Dysregulation of Indian hedgehog – Parathyroid hormone related protein signalling in cartilaginous neoplasia

Sevan Hopyan

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy, Graduate Department of the Institute of Medical Science, University of Toronto

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Dysregulation of Indian hedgehog – Parathyroid hormone related protein signalling as a mechanism of cartilaginous neoplasia

Degree of Doctor of Philosophy, 2001

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ABSTRACT

Enchondroma is a benign cartilage-forming tumour of bone that might be caused by dysregulation of growth plate signals. A paracrine feedback loop couples Indian hedgehog (IHH), which stimulates growth plate chondrocyte proliferation, to Parathyroid hormone related protein (PTHrP), which regulates chondrocyte differentiation. Here I show that these two signalling pathways were uncoupled and IHH was dysinhibited in explant cultures of human enchondromas and their malignant counterparts. Chondrosarcomas. A heterozygous variant Type I PTH/PTHrP receptor (PTHRI) was discovered in the germline of one patient and as a somatic change in another with enchondromatosis. In this condition, multiple enchondromas may lead to deformity and chondrosarcoma. The variant PTHR1 suppressed Cyclic adenosine monophosphate baseline level in a dominant fashion and abolished Inositol triphosphate accumulation in vitro. Transgenic mice expressing the variant receptor under the regulatory elements of Type II collagen developed enchondromatosis.
I show that the transcription factor Gli2 was a positive regulator of Ihh function in the murine growth plate, while Gli3 was a negative regulator. Overexpression of Gli2 was found in human enchondromas, and was sufficient to cause enchondromatosis in transgenic mice. A lack of Gli3 accelerated another condition of benign chondrocyte neoplasia, synovial chondromatosis.

Patched 1 (PTCH1), a Hedgehog receptor, formed a complex with PTHR1, and this complex was required for effective accumulation of PTHR1 second messengers. The variant PTHR1 did not associate with PTCH1, and constitutively activated Hedgehog signalling in a manner that was likely dependent upon suppression of Protein kinase A.

Dysregulation of growth plate signals causes certain benign cartilage tumours. Agents that block Hedgehog signalling in particular might be useful in preventing the deleterious sequelae of enchondromas.
ACKNOWLEDGEMENTS

My greatest thanks to God. I apologise to all the people whose love and whose needs I have neglected during my selfish drive to complete this thesis. Chief among these are my sister Talar, my mother Lucya and my father Takvor.

I am indebted to my supervisors, mentors and friends, Ben Alman and Jay Wunder. Their enthusiasm, engagement and support in good times and bad were unparalleled and moving. I am grateful to Irene Andrulis and Bob Bell for their unconditional support over many years. My heartfelt thanks to Jane Aubin and Sean Egan, the other members of my thesis advisory committee, for scholarly exchange of such great value that it has changed me profoundly and, dare I say, irrevocably. Many thanks to Chi-Chung Hui for Gli2 constructs and Gli2 and Gli3 deficient mice as well as for supportive discussions.

Kunath, Brian Ciruna, Deanna Church, Ekaterina Hadjantonakis, Hassina Benchabane, Pamela Hoodless, John Hudson, Linda Doughty, Gabriella Nagy, Lily Morikawa (for histology), Michael Ho (for immunohistochemistry), Sandra Tondat and Lois Schwartz (for pronuclear microinjection), Malgosia Kowmacka (for ES cell media and feeder cells), Aileen Davis (for statistical help), Paul Reynolds, Mingyao Liu, Jim Dennis and Janet Rossant for help, support and general love. Bless them all.

I feel deep appreciation for the eager cooperation of patients and their families: may they benefit a thousand times over in return. For sharing precious reagents, I thank U.-I. Chung (for PTHR1 ES cells), F. de Sauvage (Genentech - for SMO cDNA), W. Gaffield (for cyclopamine), C-C Hui (for Gli2 and PTCH1 cDNAs and for Gli2 and Gli3 deficient mice), T. Ingolia (Ontogeny - for Shh-N protein), H. Juppner and A. Karaplis (for PTHR1 cDNA), C. Tabin (for IHH and SHH cDNAs), and Y. Yamada (for ColII promoter/enhancer cDNA). For human specimens in addition to those obtained from the Mt. Sinai Sarcoma Tissue Bank, I thank W. Cole, A. Gross, C. Hutchison and J. Wright.
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LIST OF ABBREVIATIONS

A – adenine
AML1 – Acute myelogenous leukemia 1
APC – Adenomatous polyposis coli
AS – Asparagine synthetase
α - anti: alpha
Bmp – Bone morphogenic protein
β Gal – Beta galactosidase
β2M – Beta-2-microglobulin
bp – base pairs
BrdU – bromodeoxyuridine
cm – centimetre
C – cytosine: cysteine (as in R150C)
cAMP – Cyclic adenosine monophosphate
CBFα1 – Core binding factor alpha 1
ci – cubitus interruptus
ColII – Type II collagen
ColX – Type X collagen
Cos2 – Costal 2
CPM – counts per minute
CREB – cAMP response element binding protein
CSA – chondrosarcoma
Dhh – Desert hedgehog
DMEM - Dulbecco’s modified eagle medium

E - embryonic day

ECA - enchondroma

ES - embryonic stem

EWS - Ewing’s sarcoma

EXT - Exostosis

fM - fentomolar

FBS - fetal bovine serum

FD - fibrous dysplasia

Fgf - Fibroblast growth factor

Fu - Fused

g - gram

G - guanine

GDP/GTP - guanine diphosphate/ guanine triphosphate

Gli - Glioblastoma

GP - growth plate

$^3$H - tritium

HA - haemaglutinin

Hh - Hedgehog

Ihh - Indian hedgehog

Ig - immunoglobulin

Igf - Insulin-like growth factor

IP - immunoprecipitate
IP$_3$ – Inositol triphosphate
kD – kilo-Dalton
l – liter
M – molar
μM – micromolar
mm – millimetre
mM – milimolar
MO – myositis ossificans
N – normal
-N – amino terminus
OB – osteoblast culture
OC – Osteocalcin
OSA – osteosarcoma
pM – picomolar
PBGD – Porphobilinogen deaminase
PKA – Protein kinase A
ptc – patched (Drosophila)
PTCH1 – Patched 1 (mammalian)
PTHrP – Parathyroid hormone related protein
PTHRI – Type I PTH/PTHrP receptor
R – arginine
RLU – relative luciferase units
RT-PCR – reverse transcription-polymerase chain reaction
**SDS** – sodium dodecylsulphate

**Shh** – Sonic hedgehog

**SMO** – Smoothened

**SSCP** – single strand conformation polymorphism

**Su(Fu)** – Suppressor of fused

**T** – thymine

**TRAP** – tartrate resistant acid phosphatase

**WT** – wild type

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CHAPTER 1

Introduction
BACKGROUND

Development and neoplasia

Development is the most substantial biological undertaking of any organism. The regulation of development is necessarily exquisite. If for no other reason, the shear magnitude and complexity of the problem of developmental regulation lends this regulation susceptible to error. In particular, dysregulation of developmental mechanisms might cause neoplasia.

Neoplastic cells share many characteristics with developing cells. These characteristics include maintenance of an incompletely differentiated state, rapid proliferation, mobility and invasive capability. A variety of tumours resemble the developing stages of their tissue of origin, and this resemblance may be manifest at a morphological, behavioural or molecular level. Understanding why neoplastic cells resemble undifferentiated cells would facilitate therapeutic approaches designed to differentiate those cells, thereby causing them to lose characteristics which make them deleterious to the host, without necessarily restoring all genetic controls.

Evidence implicating the significance of developmental regulators in neoplasia has been accumulating over many years. Positive regulators of developmental signalling pathways may behave as oncogenes when they are ectopically expressed or harbour activating mutations. Examples of such positive regulators are WNT (breast cancer), \( \beta \)-catenin (aggressive fibromatosis), AMLI (leukemia), PAX3 and PAX7 (rhabdomyosarcoma), EWS and Manic Fringe (Ewing’s sarcoma), and Sonic Hedgehog (SHH), Smoothened (SMO), GLI1 and GLI2 (basal cell carcinoma and medulloblastoma). Negative developmental regulators may behave as tumour
suppressor genes when they harbour loss of function mutations. Such negative regulators include APC (aggressive fibromatosis\textsuperscript{14}, colon cancer\textsuperscript{15}) and Patched1 (PTCH1; basal cell carcinoma and medulloblastoma\textsuperscript{13,16}). What has become increasingly clear is the importance of the dysregulation of a given signalling pathway, rather than of a single gene. For example, while deregulation of either β-catenin or APC independently may give rise to aggressive fibromatosis\textsuperscript{3,14,17}, both these genes encode members of the same signalling pathway. Likewise, SHH, PTCH, SMO, and GLI encode members of another pathway\textsuperscript{18}.

Of the three general types of cancer, carcinoma, hematopoietic neoplasia and sarcoma, sarcoma is the least studied. Sarcomas and their benign precursors are tumours of connective tissue presumably derived from some mesenchymal cell type. Skeletal sarcomas are most common in children and adolescents, whereas soft tissue sarcomas are more common in adults. Although less common than other types of cancer in the general population, sarcomas have an arguably greater negative impact than most neoplastic conditions. This impact is due to the large number of life years and reproductive potential lost due to mortality at a young age as well as the often tremendous physical and non-physical trauma caused by sarcomas and their treatment\textsuperscript{19,20}.

**Cartilage-forming tumours**

There are a number of neoplasms with cells that bear some resemblance to chondrocytes and which produce a cartilaginous matrix. The various types of cartilaginous neoplasms are individually distinct based on histologic features, skeletal location and their potential for locally destructive behaviour and for metastasis, usually to
the lungs\textsuperscript{19-20}. Cartilage tumours, with the exception of chondrosarcoma, occur most commonly in children and young adults. The following tumours have recognisably chondroid elements, but also have non-chondroid features to varying degrees: 1) Chondroblastoma is a locally aggressive tumour occurring within the epiphysis of a bone. These tumours carry a risk of metastasis of less than five percent. 2) Chondromyxoid fibroma is a relatively rare, benign metaphyseal tumour that is locally destructive. 3) Chordoma is felt to arise from remnant notochordal tissue, and occurs most commonly at the base of the skull or in the sacrum. These tumours are locally aggressive and carry a less than fifteen percent risk of metastasis. 4) Osteochondroma, or exostosis, is a bony outgrowth with a central medullary cavity which is continuous with that of the host bone and has a well differentiated cartilage cap that is similar to growth plate tissue. These lesions can cause mechanical symptoms, and the cartilage cap may also undergo malignant transformation, most commonly to chondrosarcoma and rarely to osteosarcoma. Osteochondromas themselves may be aneuploid\textsuperscript{21}, and the risk of malignant change is less than one percent for a solitary osteochondroma\textsuperscript{19}, but is higher when multiple osteochondromas are present. Hereditary multiple exostoses is an autosomal dominant condition with an incidence of about 1/50000, and, as are some sporadic exostoses, is caused by loss of function mutations in one of the three \textit{EXT} family genes\textsuperscript{21-23}. \textit{EXT} genes are homologs of Drosophila \textit{tout velu}, which is necessary for long range diffusion of hedgehog ligand\textsuperscript{24-25} (see below).

Other benign tumours with easily recognizable cartilaginous differentiation include enchondromas, which occur adjacent to the metaphyseal side of the growth plate (Fig. 1), and periosteal chondromas, which occur at the surface of a bone\textsuperscript{19-26}. 

Enchondromas can occur as solitary lesions, or as multiple lesions in enchondromatosis (Ollier's and Maffucci's diseases). An enchondroma may be completely asymptomatic and be discovered as an incidental finding on a radiograph. Clinical problems that can be caused by enchondromas include pain, skeletal deformity, bony weakness leading to pathological fracture, and malignant change to chondrosarcoma\textsuperscript{19,20,27,28}. The risk of chondrosarcoma arising from an enchondroma is about 25% in Ollier's disease and virtually 100% in Maffucci's disease\textsuperscript{38}. Maffucci's disease is further distinct from Ollier's disease because of additional phenotypic features such as soft tissue hemangiomas and visceral and central nervous system tumours. The extent of skeletal involvement is variable in enchondromatosis, and may include dysplasia that is not directly attributable to enchondromas\textsuperscript{29-32}. Enchondromatosis is rare, obvious inheritance of the condition is unusual\textsuperscript{31,32} and no candidate genetic loci have been identified. Enchondromas are composed of cells cytologically similar to growth plate chondrocytes, possibly representing foci of incomplete endochondral ossification\textsuperscript{20,26}. Enchondromas may disappear with time following skeletal maturity, possibly due to gradual completion of endochondral ossification. Little is known about the molecular pathology of these lesions, and there are no animal models. Current treatments are exclusively surgical, and consist of tumour excision with bone defect reconstruction, fixation of pathological fractures\textsuperscript{38} and osteotomy for limb realignment\textsuperscript{33}.

