckens (2), Dan E. Wells (3), Glen Evans (4) and Jeffery D. Esko (1)

(1) Dept. of Cellular and Molecular Medicine, Glycobiology Research & Training Center, University of California, San Diego; (2) Dept. of Anatomy, University of California, San Francisco (3) Dept. of Biology and Biochemistry, University of Houston (4) Egea Biosciences, Inc., San Diego, California

Hereditary Multiple Exostoses (HME) is an autosomal dominant disease characterized by osteochondromas on the ends of long bones. The disease has been linked to mutations in EXT1 and EXT2. EXT1 and EXT2 encode subunits of the heparan sulphate (HS) copolymerase, which consists of GlucNAc and GlcA transferases and is responsible for the assembly of the backbone of the chain. Etiologic mutations in EXT1 reduce both enzyme activities in vitro and map the location of the GlcA transferase site in the protein to an N-terminal domain. In order to understand how a change in heparan sulfate biosynthesis might result in exostoses, null alleles of each gene have been created in mice. (Lin et al, 2000 Dev. Biol. 224:299-311; Strckens, Dominique, Zak, Beverly, Wells, Dan E., Evans, Glen, and Esko, Jeffery D., unpublished). Homozygous null embryos arrest development at gastrulation, with absence of markers for mesodermal differentiation. Cells derived from these embryos fail to make heparan sulfate, demonstrating the essential role of both genes in the assembly process. Cells derived from heterozygous embryos produce reduced amounts of heparan sulfate, but heterozygous embryos appear normal, develop to maturity, and reproduce. EXT1 heterozygotes rarely form exostoses (3/39), but EXT2 heterozygotes form exostoses more frequently (6/39). Compound heterozygotes (EXT1+/-EXT2+/-) develop exostoses at even higher frequency (26/88), but have other non-obvious defects. These findings suggest that the level of expression of heparan sulfate may contribute to the degree of penetrance of HME. Various environmental and nutritional parameters are being varied to determine how they affect the frequency and severity of exostoses. Chondrocytes derived from mutant mice exhibit differences in erk phosphorylation dependent on FGF2. Although it is unclear if this pathway plays a role in ectopic bone growth characteristic of the disease, a model can be put forward in which reduced growth factor signaling in perichondrial cells and chondrocytes affect their differentiation or division.

Session VI: EXT Function in Morphogenesis I

Rahul Warrior, PhD, Assistant Professor, University of California, Irvine, School of Biological Sciences, Department of Developmental and Cell Biology

“Modulation of growth factor signaling by the ext 2 tumor suppressor gene” -----

D. Bornemann (1), W.Staats (2), J. Duncan (1), S.B. Selleck (2) and R Warrior (1)

(1) University of California - Irvine, Department of Developmental and Cell Biology

(2) University of Minnesota - Genetics, Cell Biology and Development

There is increasing evidence that growth factor signaling is crucially affected by extracellular matrix components that regulate the diffusion and hence the distribution range of secreted ligands. Recent work in *Drosophila* and vertebrate embryos had demonstrated that mutations in several genes involved in glycosaminoglycan synthesis can dramatically affect signaling by Hedgehog, FGF, Wnt, and BMP ligands. Vertebrate EXT1 and EXT2 genes encode related proteins that are thought to interact directly and function as a heparan sulfate copolymerase. However it is currently unclear whether the two proteins also have independent or partially redundant functions. Mutations in *tout-velu* (*ttv*), the *Drosophila* ortholog of EXT1, have been characterized and shown to specifically affect Hh signaling. Biochemical and cell culture studies suggest that EXT1 and EXT2 proteins act as co-polymerases in the synthesis of glycosaminoglycans, polysaccharide chains attached to extracellular proteins. Thus it would be predicted that mutations in the EXT1 and EXT2 genes would have similar phenotypes. We have recently identified mutations in the *Drosophila* EXT2 ortholog. Surprisingly, our preliminary studies indicate that *ext2* mutants show defects in multiple growth signaling pathways in contrast to previous findings that the EXT1 ortholog *ttv* primarily affects diffusion of the growth factor Hedgehog (Hh)

Dr. Scott Selleck's group has collaborated with us to examine the effect of an allelic series of *ext2* mutations on the synthesis of heparan sulfate and chondroitin sulfate. These results will also be presented.

Session VII: EXT Function in Morphogenesis II

Scott B. Selleck, MD, PhD, Professor, Departments of Pediatrics and Genetics, Cell Biology and Development, University of Minnesota

“Proteoglycans as regulators of growth factor/morphogen distributions in tissues” -----

Jaime Reuter, Ross Waldrip, Xiaobo Chen, Will Staatz, and Scott B Selleck, Departments of Pediatrics and Genetics, Cell Biology and Development, The University of Minnesota, Minneapolis, MN - & - Department of Molecular and Cellular Biology, Arizona Cancer Center, The University of Arizona, Tucson, AZ

We have been investigating the function of glypicans, a family of heparan sulfate proteoglycans, during morphogenesis in the fruitfly, *Drosophila*. Earlier studies of dally and dally-like, the two glypicans in *Drosophila*, demonstrated that these GPI-linked proteoglycans are required for normal cellular responses to Wingless (Wg), a secreted growth factor of the Wnt family. These findings were consistent with models proposing that cell surface proteoglycans can serve as co-receptors to enhance the assembly of specific signaling complexes at the plasma membrane.