Synovial chondromatosis is another variety of cartilaginous neoplasia. In this condition, tissue resembling growth plate cartilage grows ectopically from the inner surface of the synovial lining of a joint. The cartilage growths typically undergo central ossification, analogous to endochondral ossification. The cartilage in this case fills the
joint cavity causing painful swelling and secondary osteoarthritis\textsuperscript{20}. Inheritance of synovial chondromatosis is unusual, although a familial form of the condition has been reported\textsuperscript{34,35}. The relative risk of malignant transformation to chondrosarcoma has been estimated to be 5\textsuperscript{36,37}. Current treatment consists of periodic surgical removal of the ectopic cartilage and of the synovium to relieve symptoms and prevent the destruction of articular cartilage\textsuperscript{19}.

Chondrosarcoma is a malignancy that occurs primarily in adults\textsuperscript{20}. Approximately two thirds of chondrosarcomas are currently thought to arise de novo from otherwise normal bone, and rarely from soft tissue. The remaining third of chondrosarcomas arise from some precursor lesion, most commonly an enchondroma or an osteochondroma\textsuperscript{19}. A large number of cytogenetic derangements have been discovered in chondrosarcomas\textsuperscript{38}, most notably at 9p21\textsuperscript{39}, but none are universal. The only correlation between a cytogenetic change with a histologic subtype of chondrosarcoma is the occurrence of a reciprocal t(9:22) translocation resulting in the fusion of EWS with another gene, which generates a transcription factor with increased potency, in extraskeletal myxoid chondrosarcomas\textsuperscript{40,41}. Mice overexpressing c-Fos develop chondrosarcomas and osteosarcomas\textsuperscript{42}, although expression of c-Fos has not been demonstrated in human chondrosarcoma\textsuperscript{43}. It has more recently been shown that c-Fos expression is induced by Parathyroid hormone related protein (PTHrP)\textsuperscript{44}. Unlike in osteosarcoma, Retinoblastoma (RB), p53 and 12q13-15 alterations are not common in chondrosarcoma\textsuperscript{45-47}, nor is telomerase activity\textsuperscript{48}. Loss of heterozygosity for markers linked to EXT1 and EXT2 has been observed in sporadic chondrosarcomas, in addition to those that arise in people with hereditary multiple exostoses\textsuperscript{22,49}. Genetic changes that
have been observed in a chondrosarcoma but not in an enchondroma from the same patient with enchondromatosis include deletion of 1p11-31.2 in one case and loss of heterozygosity at 9p21 and 13q14 together with overexpression of p53 in another.

Most histological subtypes of chondrosarcoma are relatively radiation and chemotherapy resistant, and are treated with wide surgical excision alone. Major ablative amputations or limb salvage procedures are often necessary, resulting in approximately 60% long-term survival from the disease.

**Bone development**

Development of a mammalian zygote proceeds by multiple rounds of cell division to generate a morula of equipotent cells. Cavitation of the morula converts it into the blastocyst, which has an eccentric inner mass of cells. The embryo proper is derived from this inner mass, which undergoes gastrulation in the third week of gestation in humans. Gastrulation establishes the three distinct germ layers of endoderm, mesoderm and ectoderm. Mesoderm is the germ layer that all connective tissues are derived from. Following the onset of neurulation at four weeks in humans, paraxial mesoderm differentiates and segments into 42-44 pairs of somites in a cranial to caudal direction. Instructed by secreted signals, in large part from the notochord, somites differentiate into sclerotome ventromedially and dermomyotome dorsolaterally. The axial skeleton is derived from sclerotome. Appendicular bones and tendons, including the shoulder and pelvic girdles, are derived from lateral plate mesoderm. Cells from lateral plate mesoderm migrate to the prospective sites of the limb buds. In contrast, cells destined to form muscles, nerves and blood vessels are derived from somitic mesoderm, which is
medial to lateral plate mesoderm, and invade the limb buds secondarily to join the growing skeleton\textsuperscript{52-53}.

The early limb bud consists of a proliferating population of mesodermal cells covered by ectoderm. Development of the limb bud is regulated by four regional organizing centres\textsuperscript{54-57}. These include 1) the mesodermal progress zone from which the skeletal elements are derived, 2) the mesodermal zone of polarizing activity which primarily establishes anterior – posterior pattern polarity, 3) the apical ectodermal ridge which is primarily responsible for limb outgrowth, and 4) the surrounding ectoderm which primarily establishes dorsal – ventral pattern polarity. These regional organizing centres are co-dependent, and together they coordinate outgrowth of the limb along the three orthogonal axes. The basic pattern of the entire skeleton is complete by the end of organogenesis, which takes place between weeks four and eight of gestation in the human.

Most of the bones of the vertebrate skeleton, with the exception of the craniofacial bones and part of the clavicle, are transformed from mesodermally-derived mesenchyme to bone by the process of endochondral ossification\textsuperscript{53-58}. In this process, condensed mesenchyme which prefigures the primitive shape of a bone first differentiates to cartilage. Ossification begins in the centre of the cartilage template and proceeds toward either end, trailing behind the receding zone of cartilage known as the growth plate. In mammals, a secondary centre of ossification forms at the two extreme ends of the cartilage template. The growth plate is responsible for longitudinal growth, and shrinks in height until it disappears following adolescence in human bones, but remains present in the long bones of other mammals including rodents. Chondrocytes in the growth plate
differentiate toward the primary centre of ossification (Fig. 2). Resting chondrocytes first enter a proliferative phase, followed by a post-mitotic hypertrophic phase at the end of which they mainly undergo programmed cell death. The cartilage matrix produced by growth plate chondrocytes changes in composition from predominantly Type II collagen (ColII) in the proliferative zone to predominantly Type X collagen (ColX) in the hypertrophic zone, where it also calcifies. This calcified cartilage is removed by chondroclasts\textsuperscript{59,60}, and forms the scaffold on top of which primary bony trabeculae are laid by osteoblasts which arrive by way of invading blood vessels. An understanding of current models of the molecular regulation of chondrocyte differentiation will be facilitated by a synopsis of two key signal transduction pathways.

**Hedgehog signal transduction**

The importance of Hedgehog signalling as well as the identity and function of key Hedgehog signal transduction mediators was first established by work in Drosophila, where it contributes to the segmentation of embryos and the patterning of imaginal-disk outgrowth\textsuperscript{61,62}. Hedgehog signalling has since been discovered to be essential for the regulation of vital vertebrate embryonic processes as well as for the development of many organ systems. These include differentiation of visceral endoderm, the establishment of left – right asymmetry\textsuperscript{63}, somite patterning\textsuperscript{64} and differentiation\textsuperscript{65,66}, central nervous system patterning and differentiation\textsuperscript{67,68}, spermatogenesis\textsuperscript{69}, specification of hematopoietic and endothelial cells\textsuperscript{70}, lung\textsuperscript{71,72}, pancreatic\textsuperscript{73} and intestinal development\textsuperscript{72,74,75}, hair cycle regulation\textsuperscript{76}, tooth development\textsuperscript{77}, limb patterning and outgrowth\textsuperscript{78}, and regulation of chondrocyte\textsuperscript{79,80} and osteoblast differentiation\textsuperscript{80,81}. 
Hedgehog signalling has been co-opted to regulate a variety of cellular functions, and dysregulation of this signalling pathway is known to cause disorders of pattern and of proliferation in humans. Patterning anomalies involve the central nervous system, viscera and limbs, and include holoprosencephaly, midline facial anomalies, defects of the kidneys, heart, and genitals, syndactyly and polydactyly. Neoplasms are caused by excessive Hedgehog signalling, and include sporadic and familial basal cell carcinoma, medulloblastoma and rhabdomyosarcoma.

The Hedgehog signal transduction pathway is conserved between species. There are three homologs of Drosophila hedgehog in mammals: Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh). Shh is the most widely expressed and best studied of these ligands. The human IHH gene is located on chromosome bands 2q33-35. It shares high sequence similarity with SHH, is processed in a similar manner, and either protein can substitute for the function of the other. Ihh is expressed by differentiating extraembryonic endoderm, kidney, gut, growth plate chondrocytes and osteoblasts. The role of Dhh is the most restricted of the Hedgehog ligands. Dhh regulates spermatogenesis and organization of the perineurium.

Hedgehog ligands are processed post-translationally by autocleavage of a 45 kD precursor, releasing a 19 kD amino (N) terminal signalling domain and a 26 kD carboxy terminal domain. The N-terminal signalling domain is further modified by covalent addition of a cholesterol moiety by the carboxy terminal domain which acts as an intramolecular cholesterol transferase. N-terminal hedgehog protein can be secreted, and can signal at short as well as long range. The cholesterol moiety may allow the ligand to be tethered to cell surfaces, and regulate the distance over which it acts.
although it is not yet clear whether cholesterol modification restricts or enhances diffusion of hedgehog\textsuperscript{18}. A twelve-pass transmembrane protein with a sterol sensing domain called dispatched has been identified in Drosophila which likely displaces hedgehog from the lipid bilayer to allow effective signalling\textsuperscript{104}. Patched (see below), a distant relative of dispatched\textsuperscript{105}, and Hedgehog interacting protein\textsuperscript{106} contribute to the spacial restriction of Hedgehog ligands. Ext/tout velu is a glycosyl transferase that induces the expression of cell surface heparan sulphate\textsuperscript{107,108}, and is essential for long range diffusion of the hedgehog ligand\textsuperscript{109}.

Hedgehog signal transduction is best understood at the genetic level (Fig. 3). Work in Drosophila has shown that \textit{hh} and \textit{ptc} have opposite functions in regulating expression of \textit{wingless} and \textit{decapentaplegic}. Mutants of \textit{patched (ptc)}, which encodes a twelve pass transmembrane protein, phenocopy hedgehog (\textit{hh}/\textit{ptc} double mutants, indicating that in the absence of \textit{ptc}, \textit{hh} is not necessary\textsuperscript{110}. Mutants of \textit{smoothened (sma)}, which encodes a seven-pass transmembrane protein with homology to G protein–coupled receptors, display similar phenotypes to \textit{hh} mutants. \textit{Smo} is required for all Hedgehog signalling in Drosophila. When embryos lack both \textit{sma} and \textit{ptc}, they phenocopy \textit{sma} single mutants, indicating that \textit{ptc} is genetically upstream of \textit{sma}. In the absence of both \textit{ptc} and \textit{hh}, \textit{sma} activity leads to constitutive activation of Hedgehog targets. In the absence of \textit{hh}, \textit{ptc} represses \textit{sma} activity. This repression is relieved by the presence of \textit{hh}, thereby allowing activation of Hedgehog targets by \textit{sma}\textsuperscript{111-113}. It was shown in vertebrate cells that labelled Hh protein binds Ptch1 (vertebrate ptc) – expressing cells with high affinity, and that Hh and Ptch1, but not Hh and Smo, can be coimmunoprecipitated and crosslinked\textsuperscript{114,115}. How this binding is communicated to Smo
is not clear. A direct mechanism is suggested by biochemical studies showing that overexpressed Smo and Ptch1 form a complex. An indirect mechanism is also likely at work, as it was shown that ptc destabilizes smo in the absence of hh, and that hh binding to ptc removes ptc from the cell surface while causing phosphorylation, stabilization and accumulation of smo at the cell surface. It was shown indirectly in a frog melanophore assay that SMO may activate Gαi and inhibit cAMP synthesis. A second mammalian Ptch gene, Ptch2, has been isolated which lacks a carboxy-terminal domain and is expressed in the testis and skin. Unlike Ptch1, Ptch2 expression is not restricted to Hh target cells, but is coexpressed by Shh-expressing cells. Mutants of Ptch2 have not yet been described.

So far, all Hedgehog signalling appears to activate zinc finger-containing transcriptional effectors of the ci/Gli family. In Drosophila, cubitus interruptus (ci) is constitutively cleaved in the absence of hedgehog signalling, generating a transcriptional repressor that binds to, and inactivates, hedgehog target genes. Hedgehog signalling inhibits this proteolytic cleavage by a phosphorylation-dependent process. As a result, full-length ci protein activates transcriptional targets. There are three vertebrate homologues of ci, known as the Gli (after glioblastoma) family of transcription factors. Gli2 and Gli3 both have an N-terminal repressor domain and a C-terminal activator domain, whereas Gli1 has only an activator domain. The activator and repressor capabilities of Gli2 and Gli3 are context-dependent. In a rat neural stem cell cell line (MNS70), full length Gli1 and Gli2 can activate whereas Gli3 can repress a Hedgehog responsive reporter gene. Expression of N-terminal truncated forms of either Gli2 or Gli3 can ectopically activate Shh-target genes in the dorsal central nervous system. In the
limb bud mesenchyme, *Gli1* is upregulated whereas *Gli3* is downregulated in response to Shh. In the presence of ectopic Ihh in the anterior limb bud of the Doublefoot mouse mutant, *Gli2* is downregulated, in contrast to what might be expected from the *in vitro* studies mentioned above. In flies, the activity of Ci is modulated by its interaction with a microtubule-associated complex containing several other proteins. These include costal2, which negatively regulates Ci nuclear import but is required for Ci activation, fused, a protein-serine/threonine kinase and an activator, and suppressor of fused, a repressor. Mouse Suppressor of fused has been shown to bind to all three Gli proteins and inhibits Gli1 action by preventing nuclear import. Protein kinase A (PKA) has been regarded as a conserved inhibitor of Hedgehog signalling. Indeed, expression of a dominant negative form of PKA mimicks ectopic Hedgehog expression and phosphorylation of Ci by PKA is required for proteolytic cleavage of Ci. However, there is contrasting data from separate groups of investigators with regard to the effect of PKA on Ci activation. Wang, G. et al showed that PKA inhibits the full length activator form of Ci, whereas Wang, Q.T. et al showed that Ci activation requires phosphorylation by PKA.

**PTHrP signal transduction**

*PTHrP* and its receptor, the *Type I PTH/PTHrP receptor* (*Pthr1*), are expressed in the pregastrulation embryo, in extraembryonic tissues and in tissues derived from all three germ layers. *PTHrP* functions can be divided into four categories. First, *PTHrP* stimulates transepithelial calcium transport in a variety of tissues. Second, *PTHrP* is a smooth muscle relaxant in the vascular system and in a broad range of viscera.
Third, PTHrP is a regulator of proliferation, differentiation and apoptosis in many cell types. Finally, PTHrP regulates a number of pre- and post-implantation developmental processes. In most sites, PTHrP and its receptor are coordinately expressed in adjacent cells, implying that these proteins are involved in paracrine signalling pathways. For example, PTHrP is expressed by the perichondrium, whereas Pthrl is present on a subset of proliferating growth plate chondrocytes. In extraskeletal sites PTHrP is predominantly expressed in the surface epithelium of developing organs, and Pthrl resides on the adjacent mesenchymal cells.

PTHrP is the product of a single gene, which is highly similar in organization and sequence to the PTH gene. Its functions, however, are more similar to those of developmental factors. Through alternative splicing, the multi-exonic PTHrP gene gives rise to three initial translation products. These products undergo extensive posttranslational processing and give rise to a family of mature secretory forms of the protein, each with its own physiological functions and its own receptor or receptors. The amino-terminal secretory form, PTHrP (1-36), is the most relevant form with regard to chondrocyte differentiation, and acts through Pthr1.

Two mammalian PTH/PTHrP receptors have been isolated. PTHR1 is the classical receptor, and binds PTH and PTHrP with equal affinity. PTHR2 binds PTH with high affinity, but not PTHrP. PTHR3, which has so far been isolated from zebrafish, binds PTHrP, but not PTH. PTHR1 is a seven-pass transmembrane protein that is linked to heterotrimeric G proteins. I have focused attention on PTHR1 because it is the key mediator of paracrine IHH-PTHrP signalling in the growth plate. A family of related proteins includes receptors for Secretin, Growth hormone-releasing
hormone, Vasoactive intestinal polypeptide, Type I gastric–inhibitory polypeptide, Glucagon, Glucagon–like peptide 1, Corticotropin–releasing factor, and Pituitary adenylate cyclase activating peptide 1.

$G$ proteins are membrane–associated, and are composed of an $\alpha$ subunit that, in the inactive state of an associated receptor, hydrolyses guanine triphosphate (GTP) to guanine diphosphate (GDP), and is loosely bound to a tightly associated dimer made of $\beta$ and $\gamma$ subunits. Upon binding ligand, the receptor acts upon the $G$ proteins, causing GDP to be replaced with GTP. As a result, the $\alpha$ subunit carrying GTP dissociates from the two other subunits. Either the monomer or the dimer then acts upon a target effector protein that is often also membrane associated, which in turn reacts with targets in the cytoplasm, generating second messengers. Various $G\alpha$ proteins define different $G$ protein trimers ($G\alpha_4$, $G\alpha_{alp}$, $G\alpha_q$, $G\alpha_s$, and $G\alpha_t$), each of which regulates a distinctive set of downstream signalling pathways. $PTHR1$ most strongly associates with $G\alpha_s$ and $G\alpha_t$. Activation of $G\alpha_s$ stimulates Adenylate cyclase, which generates the second messenger Cyclic adenosine monophosphate (cAMP) (Fig. 4). Activation of $G\alpha_t$ stimulates Phospholipase C to generate Inositol trisphosphate (IP$_3$). $PTHR1$ has been shown to weakly associate with $G\alpha_t$, which inhibits Adenylate cyclase. Cyclic AMP binds the regulatory R subunit of PKA, causing release of the catalytic C subunit. Some of these C subunits then diffuse into the nucleus, where they phosphorylate cAMP response element binding protein (CREB). Phosphorylated CREB can then bind to the response element CRE, which is found on genes whose transcription is induced by cAMP.
PTHR1 has also been shown to signal via alternate mechanisms\textsuperscript{150}. These include activation of the MAP kinase pathway\textsuperscript{151}, and an intracrine mechanism involving nucleolar targeting of PTHrP\textsuperscript{152}. Internalization of the PTHrP/PTHR1 complex has been demonstrated\textsuperscript{153,154}, but how this phenomenon relates to the intracellular signalling pathways has yet to be shown. PTHR1 is also known to elicit differing and even opposite cellular responses depending on whether it is stimulated continuously or intermittently\textsuperscript{155}.

**Regulation of chondrocyte differentiation**

Growth plate chondrocyte proliferation and differentiation are tightly regulated. Of the molecular signals that regulate these processes, Ihh and PTHrP have been shown to play major roles (Fig. 2). \textit{Ihh} is expressed by prehypertrophic and hypertrophic chondrocytes\textsuperscript{79,81}. \textit{PTHrP} is primarily expressed by perichondrial cells surrounding the growth plate, in a region near the articular surface\textsuperscript{79,81,156}. The likely \textit{Ihh} receptor \textit{Ptch1}, initially thought to be expressed exclusively in the perichondrium\textsuperscript{79}, has been shown to be expressed also by chondrocytes in the proliferative zone in mice\textsuperscript{80}.

Although they are intimately related, the individual functions of \textit{Ihh} and of \textit{PTHrP} are largely distinct in the growth plate. In mice lacking the secreted ligand \textit{PTHrP} or its receptor \textit{Pthrl}, hypertrophic differentiation of proliferating chondrocytes is dramatically accelerated, indicating that \textit{PTHrP} signalling is responsible for delaying differentiation of proliferating chondrocytes\textsuperscript{156-161}. It has been shown that the ability of \textit{PTHR1} to induce cAMP accumulation by activating \textit{G}_{\alpha_{s}} is responsible for chondrocyte differentiation delay\textsuperscript{24}. In contrast, the ability of \textit{Pthrl} to cause IP\textsubscript{3} accumulation by activating \textit{G}_{\alpha_{q}} is associated with accelerated hypertrophic differentiation. This was shown in a knock-in
study where mutation of Pthrl that selectively inactivated its ability to stimulate \( \alpha_q \) resulted in delay of hypertrophic differentiation\(^{162}\). Therefore, Pthrl has a dual role in regulating chondrocyte differentiation, although its ability to delay hypertrophy seems to predominate. Mice lacking Ihh have very short limbs and a profound defect in chondrocyte proliferation\(^{80}\). Although hypertrophic differentiation is also accelerated in these mice, it is associated with a loss of PTHrP in the periarticular perichondrium, indicating that PTHrP is downstream of Ihh\(^{10}\). In fact, \( Ihh,PTHrP \) double homozygous mutant mice phenocopy \( Ihh \) single mutant mice, demonstrating that \( Ihh \) is required for \( PTHrP \) function\(^{163}\). Upregulation of \( PTHrP \) expression in the periarticular perichondrium by Ihh is probably indirect, since \( PTCH1 \) is not expressed in that region of the perichondrium. Ihh driven chondrocyte proliferation is largely independent of PTHrP, since expression of a constitutively active \( PTHR1 \) in \( Ihh^{\Delta} \) mice could not rescue the defect in proliferation\(^{163}\). Therefore, the mitogenic function of Ihh is likely mediated directly by the Hedgehog receptor complex expressed by proliferating chondrocytes. PTHrP downregulates \( Ihh \), likely by both direct and indirect means. Delay of chondrocyte differentiation by PTHrP may decrease the number of prehypertrophic and hypertrophic cells, and thereby indirectly downregulate \( Ihh \). A direct mechanism is implied by the finding that inhibition of \( Ihh \) expression by PTHrP \textit{in vitro} does not require protein synthesis\(^{164}\).

Many other signals contribute to growth plate regulation. For example, chondrocyte proliferation is stimulated by Insulin-like growth factor 1 (Igf-1)\(^{165}\), basic Fibroblast growth factor (Bgf)\(^{166}\) and CREB\(^{167}\). Transforming growth factor beta\(^{168}\) as well as Delta1 – activated Notch signalling\(^{169}\) delay hypertrophic differentiation, whereas
Core binding factor alpha 1, Igf-1, Thyroid hormone, Connective tissue growth factor and CREB promote this process. There are conflicting reports as to the effect of Retinoic acid on chondrocyte hypertrophy. Bone morphogenic proteins (Bmp) 2 and 4 may mediate the induction of PTHrP by Ihh by means of the Bmp receptor IA. Bmp-6, which contributes to hypertrophic differentiation, may partially mediate the inhibition of IHH by PTHrP. Sox-9 likely partially mediates the effects of PTHrP with regard to delay of chondrocyte hypertrophy. Activation of the Fgf receptor 3 inhibits expression of Ihh. Matrix metalloproteinase 9/Gelatinase B is expressed by chondroclasts and regulates apoptosis of hypertrophic chondrocytes. Removal of cartilage at the chondro-osseous junction and, together with Vascular endothelial growth factor, regulates growth plate angiogenesis. Galectin 3 is important for chondrocyte survival and may help coordinate chondrocyte death with vascular invasion.

**HYPOTHESIS**

Cartilaginous neoplasia is due, at least in part, to dysregulation of the IHH-PTHrP signalling pathway.

**RATIONALE**

I’ve chosen to focus on the role of the IHH-PTHrP pathway in particular in cartilaginous neoplasia for three main reasons. First, as noted above, certain cartilage tumours, including enchondromas and chondrosarcomas, share cytological and matrix features with growth plates, albeit in an unorganised fashion. Morphological similarity naturally implies some degree of molecular similarity, and these molecules might include
regulators of cell behaviour. I find it difficult to conceive of enchondromas and chondrosarcomas as not being responsive to IHH and PTHrP, the most potent regulators of chondrocyte proliferation and differentiation discovered to date. Second, from a therapeutic perspective, whether activation of the IHH-PTHrP pathway plays a causal role or is merely a phenomenon secondary to some other factor causing expression of a chondrocyte phenotype in cartilage tumours might be largely irrelevant. Differentiation of neoplastic chondrocytes with agents that interfere with IHH and PTHrP signalling might be possible in either case. Third, blockade of developmentally important signalling pathways is attractive because of the relatively selective effect such blockade would have beyond the stage in an individual when that pathway is no longer as important. For example, one particular agent which specifically blocks Hedgehog signalling, cyclopamine\textsuperscript{183,184}, is known to be harmless to adult ewes who eat the substance, while it is toxic to their embryos. This agent and others like it might differentiate cartilage tumours with minimal side effects.

**OBJECTIVES**

To investigate the validity of the hypothesis, the following questions will be addressed:

1) Is the IHH-PTHrP signalling pathway expressed and active in cartilage – forming tumours?

2) If so, is activation of IHH-PTHrP signalling merely a secondary phenomenon in cartilage tumours, or can dysregulation of this pathway initiate neoplasia?

3) Can knowledge of how IHH-PTHrP signalling is altered in pathological conditions contribute to the understanding of normal growth plate development?
Enchondromas are most commonly found adjacent to the metaphyseal side of a growth plate, and may be connected to the growth plate by a core of cartilage. Enchondromas might be caused by a focal failure of endochondral ossification.
Growth plate chondrocytes differentiate in a columnar fashion, toward the centre of ossification (downward). A fixed number of reserve cells first enter a proliferative state, followed by a post-mitotic state of hypertrophy, at the end of which they undergo programmed cell death. Chondroclasts resorb the calcified matrix at the chondro-osseous junction. Blood vessels invade the distal growth plate, allowing osteoblasts to access the calcified matrix and lay new bone.