We report here a previous unknown function of dally-like, the capacity to inhibit long range signaling of Wg. Wg serves as both a short and long range signaling molecule during patterning of the wing, controlling the specification of sensory bristles along the anterior wing margin. *dly* mutants show wing margin defects indicative of elevated levels of long range Wg signaling. *achaete*, a gene known to require a high threshold of Wg signaling, is ectopically expressed in *dly* mutants, at positions distant from the Wg secreting cells. In preliminary experiments, we have found that the distributions of extracellular Wg are altered by reduction in *dly* function, consistent with a role for *dly* in limiting the distribution of Wg. Analysis of genetic mosaics confirm that *dly* acts 1) cell autonomously to promote Wg signaling and 2) non cell autonomously to restrict the range of WG activity. These findings support previous studies documenting that glypicans can promote Wg responses, but suggest that an additional critical function of these proteoglycans is to limit the distribution of secreted growth factors across tissues.

Our findings with *dly* suggest the possibility that changes in the levels of heparan sulfate in patients with MHE could alter the distributions of secreted growth factors at the growth plate. In particular, our results provide precedence for the ability of specific heparan sulfate proteoglycans to limit the distribution of growth factors, ensuring that only specific subsets of cells receive these cues. Reductions in heparan sulfate levels could permit the release of growth factors to cells that normally do not receive these signals. Thus, the growth of bony tumors in MHE patients could be the result of aberrant distributions of growth promoting signals.

In separate studies done in collaboration with Rahul Warrior (University of California – Irvine), we will report on the structural characterization of glycosaminoglycans from fruitflies bearing mutations in *ext2*. These findings support the requirement for both EXT1 and EXT2 proteins in generating normal levels of heparan sulfate during development.

This work is supported by funding from the NIH and NCI to Scott B. Selleck

Session VII: EXT Function in Morphogenesis II

Gyeong-Hun Baeg, Department of Genetics, Howard Hughes Medical Institute, Harvard Medical School, Perrimon Laboratory

“The role of HSPGs in the formation of the Wingless gradient in *Drosophila*” -----

Gyeong-Hun Baeg (1), Erica Selva (1), Norbert Perrimon (1,2)

(1) Department of Genetics; (2) Howard Hughes Medical Institute, Harvard Medical School

Many biochemical studies and genetic analyses have implicated Heparan sulfate proteoglycans (HSPGs) in Wingless signaling. Wingless proteins are tightly associated with cell membranes and extracellular matrix, and mutations in three genes required for HS glycosaminoglycan (GAG) biosynthesis and modification; *sugarless* (*sgl*; UDP-glucose dehydrogenase), *fringe connection* (*frc*; UDP-glucose transport) and *sulfateless* (*sfl*; N-deacetylase/N-sulphotransferase) were identified in screens for mutants that exhibit *wg*-like segment polarity phenotypes. The *Drosophila* protein core of HSPG, Dally and Dally-Like (Dly), are substrates for these enzymes and furthermore, loss of *dally* or *dly* gene activity is associated with defects reminiscent of loss of Wingless activity. Recently, a secreted enzyme *Notum*, the *Drosophila* homologue of *cx/B* hydrolase, has been implicated as a proteoglycan modifying enzyme which contributes to shaping Wingless gradient by altering the ability of cell surface Dally and Dly to stabilize extracellular Wingless. Together, these results strongly support the idea that HSPGs are involved in Wingless signaling by regulating its distribution. We have further characterized the role of HSPGs in Wingless distribution in *Drosophila* and found that both Wingless-expressing and –receiving cells require HSPG activity for its normal distribution. We also found that there are distinct roles for HSPGs and Frizzled receptors in modulating the distribution of Wingless. Furthermore, our data suggest that there is a possible role for HSPGs in Argosome-mediated Wingless transport.

Session VII: EXT Function in Morphogenesis II

Siu Ing (Inge) The, PhD, Assistant Professor, University of Massachusetts Medical School

“The role of *Drosophila* EXT I homolog *tout velu* in Hedgehog distribution” -----

We have discovered a *Drosophila* gene, *tout velu*, that is essential for the proper distribution of the Hedgehog molecule. *tout velu* is a member of the *EXT* gene family which is involved in the Multiple Exostoses Syndrome. *tout velu* mutants have been shown to have a defect in Heparan Sulfate Proteoglycans (HSPG) biosynthesis in *Drosophila melanogaster*. Our analysis is the first evidence that HSPGs are important for correct distribution of Hedgehog. The levels of secreted Hedgehog can induce different cell fates.

Secreted proteins, such as Hedgehog, need to find their way through a dense extracellular matrix of collagen, hyaluran, and proteoglycans. This transport is further complicated by the fact that some secreted proteins, like members of the Hedgehog family, are tethered to the membrane via a cholesterol moiety. Despite this membrane “anchor” they can be secreted and signal to cells several cell diameters away. We are interested in the role that HSPGs play in Hedgehog secretion and distribution and have begun to investigate the roles of *tout velu* in Hedgehog localization and distribution.