Ihh is secreted by prehypertrophic and hypertrophic chondrocytes. Ihh induces proliferation of chondrocytes as well as secretion of a second secreted signal, PTHrP, from the periaricular perichondrium. PTHrP signals its receptor on proliferating chondrocytes to delay hypertrophic differentiation.
Hedgehog signal is conserved across species. Binding of a hedgehog ligand (Hh) with Ptch relieves the otherwise constitutive inhibition of Ptch on Smo. Smo then induces expression of Hedgehog target genes by way of the Gli family of transcription factors.
Stimulation of PTHR1 by PTHrP acts on membrane associated G proteins to replace GDP with GTP, allowing the α subunit to dissociate from the βγ dimer. Gαs (pictured here) stimulates adenylate cyclase to produce cAMP, which in turn releases the catalytic subunit of PKA, allowing it to diffuse into the nucleus and phosphorylate CREB. Phosphorylated CREB then binds to response elements on genes whose transcription is induced by cAMP.
CHAPTER 2

Uncoupled Indian hedgehog – Parathyroid hormone related protein signalling in cartilage tumours and a mutant Type I PTH/PTHrP receptor in enchondromatosis


*Performed all procedures other than SSCP, sequencing, pronuclear microinjection and immunohistochemistry.
SUMMARY

Enchondromatosis is a condition in which multiple enchondromas predispose patients to developing skeletal deformity and chondrosarcoma. I investigated whether dysregulation of Indian hedgehog – Parathyroid hormone related protein signalling (IHH-PTHrP), which regulates chondrocyte proliferation and differentiation, contributes to the genesis of enchondromas. Here I show that IHH/PTHrP signalling is functional but dysregulated in enchondromas and chondrosarcomas. A mutant Type I PTH/PTHrP receptor (PTHR1) was discovered in human enchondromatosis that signals abnormally in vitro and causes enchondromatosis in transgenic mice.
INTRODUCTION

Enchondromas are common benign cartilage tumours 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. Enchondromatosis is rare, obvious inheritance of the condition is unusual, and no candidate loci have been identified. 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. I speculated that dysregulation of IHH/PTHrP signalling contributes to the genesis of enchondromas.

RESULTS

Expression of IHH/PTHrP pathway members in cartilage tumours

In order to test for expression of key IHH/PTHrP signalling pathway members, I utilized reverse transcription – polymerase chain reaction (RT-PCR) analysis. I found that IHH and PTHrP were both expressed in 4/4 human enchondroma and 22/23.
chondrosarcoma specimens, but less consistently in human articular cartilage (IHH 0/4, PTHrP 2/4), cortical bone (IHH 2/11, PTHrP 11/11) and other benign cartilage tumours including chondroblastomas (IHH 2/7, PTHrP 2/7) and mature osteochondromas (IHH 0/2, PTHrP 2/2). I therefore tested for expression of PTHR1, the IHH receptor Patched 1, and the Hedgehog responsive transcription factors GLI1, GLI2 and GLI3 in enchondromas and chondrosarcomas, and found that they were all consistently expressed in these lesions (Table 1). Semiquantitative RT-PCR analysis revealed that the ratio of expression of (GLI1 + GLI2)/GLI3 was higher in enchondroma (1.5, n=4) and chondrosarcoma specimens (1.5, n=11) than in normal articular cartilage (0.34, n=4) and cortical bone (0.40, n=11) (Table 2), suggesting that cells in these pathologic tissues are transducing the IHH signal, since GLI1 and GLI2 are generally upregulated and GLI3 is downregulated in response to Hedgehog ligand.

### Functional but uncoupled IHH/PTHR signalling in cartilage tumour explants

To test whether the PTHrP and IHH signalling pathways are functional in cartilage tumours, short term primary organ cultures of enchondromas from two patients and chondrosarcomas from ten patients were established. Treatment of neoplastic cultures for four days with 10⁻⁷M PTHrP (1-34) resulted in delayed hypertrophic differentiation as assessed by decreased expression of Type X collagen (COLX), an exclusive marker of hypertrophic chondrocytes (p=0.01, Fig. 1a). PTHrP treatment had no effect on neoplastic chondrocyte proliferation, as measured by tritiated thymidine (³H-T) uptake (p=0.53). Conversely, treatment of cultures with 10 μg/ml recombinant Sonic hedgehog (Shh-N, which is functionally redundant with Ihh in culture) increased
\[^{3}\text{H}\] uptake (p=0.02, Fig. 1b) without effecting \textit{COLX} expression level (p=0.39).

Treatment of cultures with agents that specifically block Hedgehog signalling, namely 10^{-3} M cyclopamine\(^{183,184}\) (Fig. 1c) and 10 μg/ml neutralizing anti-pan-Hh-N 5E1 antibody\(^{185}\) partially decreased \[^{3}\text{H}\] uptake (n=6, p=0.07 for cyclopamine and n=12, p=0.11 for anti-pan-Hh-N antibody) but had no effect on \textit{COLX} expression (p=0.32, p=0.39, respectively). These findings are consistent with the known consequences of \textit{IHH}\(^{80,163}\) and \textit{PTHR1}\(^{161,186}\) signalling on normal chondrocyte differentiation and proliferation, respectively. In the growth plate, these processes are co-regulated because \textit{IHH} induces \textit{PTHR1} expression, and \textit{PTHR1} in turn feeds back negatively on \textit{IHH} expression\(^{79,156}\). I did not detect downregulation of \textit{IHH} following treatment with \textit{PTHR1} in any of the tumour cultures. Downregulation of \textit{IHH} was, however, observed in 4/4 growth plate cultures (Fig. 1d). This feedback mechanism is therefore not operative in enchondromas and chondrosarcomas.

**A mutant \textit{PTHR1} in enchondromatosis**

A mutant \textit{PTHR1} could account for the lack of downregulation of \textit{IHH} by \textit{PTHR1} in enchondroma and chondrosarcoma. To test for this possibility, single strand conformation polymorphism analysis and manual sequencing was undertaken to screen cartilage tumour specimens for \textit{PTHR1} sequence alterations. A heterozygous cytosine to thymine change, resulting in the substitution of arginine with cysteine in the extracellular domain (R150C) of the \textit{PTHR1} in enchondroma specimens from two of six patients with enchondromatosis (Ollier's disease), was discovered (Fig. 2a). Both of these patients were male, with mild to moderate disease severity. In one of these patients, the variant \textit{R150C PTHRI} allele was carried in the germline, and was inherited from the father who
has mild spondyloepiphysial dysplasia, but no evidence of enchondromas on radiographs, similar to a scenario reported previously. In the other patient, the R150C PTHRI allele represented a somatic change, since normal bone adjacent to the enchondroma specimen did not contain the variant allele. It is possible that this second patient is a mosaic carrier of the R150C PTHRI allele, but I was not able to test for this possibility since enchondroma tissue from only one site was available for study. The R150C change was not found in DNA samples from 100 unaffected individuals, nor in 50 sporadic chondrosarcoma specimens.

**Mutant PTHR1 signalling is impaired in vitro**

The signalling ability of the PTHR1 has been most extensively studied in the context of its G protein – linked ability to induce Cyclic adenosine monophosphate (cAMP) and Inositol triphosphate (IP3) accumulation upon ligand binding. The functional relevance of the arginine 150 residue is not known. Cyclic AMP and IP3 assays were performed using COS-7 cells and embryonic stem (ES) cells lacking both copies of the native Pthrl gene, respectively. Wild type (WT) and R150C PTHRI cDNAs were generated by site – directed mutagenesis and transiently transfected for analysis.

Other PTHR1 mutants, known to cause skeletal dysplasias with phenotypes distinct from that of enchondromatosis, include cAMP activating mutations in Jansen metaphyseal chondrodysplasia, and a cAMP loss of function mutation in Blomstrand chondrodysplasia. These mutants were also generated and tested in the cAMP assays as internal controls. Surprisingly, expression of R150C PTHRI in COS-7 cells lowered the basal level of cAMP in comparison to empty vector or WT PTHRI - transfected cells (Fig. 2b, d). R150C PTHRI - transfected cells could still accumulate
cAMP in response to ligand in proportion to WT PTHRI – transfected cells. Importantly, R150C PTHRI abolished the ability of transfected cells to accumulate IP$_3$ in response to ligand (Fig. 2c). When increasing amounts of the R150C PTHRI variant was cotransfected with a fixed amount of WT PTHRI, the variant demonstrated a dominant negative effect on cAMP level (Fig. 2e). I next tested the expression level and subcellular localization of c-Myc-tagged WT and R150C PTHRI proteins by Western analysis and confocal immunofluorescence. With these tests, I found no gross change in mutant receptor expression or localization (Fig. 2f).

**Transgenic mouse model of enchondromatosis**

To evaluate the effect of the R150C PTHR1 mutation in vivo, I used the murine Type II collagen (ColII) promoter and enhancer$^{196,197}$ to drive expression of WT PTHR1 or R150C PTHR1 in the growth plates of transgenic mice (Fig. 3d). Mice expressing WT PTHR1 in two separate founder lines did not display any morphological abnormalities compared to wild-type littermates. At birth, growth plates of mice from three separate founder lines expressing R150C PTHR1 were of similar overall size, but had shorter hypertrophic zones, than those of mice expressing WT PTHR1 (Fig. 4a, b). This difference was greatest in growth plates which contribute the most to longitudinal growth, such as the proximal humerus compared to the distal humerus, and was confirmed by immunostaining against COLX to highlight the hypertrophic zone (Fig. 4c, d). Beyond eight weeks of age, features consistent with human enchondromatosis were evident in two of these R150C PTHR1 founder lines. Paraffin embedded sections stained with safranin-O to highlight cartilage revealed cellular cartilage islands, or rests, in the metaphyses of multiple long bones (Fig. 5), including the proximal humerus (Fig. 5b-e),
distal femur (Fig. 5f) and tibia. Columns of cartilage commonly connected the rests to the adjoining growth plate, as is often found in human enchondromatosis (Fig. 5b, c). Regions of varying cellularity, with proliferating cells and occasional binucleate lacunae, arranged in lobular patterns in a hyaline cartilage matrix that was partially or completely surrounded by bony matrix (Fig. 5d, e), similar to human enchondromas, were observed. Another similarity to human enchondromatosis was the persistence of these lesions well into adulthood (Fig. 5c, e). The paucity of hypertrophic cells in the growth plates of R150C PTHR1 mice persisted beyond adolescence (Fig. 6a, b), and may have resulted in the inclusion of transitional cells, which were still competent to proliferate within the trabecular cores of cartilage (evident as thin, red longitudinal streaks in Fig. 5a, b) to give rise to metaphyseal enchondromas. Further, the origin of the enchondromas was likely due to abnormal proliferation/differentiation rather than to a defect of resorption, since chondroclasts, cells which resorb calcified cartilage at the cartilage – bone interface and are distinguished by their ability to retain an acid phosphatase stain following tartrate challenge (TRAP stain), were found in equal numbers in WT PTHR1 and R150C PTHR1 mice (Fig. 6c, d).

DISCUSSION

Enchondroma formation

The results implicate abnormal growth plate development in the formation of enchondromas. In particular, some cases of enchondromatosis are likely caused by a heterozygous, dominant mutant PTHR1. The cysteine residue in position 150 might form new or alternate disulphide linkages with other cysteines that are present in the
extracellular amino terminus or in the extracellular loops between the transmembrane portions of PTHR1\textsuperscript{191}. In so doing, the R150C change might alter the structure of PTHR1 in a way that changes its signalling characteristics.

The R150C PTHR1 variant may be involved in the development of enchondromatosis by altering chondrocyte differentiation and proliferation. Prevention of IP\textsubscript{3} accumulation in response to ligand is consistent with delayed chondrocyte hypertrophy\textsuperscript{162}. Baseline cAMP level, which has the opposite effect as IP\textsubscript{3}, was suppressed by the R150C variant, possibly due to strengthened association with G\alpha\textsubscript{i}. However, accumulation of cAMP was still observed in response to PTHrP. The intracellular balance of second messengers is therefore tipped in favour of cAMP over IP\textsubscript{3} in the presence of ligand, which is likely responsible for the more distal transition from proliferation to hypertrophy seen in \textit{R150C PTHR1} mice. Cyclic AMP, through activation of protein kinase A (PKA), is a conserved inhibitor of Hedgehog signalling\textsuperscript{132}. Therefore, overactive IHH signalling might be caused directly by the R150C PTHR1 variant, leading to inappropriate chondrocyte proliferation. Both delay of growth plate chondrocyte differentiation and maintainance of proliferative ability are conceivably necessary for enchondroma formation. In \textit{R150C PTHR1} transgenic mice, proliferating chondrocytes were abnormally close to the chondro-osseus junction, increasing the likelihood that some of them would be trapped in cartilage cores that normally extend into the metaphysis, where they could give rise to enchondromas.

\textbf{Genetics of enchondromatosis}

Different PTHR1 mutations cause distinct phenotypes, which underlines the importance and complexity of this protein. Delay of chondrocyte hypertrophy can also
be achieved by PTHRI mutations which augment cAMP level in the absence of ligand\textsuperscript{163,187}. However, these mutations are not consistent with the enchondromatosis phenotype. Elevation of cAMP level causes systemic hypercalcemia and hypophosphatemia which are found in Jansen's metaphyseal chondrodysplasia\textsuperscript{188}, but not in enchondromatosis\textsuperscript{198}. The R150C mutation alters signalling in a manner that is apparently specific to enchondromatosis.

It is possible that enchondromatosis is more commonly familial than previously thought. Variable penetrance or anticipation of the condition may mask inheritance, as in the case reported here of father – to – son transmission of the \textit{R150C PTHRI} allele. In the other case reported above, the \textit{R150C PTHRI} change was somatic. Mutations that cause enchondromatosis might arise as somatic mutations during embryogenesis, and subsequently be distributed in a mosaic fashion in the host. The distribution of the mutant allele might determine the extent and severity of the phenotype. If the mutant allele is distributed outside of the musculokeletal system, germline transmission might occur.

Cases of enchondromatosis without a demonstrable PTHRI mutation, as well as solitary enchondromas, likely arise from similar molecular mechanisms, except that the primary gene defect may involve other members of the IHH/\textit{PTHrP} signalling pathway. Active Hedgehog signalling, in particular, is likely an important component in the genesis and maintenance of enchondromas. Evidence for this notion is found in the observations that 1) \textit{IHH} could not be downregulated in the tumour explant cultures, 2) tumour proliferation was dependent upon Hedgehog signalling, and 3) the R150C PTHRI mutant suppressed cAMP level, and therefore inhibited activation of the Hedgehog -
inhibitory PKA. Together with these findings, observation of elevated levels of GLI1 and GLI2 and depressed levels of GLI3 expression in the cartilage tumours suggest that the former are positive regulators while the latter is a negative regulator of Hedgehog signal transduction in chondrocytes. Whether this suggestion is true and whether excessive Hedgehog signalling is sufficient to generate enchondromas are testable hypotheses.

The persistence of growth plate tissue in the form of enchondromas beyond adolescence, when growth plate tissue has normally disappeared in humans, may allow accumulation of secondary genetic events which cause chondrosarcoma to arise within a preexisting enchondroma. One way a chondrosarcoma might arise is through a mutation that establishes a clonal population of chondrocyte stem cells. These stem cells would, by definition, be capable of self-replication as well as generation of daughter cells that could differentiate as growth plate chondrocytes do. These latter cells would then be dependent upon IHH and PTHrP, consistent with the findings in the explant cultures described above.

**METHODS**

**Tissue specimens**

Tissues were obtained from the National Cancer Institute of Canada Sarcoma Tissue Bank housed at Mount Sinai Hospital, the Mount Sinai Hospital Bone Bank and from The Hospital for Sick Children. Consent was obtained for each specimen according to each institution's policies.
RNA extraction

Approximately 50 mg of each specimen was crushed under liquid nitrogen and total RNA extracted using 4mL of an extraction reagent (Trizol Reagent. Gibco BRL, Grand Island, New York) according to the manufacturer's directions. The specimens in Trizol were initially homogenized following crushing and centrifuged at 12000 X g for 10 min. at 4°C to remove matrix. The concentration and purity of the RNA samples were determined using spectroscopy and electrophoresis to visualize 18s and 28s rRNA bands.

Semi-quantitative RT-PCR

Two hundred nanograms of RNA template were reverse transcribed into cDNA per 8 µl reaction using cDNA synthesis reagents (Perkin Elmer/Roche, Branchburg, New Jersey). One microlitre of cDNA from the above RT reaction was used for each PCR reaction. Primer sequences are listed in Table 3. Each primer pair was designed to flank at least one intron. in order to prevent and distinguish amplification of contaminating genomic DNA. Genes of interest were matched to internal control genes that had similar PCR kinetics in order to allow near 1:1 expression level compared to the gene of interest in the linear range of amplification under optimized PCR conditions.

PCR reactions were carried out in a DNA thermal cycler (Gene Amp PCR System 9600. Perkin Elmer, Branchburg, New Jersey) at an annealing temperature of 58°C. and the linear range of amplification was determined over a range of cycles for each primer pair. PCR products were electrophoresed on 12% polyacrylamide gels and stained with ethidium bromide. Positive and negative controls for each primer pair, including a no-template water control carried over from the RT step, were run on each gel. The gels were photographed and negatives used for densitometry (ImageQuant Software v3.0.
Molecular Dynamics, Sunnyvale, California). Relative levels of band intensity of the gene of interest were compared to the internal control house keeping gene within the linear range of amplification, and normalized to a positive control sample on each gel.

**Western blot**

Protein was extracted from tissue samples by homogenization in lysis buffer (1% SDS, 10 mM/L Tris/HCl, pH 7.4), followed by 15 seconds in a boiling water bath and 5 minutes of centrifugation (12 000 X g). Sample proteins were electrophoresed on an SDS-polyacrylamide gel, transferred to a polyvinylidene difluoride membrane and stained to verify successful transfer. Western blot was performed using 5E1\textsuperscript{14}, a monoclonal antibody to the amino terminus of rat Sonic hedgehog developed by T.M. Jessel. Columbia University and obtained from the Developmental Studies Hybridoma Bank. University of Iowa (Western blot confirmed binding to human IHH, and note that SHH transcripts were undetectable – data not shown), and the anti-c-Myc 9E10 monoclonal was obtained from Santa Cruz Biotechnology, Inc. Hybridization was carried out overnight at 4°C and detected using an anti-mouse IgG – horseradish peroxidase secondary antibody and chemiluminescence.

**Explant culture**

Primary tissue specimens were cut into 1-3mm thick sections and maintained in Dulbecco’s modified eagle medium (DMEM) with high glucose, as described elsewhere\textsuperscript{199} for growth plate explants, for four days. Cultures were treated with either 0.1% FBS (agonist control), 10 µg/ml Shh-N (a gift from Ontogeny Inc.), 100\textsuperscript{7}M PTHrP (1-34, Bachem), 100\textsuperscript{4}M cyclopamine (a gift from William Gaffield - compared to
treatment with equal volume ethanol carrier), or 10 μg/ml neutralizing monoclonal anti-
Shh-N antibody (5E1) (compared to treatment with 10 μg/ml anti-mouse IgG).

Statistical comparisons were made using the Wilcoxon Signed Ranks Test, which
is a nonparametric test suitable for paired data. A parametric test could not be used
because it could not be assumed that each of the human tumour specimens were identical
prior to the start of the experiments.

**Tritiated thymidine assays**

Explant specimens were incubated with 10 μCi/ml tritiated (3H) thymidine for the
final 20 hours of culture, then digested with 0.5% Pronase (Boehringer Mannheim, Laval,
Québec) for 1 hour, followed by 0.125% Collagenase A (Boehringer Mannheim, Laval,
Québec) for 8 hours. Cells were counted and resuspended in 0.5 ml 1% SDS/0.005M
EDTA. Nucleic acids were precipitated with 1 ml ice cold 25% trichloroacetic acid
(TCA), and pipetted onto 2.4 cm glass microfiber filters (Whatman GF/C) over a suction
apparatus. The filters washed twice with 2 mL 5% TCA, air dried and placed in
scintillation vials with 5 ml scintillation cocktail, and counts per minute averaged over 5
minutes.

**Mutational analysis**

Single strand conformation polymorphism (SSCP) analysis was used to screen for
mutations, as previously described in the PTHR1 gene. Genomic DNA was extracted
from specimens using a kit (Qiagen) and used as template for PCR amplification of
fragments containing an exon and its adjacent intronic boundaries. Primer sequences are
listed in Table 4. The 33P-ATP incorporated PCR product was denatured and
electrophoresed on a native polyacrylamide gel containing 10% glycerol. Band shifts
were further analyzed using a sequencing kit (Thermo Sequenase Sequencing Kit, Amersham Life Science, Cleveland, Ohio).

**PTHR1 functional analysis**

Non-PCR based site-directed mutagenesis (Site-Directed Mutagenesis Kit, Clontech) of the *WT PTHRI* cDNA in the pcDNA1 vector was used to generate the R150C and other published mutations of the *PTHRI* gene\(^ {187-189,193-195}\). The mutant constructs were verified by automated sequencing. COS7 cells were plated at a density of 5 X 10^4 cells/ml and transfected the following day with the aid of a transfection reagent (Fugene 6, Roche). Forty hours following transfection, cells were serum starved for two hours and then treated with either PTHrP (1-34) in DMEM containing 0.1% FBS carrier or carrier alone for 15 minutes for cAMP assays and for 40 minutes for IP3 assays. Cyclic AMP assays were performed using an "enzymeimmunoassay" kit (RPN 225, Amersham), and IP3 assays were performed using a tritiated IP3 assay system (TRK 1000, Amersham). Transfection efficiency, which was typically over 40% as assessed by cotransfection with green fluorescent protein cDNA, was controlled by cotransfection of cells with β galactosidase cDNA and normalization of assay results to β galactosidase activity measured at 415λ (β Galactosidase Assay Kit, Stratagene). Immunofluorescent subcellular localization was carried out using carboxy terminal c-Myc-tagged versions of the wild type and variant *PTHR1* and anti-c-Myc 9E10 antibody (Santa Cruz Biotechnology, Inc.) following cell membrane permeabilization with 0.2% Triton X100 for 3 minutes at room temperature. (Tagging the carboxy terminus of PTHR1 has been shown not to interfere with protein expression, ligand binding or signalling capability\(^ {190}\).
Transgenic mice

The WT PTHRI or R150C PTHRI constructs were cloned by blunt ligation into the NotI sites of the mouse CollI promoter and enhancer-containing construct pKN185 (a gift from Y. Yamada) and linearized by digestion with NdeI and HindIII prior to microinjection into the pronucleus of fertilized ICR eggs. Genomic DNA was extracted from tails with the aid of an extraction kit (Qiamp, Qiagen) and screened by Southern blot for integration of the transgene. Heterozygous founder (Fig. 3a) and F1 generation (Fig. 3b) mice were used for phenotypic analysis.

For Southern blot, approximately 5μg of DNA was digested with EcoRI and BamHI, electrophoresed through a 0.7% agarose gel and depurinated in 0.2N HCl for 10 min. followed by a distilled water wash. Capillary transfer of DNA to a nylon membrane (Z. Roche) was carried out overnight using 0.4N NaOH. The nylon membrane was washed briefly in 2% sodium chloride/sodium citrate solution (SSC), air dried and crosslinked with ultraviolet light. Radioactive probe was generated by PCR amplification of ~20ng transgene template using 3.3μM 32P-labelled dCTP (without any cold dCTP) with the following primers: forward (β Globin F): CTCTGCTAACCATGTTCATG, and reverse (Sev8): GCAGGAAGATCTGTCCTC; amplicon=223bp. The probe was purified using a Sephadex G-50 column (Amersham), and quantified by scintillation counter assessment of sequential eluant samples. The nylon membrane was prehybridised with Ultrahyb (Ambion) at 42° for 1h, rotating, in an oven, followed by addition of 10⁶ counts of radioactive probe per ml hybridisation solution overnight at 42°. The membrane was then washed twice in 2% SSC, 0.1% SDS, and twice in 0.1% SSC, 0.1% SDS at 42° followed by exposure to a phosphor screen overnight. Bands were
visualised using a phosphorimager apparatus. Southern blot genotyping was often complemented with PCR genotyping (Fig. 3c), using the following primers: forward (Sev4): GGGTTCCTCAACGGCTCCT, and reverse (Sev7): CAGGTCCTCTTCGCTAATC; amplicon=133bp.

**Immunohistochemistry**

Anti-c-Myc: Five micron 4% paraformaldehyde fixed, paraffin embedded sections were dewaxed and hydrated, and epitope exposed using heat induced epitope retrieval. Sections were blocked for endogenous peroxidase and biotin, incubated for 1 hour at room temperature with primary antibody (9E10 anti-c-Myc), detected with a mouse – on – mouse immunostaining kit (Vector Labs), and visualized using diaminobenzidene.

Anti-COLX: Deparaffinized sections were incubated with 0.3% H$_2$O$_2$ / MeOH at room temperature (RT) for 40min. to quench endogenous peroxidase, then treated with 0.1% pepsin in 0.5M acetic acid at 37°C for 2h and demasked with 2mg/ml hyaluronidase PH5 at 37°C for 30min. Samples were preblocked with 3% BSA, 1% BM blocking solution (10mM maleic acid, 15mM NaCl in H2O), 30mM MgCl$_2$, 0.3% Tween-20 in PBS overnight at 4°C. Samples were incubated with a 1:40 dilution of anti-human recombinant COLX (Quartett Immunodiagnostika Biotechnologie GMBH) overnight at 4°C in a humid chamber, then washed with PBS and incubated with biotinylated anti-mouse IgG (1:200 dilution) for 1h at RT. Samples were incubated with ABC solution for 45min. at RT for colour subtraction and colour was developed using 0.1M Tris pH 7.5, 0.5mg/ml DAB, 0.03% H$_2$O$_2$. 
**TRAP (tartrate resistant acid phosphatase) staining**

A TRAP staining kit (Sigma) was used on paraffin embedded sections to identify chondroclasts.
TABLE 1: The fraction of a given specimen type for which transcripts were detectable by RT-PCR. \( SHH = \text{Sonic hedgehog}, IHH = \text{Indian hedgehog}, PTC = \text{Patched}, PTHrP = \text{Parathyroid hormone related protein}, PTHR1 = \text{Type 1 PTH/PTHrP receptor} \)

<table>
<thead>
<tr>
<th>Specimen Type</th>
<th>SHH</th>
<th>IHH</th>
<th>PTC</th>
<th>GLI1</th>
<th>GLI2</th>
<th>GLI3</th>
<th>PTHrP</th>
<th>PTHR1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Articular Cartilage</td>
<td>0/4</td>
<td>0/4</td>
<td>4/4</td>
<td>2/4</td>
<td>2/4</td>
<td>4/4</td>
<td>2/4</td>
<td>4/4</td>
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<tr>
<td>Osteochondroma (mature)</td>
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<td>0/2</td>
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<td>2/2</td>
<td>2/2</td>
<td>2/2</td>
<td>2/2</td>
</tr>
<tr>
<td>Chondroblastoma</td>
<td>0/7</td>
<td>2/7</td>
<td>7/7</td>
<td>7/7</td>
<td>7/7</td>
<td>7/7</td>
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<tr>
<td>Osteosarcoma</td>
<td>0/10</td>
<td>0/10</td>
<td>10/10</td>
<td>10/10</td>
<td>10/10</td>
<td>10/10</td>
<td>10/10</td>
<td>10/10</td>
</tr>
</tbody>
</table>

TABLE 2: Average expression ratios, by semiquantitative RT-PCR, of the \( GLI \) gene of interest over the internal housekeeping gene Asparagine synthetase (AS) in normal and lesional tissues.

<table>
<thead>
<tr>
<th>Specimen Type</th>
<th>GLI1/AS</th>
<th>GLI2/AS</th>
<th>GLI3/AS</th>
<th>( (GLI1 + GLI2) / GLI3 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal bone (n=11) +</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>articular cartilage (n=4)</td>
<td>0.60</td>
<td>0.60</td>
<td>3.23</td>
<td>0.37</td>
</tr>
<tr>
<td>enchondromas (n=4) +</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>chondrosarcomas (n=11)</td>
<td>0.74</td>
<td>0.93</td>
<td>1.11</td>
<td>1.5</td>
</tr>
</tbody>
</table>
### TABLE 3: Forward (F) and Reverse (R) RT-PCR Primer Sequences

*IH*H*=Indian hedgehog, SHH=Sonic hedgehog, PTCH=Patched, GLI=Glioblastoma

*PTHrP*=Parathyroid hormone related protein, *PTHR1*=Type 1 PTH/PTHrP receptor.


*β2M*=Beta-2-microglobulin

Primer pairs used were *IH*/ASa, *SHH*/ASa, PTCH/ASb, GLI1/ASa, GLI2/ASa, GLI3/ASC.

*PTHrP*/PBGD, *PTHR1*/PBGD, and COLX/β2M.

<table>
<thead>
<tr>
<th>GENE</th>
<th>SEQUENCE (5' - 3')</th>
<th>AMPLECTON (BP)</th>
<th>GENBANK ACCESSION</th>
</tr>
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<tr>
<td><em>IH</em>H</td>
<td>F CGGCGCCTCATGACCCA</td>
<td>278</td>
<td>L38517</td>
</tr>
<tr>
<td></td>
<td>R GACTTGACGGAGCAATGCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>SHH</em></td>
<td>F GCGGACAGGCTGATGACT</td>
<td>260</td>
<td>L38518</td>
</tr>
<tr>
<td></td>
<td>R TGTGCCCTTGAGACTCGTAGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>PTCH</em></td>
<td>F GGTCATGGTTACATGGACC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R TGCTGTCTGACTGGCGCC</td>
<td>190</td>
<td>U59464</td>
</tr>
<tr>
<td><em>GLI1</em></td>
<td>F GAGAAGCCACACAAGTGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R AGGGAGCGCTACATACATACG</td>
<td>210</td>
<td>X07384</td>
</tr>
<tr>
<td><em>GLI2</em></td>
<td>F GGAGTACGACACCCAGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R CCCTCGAACGTGCACTTTG</td>
<td>189</td>
<td>AB007298</td>
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<tr>
<td><em>GLI3</em></td>
<td>F CCTTCTGAGTCTCCTACAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R GGTGCTTCCTCGGCTGTT</td>
<td>152</td>
<td>M57609</td>
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<td><em>PTHrP</em></td>
<td>F GTGACACACGGCGACAGT</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>R CTCGCCATTGCAGAAACAG</td>
<td>156</td>
<td>M57293</td>
</tr>
<tr>
<td>Gene</td>
<td>Forward</td>
<td>Reverse</td>
<td>Length</td>
</tr>
<tr>
<td>------</td>
<td>---------</td>
<td>---------</td>
<td>--------</td>
</tr>
<tr>
<td>PTHRI</td>
<td>TTCCTGCTGAGCTACGCG</td>
<td>TGCGATCAGATGGTGAAGG</td>
<td>206</td>
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<tr>
<td>COLX</td>
<td>CCATCTCCAGGAACTCCC</td>
<td>CTTGCTCTCCTCTTTACTGC</td>
<td>215</td>
</tr>
<tr>
<td>ASa</td>
<td>ACATTGAAGCACTCCCCGCGAC</td>
<td>AGAGTGGCAGCAACCAAGCT</td>
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</tr>
<tr>
<td>ASb</td>
<td>GAGGCTTCTGAGGGAACTCT</td>
<td>TTTCTGGTGCGAGAGACAAAG</td>
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<tr>
<td>ASc</td>
<td>ACATTGAAGCACTCCCCGCGAC</td>
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<tr>
<td>PBGD</td>
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<td>GCTGGTGGCTAGGATGATGG</td>
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</tr>
<tr>
<td>β2M</td>
<td>ACCCCCACGTAAAAAGATGA</td>
<td>GGAGACACGAATCTCAAAGTAG</td>
<td>267</td>
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</table>
**TABLE 4:** Forward (F) and Reverse (R) SSCP Primer Sequences for all PTHR1 exons

<table>
<thead>
<tr>
<th>Exon</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1F</td>
<td>CAGCCTGACGCAGCTCT</td>
<td>GTAACCCGCAGCCTGTC</td>
</tr>
<tr>
<td>1R</td>
<td>GTAACCCGCAGCCTGTC</td>
<td>CAGCCTGACGCAGCTCT</td>
</tr>
<tr>
<td>2F</td>
<td>GAAATTGGGATGTCTTTGGG</td>
<td>TCCCTCACCTGACCTCATA</td>
</tr>
<tr>
<td>2R</td>
<td>TCCCTCACCTGACCTCATA</td>
<td>GAAATTGGGATGTCTTTGGG</td>
</tr>
<tr>
<td>3F</td>
<td>GTTGTAGCAGCTGACAG</td>
<td>TCCCTCACCTGACCTCATA</td>
</tr>
<tr>
<td>3R</td>
<td>TCCCTCACCTGACCTCATA</td>
<td>GTTGTAGCAGCTGACAG</td>
</tr>
<tr>
<td>4F</td>
<td>CTTGACTCTCCTTTGGTAT</td>
<td>GGAGTGAAATTTATCTGGTCA</td>
</tr>
<tr>
<td>4R</td>
<td>GGAGTGAAATTTATCTGGTCA</td>
<td>CTTGACTCTCCTTTGGTAT</td>
</tr>
<tr>
<td>5F</td>
<td>AGTGCCTCGAGACCTCC</td>
<td>TTTACCCTGTGCTCTCC</td>
</tr>
<tr>
<td>5R</td>
<td>TTTACCCTGTGCTCTCC</td>
<td>AGTGCCTCGAGACCTCC</td>
</tr>
<tr>
<td>6F</td>
<td>GCATCAGACCTCGGCCA</td>
<td>ACCCAGGTCATGGGCA</td>
</tr>
<tr>
<td>6R</td>
<td>ACCCAGGTCATGGGCA</td>
<td>GCATCAGACCTCGGCCA</td>
</tr>
<tr>
<td>7F</td>
<td>ACCTTCCGGAGGCAG</td>
<td>GAGGCCAGTGCCGCA</td>
</tr>
<tr>
<td>7R</td>
<td>GAGGCCAGTGCCGCA</td>
<td>ACCTTCCGGAGGCAG</td>
</tr>
<tr>
<td>8F</td>
<td>TGACTTCCTCCGGAGGCAG</td>
<td>CTCTTTCTGTCACCCAC</td>
</tr>
<tr>
<td>8R</td>
<td>CTCTTTCTGTCACCCAC</td>
<td>TGACTTCCTCCGGAGGCAG</td>
</tr>
<tr>
<td>9F</td>
<td>GAATGACCTTTGAGACAG</td>
<td>CTCACATGCTTCTGGAAG</td>
</tr>
<tr>
<td>9R</td>
<td>CTCACATGCTTCTGGAAG</td>
<td>GAATGACCTTTGAGACAG</td>
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<tr>
<td>10F</td>
<td>TCAACAGCTAATGTCAGACC</td>
<td>GAGGCCGCACATCCCA</td>
</tr>
<tr>
<td>10R</td>
<td>GAGGCCGCACATCCCA</td>
<td>TCAACAGCTAATGTCAGACC</td>
</tr>
<tr>
<td>11F</td>
<td>GACAGAGCGAGAGCTATG</td>
<td>TCAACAGCTAATGTCAGACC</td>
</tr>
</tbody>
</table>
11R: ATACATGGTACCAGGACTC
12F: GAAGTGGCCTTTGGCCCT
12R: GGTGCTCTGAATCAGGC
13F: CTTCCAGGTGCACGATG
13R: CTTCCAGGTGCACGATG
14-1F: GGAGACACACTGACTG
14-1R: TTGGCATGGGCAAGCCAG
14-2F: TACTGCCCCACTGCCACCA
14-2R: TGGTCCATCTGTCATCCA
Chondrosarcoma (CSA, n=10) and enchondroma (ECA, n=2) explant cultures. 

**a.** Downregulation of Type X collagen (COLX) compared to internal control β-2-microglobulin (B2M) by semiquantitative RT-PCR following treatment with 10^{-7}M PTHrP (1-34) compared to treatment with carrier alone (p=0.01, n=12 - Wilcoxon Signed Ranks Test). 

**b.** Increased incorporation of tritiated thymidine (³H-T) following treatment with 10 µg/ml recombinant Shh-N protein (p=0.02, n=12 - Wilcoxon Signed Ranks Test). 

**c.** Decreased incorporation of ³H-T following treatment with 10^{-4}M cyclopamine dissolved in ethanol (p=0.07, n=6 (2 enchondroma and 4 chondrosarcoma specimens) – Wilcoxon Signed Ranks Test). 

**d.** Semiquantitative RT-PCR assay showing IHH is downregulated compared to internal control Asparagine synthetase (AS) in growth plate (GP) explants (n=4), but not in chondrosarcoma (CSA) (nor in enchondroma – data not shown) explants, following treatment for four days with 10^{-7}M PTHrP (1-34).
Figure 2

(a) normal enchondromatosis

C/T
A C G T A C G T

(b) Figure showing cell proliferation

(c) Figure showing cell proliferation

(d) Figure showing cell proliferation
PTHRI mutation and functional analysis. a. C to T change and resulting amino acid substitution. b. Cyclic AMP “enzymeimmunoassay” (Amersham) in COS-7 cells comparing R150C variant with empty vector (pcDNA1), wild type (WT) PTHRI, other known loss of function (P132L) and activating mutants (H223R, T410P) and a polymorphism we identified (E546K), in the presence of $10^{-6}$M PThrP (1-34) compared to carrier alone. c. IP$_3$ $^3$H-T assay (Amersham) in ES cells which lack the native PTHRI gene. d. Cyclic AMP accumulation in response to increasing PThrP dose. Assays were performed three times in duplicate with DNA from two separate bacterial colonies. e. Dominant negative effect of R150C PTHRI over WT PTHRI with regard to cAMP level. f. Confocal immunofluorescent subcellular localization of carboxy terminal c-Myc-tagged WT and R150C PTHRI in COS-7 cells using 9E10 antibody following cellular permeabilization.
Southern blot of founder (a) and F1 generation (b) mouse genomic DNA. c, PCR genotyping of F1 generation mice. d, Anti-c-Myc immunohistochemistry revealed expression of the c-Myc tagged R150C (and WT- not shown) PTHRI transgene (brown colour) under the control of ColII regulatory elements in growth plate chondrocytes, but not in the surrounding perichondrium where ColII is not expressed, nor in a negative control growth plate (e).
Newborn mouse growth plates. Trichrome stained WT PTHR1 (a) and R150C PTHR1 (b) transgenic proximal humeral growth plates at identical magnification demonstrated a difference in the height of the hypertrophic zone (between dark lines). Anti-COLX immunostaining of WT PTHR1 (c) and R150C PTHR1 (d) transgenic distal femoral growth plates highlighted the relatively short hypertrophic zone (dark brown colour and arrows) in R150C PTHR1 mice.
Murine enchondromas were evident in R150C PTHR1 (b-arrows, d), but not WT PTHR1 (a), proximal humeri at 8.5 weeks of age as multiple metaphyseal cartilage rests. These rests persisted to at least 38 weeks of age (c,e), occurred in multiple bones including the distal femur (20 weeks old - f), and consisted of islands of chondrocytes in a hyaline cartilage matrix, similar to human enchondromas.
A paucity of hypertrophic chondrocytes in R150C PTHR1 growth plates (b) persisted relative to WT PTHR1 growth plates (a) and may have allowed proliferating chondrocytes to have been left behind in the metaphysis to form enchondromas. Tartrate resistant acid phosphatase staining specifically highlighted equal numbers of chondroclasts (red-brown colour) at the cartilage-bone interface in WT PTHR1 (c) and R150C PTHR1 (d) bones, suggesting the metaphyseal cartilage rests were not caused by a resorption defect.
CHAPTER 3

Gli2 and Gli3 in growth plate regulation and pathology
SUMMARY

The Hedgehog responsive transcription factors Gli2 and Gli3 have specific and redundant functions during limb patterning\(^{181}\), but their roles in later growth plate regulation have not been elucidated. Growth plate chondrocyte proliferation is regulated in large part by Indian hedgehog (Ihh) signalling\(^{60,163}\). Here I show how the likely Ihh transcription factors Gli2 and Gli3 contribute to the regulation of chondrocyte proliferation, as well as how these molecules modify regulation of differentiation by PTHrP. Based on relative growth plate size and BrdU uptake of proliferative zone chondrocytes in long bone cultures from mice lacking Gli2 and Gli3 and from those overexpressing Gli2 under the regulatory elements of Type II collagen (CollII). I show that chondrocyte proliferation is positively regulated by Gli2 and negatively regulated by Gli3. PTHrP treatment of organ cultures inhibited proliferation in addition to having delayed differentiation of chondrocytes. Both of these effects of PTHrP were dampened by Gli2 and augmented by Gli3. Dysregulation of Hedgehog signalling in fetal bone explants of mice which overexpress Gli2 or lack Gli3 is analogous to that caused by the mutant PTH/PTHrP receptor (PTHR1) which causes enchondromatosis in humans (Chapter 2). These mice developed chondrocyte neoplasia equivalent to the human conditions enchondromatosis and synovial chondromatosis, respectively.
INTRODUCTION

Growth plate chondrocyte proliferation and differentiation are regulated in part by Indian hedgehog (Ihh) and Parathyroid hormone related protein (PTHrP). Expression of PTHrP, which results in delay of hypertrophic differentiation of chondrocytes, is dependent upon Ihh. Ihh-driven chondrocyte proliferation, however, is largely independent of PTHrP. Therefore, the mitogenic function of Ihh is likely mediated directly by the Hedgehog signalling pathway in proliferating chondrocytes. The Gli family of transcriptional regulators likely mediates Ihh function in the growth plate.

The skeletal phenotype of mice lacking either of the Hedgehog transcription factors Gli2 or Gli3 is less severe than that of Ihh null mice. while Gli1 single homozygous mutant mice are apparently normal. Gli2+/- mice develop short long bones, while Gli3+/- mice display regional, mainly anterior abnormalities such as shortened or deleted tibias and preaxial polydactyly. Some of these phenotypes are exacerbated in double mutants, indicating that Gli2 and Gli3 have redundant as well as specific functions during limb patterning. These functions may be attributable to the patterns of expression of Gli2 and Gli3 and to the distinct biochemical properties of the different Gli proteins. Gli2 and Gli3 both have an N-terminal repressor domain and a C-terminal activator domain, whereas Gli1 has only an activator domain. The activator and repressor capabilities of Gli2 and Gli3 are context-dependent. Whether Gli2 and Gli3 are positive or negative regulators of proliferation and differentiation in the growth plate remains to be elucidated.

Ectopic tissue resembling growth plate cartilage is found in certain benign cartilage tumours. These include enchondromas and synovial chondromatosis.
Such cartilage tumours might be caused by inappropriate expression or regulation of growth plate signalling proteins. I recently showed that Hedgehog signalling is overactive in enchondromas relative to normal mesenchymal tissues (Chapter 2). Whether excessive Hedgehog signalling is sufficient to cause cartilage tumours is unclear. Here I show that Gli2 acts as an activator and Gli3 as a repressor of Ihh function in the murine growth plate. Overexpression of Gli2 or a lack of Gli3 led to ectopic proliferation of cartilage in mice, resembling the human conditions enchondromatosis and synovial chondromatosis, respectively.

RESULTS

Growth plate proliferation is increased by Gli2 and diminished by Gli3

Relative growth plate size and proliferative zone bromodeoxyuridine (BrdU) uptake were compared between mice with genetic alterations of Gli2 and Gli3. Overall growth plate height at E16.5 was most notably altered in bones from Gli2−/− mice, where the distal femoral growth plate was 17% shorter (1.28 +/- 0.8mm. n=4) than in WT mice (1.54 +/- 0.15mm. n=4). All long bones of Gli2−/− mice were previously reported to be shorter than those of WT mice181. and I measured a difference of 10% in femoral length between these animals (Gli2−/− = 3.0 +/- 0.02mm. n=4 vs. WT = 3.35 +/- 0.12mm. n=4). At E16.5, there was no alteration of growth plate height in Gli2+/−, Gli3+/−, Gli3−/− mice, nor in transgenic mice overexpressing Gli2 under the regulatory elements of mouse Type II collagen (ColII). Offspring of three out of four founder mice expressing the ColII-Gli2 transgene did, however, develop growth plates that were longer than those of WT littermates by four weeks of age. Proximal tibial growth plate height, for example, was
49% taller in ColII-Gli2 animals (380 +/- 10\mu m, n=3 vs. 255 +/- 25, n=2 in WT animals – Fig. 1c,d). Both the proliferative zone and the hypertrophic zone were greater in size, which was most clearly demonstrated by anti-COLX immunostaining to highlight the hypertrophic zone (Fig. 1e,f). This growth plate height difference between ColII-Gli2 and WT mice diminished as the growth plate thinned throughout adolescence, and was barely perceptable by eight weeks of age (Fig. 1g,h). All long bones were also taller in these transgenics. Tibias of ColII-Gli2 mice were 11% longer (14.4 +/- 0.4 mm, n=3) than those of WT littermates (13 +/- 0.2 mm, n=3).

Lower extremity long bones of EL6.5 mice were dissected free of soft tissues and maintained in culture for seven days. BrdU was added to the media for the final 20 hours of culture before the limbs were fixed, paraffin embedded, sectioned and stained to detect BrdU. The proportion of proliferative zone BrdU positive cells in the growth plates of these mice was decreased by a total lack of Gli2 and increased in mice overexpressing Gli2 compared to WT littermates. BrdU uptake was also increased by a total lack of Gli3 (Fig. 2b, 4).

**PTHrP stimulation decreases proliferation in addition to delaying hypertrophic differentiation, and these responses are diminished by Gli2 and augmented by Gli3**

Right and left limbs from a single animal were maintained in organ cultures described above and underwent daily changes of media containing either 10^{-6}M PTHrP (1-34) or 0.1% bovine serum albumin (BSA). Immunostaining of sections with anti-COLX antibody to highlight the hypertrophic zone of the growth plate revealed that PTHrP treatment of cultures resulted in diminution of this zone, as anticipated (Fig. 2a,
Surprisingly, PTHrP treatment of cultures also resulted in a small decrease in the proportion of BrdU positive cells (Fig. 2b, 4).

The extent to which PTHrP treatment of organ cultures altered differentiation and proliferation varied according to genotype. Growth plates from $Gli2^{+/+}$ mice were the most sensitive to PTHrP with respect to hypertrophic differentiation delay and decrease in BrdU uptake compared to growth plates from $Gli2^{++}$ and $Gli2^{+/+}$ mice (Fig. 2a, b, 3, 4). Consistent with these findings, these changes were smaller in $ColII$-$Gli2$ transgenic growth plates compared to those of WT littermates (Fig. 2a, b, 3, 4). Sensitivity to PTHrP was also diminished in $Gli3^{+/+}$ growth plates compared to growth plates from $Gli3^{+/+}$ and $Gli3^{++}$ mice (Fig. 2a, b, 3, 4).

$ColII$-$Gli2$ mice develop enchondromatosis

Since transgenic mice expressing $ColII$-$R150C PTHR1$ develop enchondromatosis, and since the growth plates from these mice behaved similar to those of $ColII$-$Gli2$ mice in explant culture, I investigated whether enchondromas arose in $ColII$-$Gli2$ transgenic mice. Histological sections beyond twelve weeks of age of long bones were stained with safranin-O to highlight cartilage. Islands or rests of cartilage on the metaphyseal side of the growth plate were evident in three founder lines expressing the $ColII$-$Gli2$ transgene, but not in WT littermates (Fig. 5). The distal femur (Fig. 5a, c), tibia, proximal humerus (Fig. 5f) and distal radius were the most common locations of the lesions, which persisted well into adulthood (Fig. 5f). These rests of cartilage were similar to growth plate tissue (Fig. 5b, d, f), and similar to those found in the $ColII$-$R150C PTHR1$ transgenic mouse model of enchondromatosis. In order to determine whether these cartilage rests resulted from a multifocal lack of resorption of
the growth plate rather than from altered growth plate proliferation and differentiation, sections were stained with acid phosphatase and challenged with tartrate to highlight chondroclasts. Chondroclasts are responsible for resorption of the calcified cartilage matrix at the bone – cartilage junction\textsuperscript{59,60}, and were found in equal numbers in both ColII-Gli2 and WT littermate bones (Fig. 5g, h).

**Gli3 heterozygous mice develop accelerated synovial chondromatosis**

Given the similarity in response to PTHrP of fetal long bone cultures from mice overexpressing Gli2 and those lacking Gli3, I investigated whether Gli3\textsuperscript{+/−} mice develop skeletal pathology. Although no enchondromas were discovered in Gli3\textsuperscript{+/−} mice, the males in particular developed cartilaginous neoplasia similar to the human condition synovial chondromatosis in their knees, which is the most common site in humans. Ectopic cartilage containing chondrocytes cytologically similar to growth plate chondrocytes was seen extending directly off the synovial lining of the joint (Fig. 6c). Binuclear lacunae and occasional mitotic figures indicated active proliferation within the lesion (Fig. 6e). This ectopic cartilage was sometimes separated from the synovial lining and present as loose bodies, and was apparently eroding into the articular surfaces of the femur and tibia, similar to late stage human synovial chondromatosis. Inflammatory cells were not seen in or around the joint lining. Calcification within the ectopic cartilage was seen as speckled opacities on radiographs (Fig. 6a, b). Synovial chondromatosis occurred predominantly in males, and was often bilateral. The occurrence of synovial chondromatosis increased with age in all genotypes examined (WT, Gli2\textsuperscript{+/−}, Gli3\textsuperscript{+/−} and Gli2\textsuperscript{+/−}/Gli3\textsuperscript{+/−}), but was significantly accelerated in males lacking one Gli3 allele at 13 months of age (p=0.012 for Gli3\textsuperscript{+/−} and p=0.047 for Gli2\textsuperscript{+/−}/Gli3\textsuperscript{+/−}, Fisher’s Exact Test)
(Fig. 6f). By 21 months, the incidence of synovial chondromatosis in Gli3−/− mice was diminished by the additional lack of a single Gli2 allele (Fig. 6f).

DISCUSSION

Gli2 mediates and Gli3 inhibits Ihh function in the growth plate

Ihh is largely responsible for growth plate chondrocyte proliferation and induction of PTHrP expression, which in turn delays hypertrophic differentiation80,81,163. In this study, the absence of Gli2 was associated with a decrease in proliferation and an increase in hypertrophic differentiation, while overexpression of Gli2 and a lack of Gli3 were associated with the opposite results. Therefore Gli2 is a positive mediator and Gli3 is a negative regulator of Ihh function in the proliferative zone of the growth plate.

Evidence of functional antagonism between Gli2 and PTHrP

It is known that, in addition to signalling the perichondrium to induce PTHrP expression, Ihh likely signals proliferating chondrocytes80. Therefore, a subset of Ihh and PTHrP target cells are likely the same. The nature of the interaction between these signals in the proliferative zone has not been elucidated. I found that chondrocyte proliferation, which is positively regulated by Gli2, was inhibited by exogenous PTHrP in organ culture. This finding is in contrast to previous reports which showed that a lack of PTHrP157, as well as exogenous PTHrP in explant cultures186, did not alter the proliferation of growth plate chondrocytes. My cultures were treated with a saturating dose of PTHrP for a prolonged period of seven days, and the result may be due to at least two factors. Prolonged superphysiologic PTHrP treatment of cultures may have decreased the number of hypertrophic chondrocytes, a source of the mitogenic Ihh signal,
enough to alter proliferation and thereby indirectly antagonize Ihh function. However, Ihh is also expressed by a population of prehypertrophic chondrocytes, which is probably expanded by PTHrP treatment, and it is difficult to estimate the relative importance of the sources of Ihh in inducing chondrocyte proliferation. An alternate possibility is that PTHrP directly antagonizes Ihh function in cells of the proliferative zone. Consistent with this possibility is the fact that protein kinase A (PKA), which is activated by PTHrP signal transduction\textsuperscript{143}, is a conserved inhibitor of Hedgehog signalling\textsuperscript{132}.

Antagonism by \textit{Gli2} on PTHrP function was also evident. The degree to which PTHrP inhibited both proliferation and differentiation was inversely related to \textit{Gli2} level and directly related to \textit{Gli3} level, which shows that \textit{Gli2} and \textit{Gli3} have distinct roles as mediators in the interaction between Ihh and PTHrP signalling, although the effects of \textit{Gli3} were weak in this regard. These findings suggest that \textit{Gli2} antagonizes PTHrP function because the presence of a higher number of \textit{Gli2} alleles was associated with a diminished ability of PTHrP to inhibit proliferation and differentiation. An alternate possibility is that endogenous PTHrP levels were raised with increasing numbers of \textit{Gli2} alleles, consequently delaying differentiation with greater effectiveness and thereby minimizing the effect of exogenous PTHrP. A problem with this alternative possibility is that endogenous PTHrP produced by the perichondrium may diffuse into the media without reaching the proliferative zone in a concentration sufficient to explain the result. Interestingly, others have shown that Hedgehog stimulation of growth plate chondrocytes exerts a pro-hypertrophic differentiation effect \textit{in vitro}\textsuperscript{202}. One can explain these findings and my data by invoking a model whereby Ihh - stimulated \textit{Gli2} antagonizes PKA – stimulated delay of chondrocyte differentiation.
Excessive Hedgehog signalling is sufficient to generate cartilaginous neoplasia

As previously noted, enchondromatosis, a human condition where multiple benign cartilage tumours form on the metaphyseal side of the growth plate as a result of abnormal growth plate development, is caused in some cases by a mutant PTHR1 (Chapter 2). Other genetic alterations that cause similar biochemical derangement would also be expected to cause benign cartilage tumours. I found that overexpression of Gli2 in the growth plate has effects on the regulation of proliferation and differentiation similar to those caused by the enchondromatosis mutant PTHR1, and does in fact result in multiple enchondromas in mice. Mice lacking a single Gli3 allele did not develop enchondromas, despite the functional approximation between Gli3"/+" and ColII-Gli2 growth plates in culture. Rather, these mice developed a different condition of benign chondrocyte neoplasia which was similar to human synovial chondromatosis. The difference in location and in the time of appearance of this condition suggests that Gli2 and Gli3 do not simply perform opposite functions, and reflects the regional importance of Gli3, especially around the periarticular perichondrium of the proximal tibia. Consistent with this hypothesis, lack of a single Gli2 allele did not prevent the emergence of synovial chondromatosis in Gli3"/+" mice, at least until 13 months of age. In both Gli3"/+" and ColII-Gli2 mice, an alteration of Hedgehog signalling resulted in ectopic chondrocyte proliferation, which underlines the importance of tight regulation of growth plate signals.
METHODS

Embryonic limb organ culture

Hind limbs from embryonic day (E) 16.5 mice were dissected free of soft tissues, leaving the perichondrium intact. These limbs were maintained in culture in 24 well plates, one limb per well, for 3 days for proliferation assays and for 7 days for differentiation assays, with daily changes of 300 μl media which just covered the explants. Right and left limbs from each mouse were compared as treatment (10^{-7} M PTHrP (1-34) in DMEM + 0.1% bovine serum albumin (BSA)) or control (DMEM + 0.1% BSA) groups.

Sample variance is provided in Figure 2 in the form of error bars. Calculation of Type I error (false positive) was not carried out because the low and varying sample number would have rendered accurate assessment of the complimentary and equally important Type II error (false negative) impossible.

COLX immunohistochemistry

Anti-COLX immunohistochemistry was carried out as described in Chapter 2.

BrdU uptake analysis

Explant cultures were treated with 100μM bromodeoxyuridine (BrdU) for the final 20h of culture. Limbs were then fixed in 10% formalin, paraffin embedded and sectioned. BrdU uptake was detected by anti-BrdU immunohistochemistry (similar to COLX above).

Generation of CollI-Gli2 transgenic mice

Full length 5' flag-tagged murine Gli2 cDNA was cloned by blunt ligation into the NotI sites of the murine CollI promoter and enhancer - containing construct
pKN185\textsuperscript{196,197} and linearized by digestion with NdeI and HindIII prior to microinjection into a pronucleus of fertilized ICR eggs. Genomic DNA was extracted from tails and screened by Southern blot for integration of the transgene. Southern blot was carried out as described in Chapter 2, except that genomic DNA was digested with EcoRI. Primer sequences for generation of the radioactive Southern blot probe as well as for PCR genotyping were: forward (β Globin F): CTCTGCTAACCATGTCATG, and reverse (mGli2R): CAGAGGACAGGCTTTTTTC; amplicon=244bp.

**Radiology and histology**

Mice were killed and radiographed using a Faxitron X-ray machine using 45V for a 33 second exposure on Kodak X-OMAT film. Limbs were dissected free of soft tissues and fixed in 4% paraformaldehyde, paraffin embedded, sectioned and stained with safranin-O to highlight cartilage.
Anti-flag immunohistochemistry revealed expression of the flag-tagged Gli2 transgene (brown colour) under the control of ColII regulatory elements in growth plate chondrocytes (b), but not in a negative control growth plate (a). Proximal tibial growth plates of WT (c,e,g) and transgenic ColII-Gli2 (d,f,h) littermates show that overexpression of Gli2 resulted in a postnatal increase in total growth plate height (safranin-O red), which was most noticeable at 4 weeks of age (c,d). Anti-COLX staining highlighted the fact that both the proliferative zone (flat, blue nuclei, between dark lines) and the hypertrophic zone (brown matrix) were taller in ColII-Gli2 mice (f) compared to WT mice (e). The difference in height between the growth plates virtually normalized by 8.5 weeks (g,h).
Growth plate chondrocyte differentiation and proliferation in short term explant cultures of skeletonised lower extremities from E16.5 mice. a, Height of the hypertrophic zone, as measured by the number of cell diameters counted longitudinally within the anti-COLX - stained area. b, Proportion of proliferative zone chondrocytes that have taken up BrdU. CollI- transgenic mice (the 4 groups on the far right in a and b) were assessed by comparison to separate controls since they are of a genetic background distinct from the remainder, and were investigated at a later time.
Anti-COL2 immunostained sections of proximal tibial growth plates following explant culture of E16.5 limbs (summarized in Fig. 2a). Compared to WT mice (a), hypertrophic cells (surrounded by brown anti-COL2-stained matrix) were more numerous in limbs which lack Gli2 (c) and fewer in mice which lacked Gli3 (e) or which overexpressed Gli2 (g). PTHrP treatment of cultures, which delayed hypertrophic differentiation of WT growth plate chondrocytes (b vs. a), was more potent in the absence of Gli2 (d vs. c) and less potent when Gli3 was lacking (f vs. e) or when Gli2 was excessive (h vs. g).
Figure 4

Anti-BrdU - stained sections of distal femoral growth plates following explant culture of E16.5 skeletonized limbs (summarized in Fig. 2b). Compared to WT mice (a), fewer proliferative zone chondrocytes (between dark lines) have taken up BrdU (brown stain) in mice lacking Gli2 (c). The opposite was true of mice which lacked Gli3 (e) or which overexpressed Gli2 (g). PTHrP decreased the number of BrdU positive cells in most samples. Sensitivity to PTHrP in this regard was greatest in the absence of Gli2 (d vs. c) and less in the absence of Gli3 (f vs. e) or in the presence of excess Gli2 (h vs. g).
Enchondromas in CollII-Gli2 mice. Ectopic rests of cartilage (arrows) are evident in CollII-Gli2, shown here in the distal femurs at 13 weeks (a, b) and 16 weeks (c, d) and in the proximal humerus at 43 weeks (f), but not wild type (e) mice. The rests are similar to growth plate tissue and to human enchondromas (b, d, f). TRAP staining highlights chondroclasts (red-brown, arrows) in equal numbers at the chondro-osseous junction in wild type (g) and CollII-Gli2 mice (h).
Figure 6

Synovial chondromatosis in Gli3+/- mice. a. Calcification in the knee of a Gli3+/-, but not WT (b), mouse at 13 months. c. Massive ectopic cartilage (arrows) within the knee joint of a 13 month old Gli3+/- mouse is contiguous primarily with the synovial lining, but also with the articular surfaces of the femur (F) and the tibia (T) where it is associated with destructive osteoarthritis. d. A knee joint from a 13 month old wild type mouse demonstrates no ectopic cartilage. e. The ectopic cells resemble growth plate chondrocytes. f. The incidence of synovial chondromatosis increases with age, and is highest in Gli3 heterozygotes at all time points.
CHAPTER 4

Patched is a co-receptor with Smoothened and PTHR1, two seven-pass transmembrane proteins with opposite effects on cAMP accumulation.
Note: The data contained in this chapter are preliminary. The model proposed is intended as a template for future experiments.

SUMMARY

The Indian hedgehog (Ihh) and Parathyroid hormone related protein (PTHrP) signalling pathways are thought to be co-regulated by means of a paracrine signalling relay. It has been shown that the Ihh receptor Patched1 (Ptc1), in addition to being expressed in the perichondrium, is coexpressed with the Type I PTH/PTHrP receptor (Pthr1) in the proliferative zone of the growth plate. Here I investigate the potential cell autonomous relationship between the Hedgehog and PTHrP signalling pathways. I show that coexpression of PTCH1 with WT, but not with the R150C enchondromatosis mutant, PTHR1 augments accumulation of intracellular cAMP and of IP3. R150C PTHR1 constitutively activates Hedgehog signalling in vitro, in a manner that is likely dependent upon suppression of PKA. PTCH1 forms a complex with WT, but not R150C, PTHR1. Therefore, augmented accumulation of cAMP and IP3 is dependent upon the ability of PTHR1 to form a complex with PTCH1. In the proliferative zone, where PTCH1 and PTHR1 are both expressed, the interaction of these signalling pathways may instruct the appropriate proliferation and differentiation readout of a given chondrocyte.
INTRODUCTION

The current model of interaction between the IHH and PTHrP signalling pathways in the growth plate involves a paracrine feedback loop. IHH, expressed by prehypertrophic and hypertrophic chondrocytes, induces expression of PTHrP by the periarticular perichondrium, which in turn downregulates IHH. The PTHrP receptor, Pthrl, is expressed by chondrocytes within the proliferative zone of the growth plate. The likely Ihh transmembrane receptor Ptchl, in addition to being expressed by the perichondrium, has been shown to be expressed also by chondrocytes in the proliferative zone in mice. Therefore, a subset of Ihh and PTHrP target cells are likely the same.

Since Ptchl and Pthrl are both expressed in the proliferative zone of the growth plate, it is conceivable that they modulate proliferation and differentiation in a cell autonomous manner. Transmembrane proteins, including G protein - coupled seven-pass transmembrane receptors, often form heterodimers that modify the signalling ability of either protein. Ptchl is a twelve pass transmembrane receptor which can form a complex, at least when overexpressed, with Smoothened (Smo), a seven pass transmembrane protein. Hedgehog ligand binding to Ptchl relieves the otherwise constitutive inhibition of Ptcchl on Smo, which can then activate downstream signalling events. It was shown indirectly in a frog melanophore assay that SMO likely inhibits cAMP synthesis, and therefore may activate Goq. PTHRl is also a seven pass transmembrane protein that couples with heterotrimeric G proteins. Unlike SMO, PTHRl most strongly activates Goq and Goq. I show here that coexpression of Ptcchl and WT PTHRl augments accumulation of cAMP and of IP3, but limits Hh signal...
transduction. These modifications of signal transduction intensity are dependent on the ability of PTHR1 to form a complex with PTCH1, since coexpression of PTCH1 with the R150C mutant PTHR1, which does not complex with PTCH1, is not associated with altered signal transduction.

RESULTS

Coexpression of PTCH1 with PTHR1 augments cAMP accumulation and limits Hedgehog signal transduction

In order to test the functional consequences of PTCH1-PTHRL coexpression, I performed cAMP, IP3, and Hedgehog signalling assays. Consistent with prior assay results, cAMP baseline level was suppressed (Fig. 1a) and IP3 accumulation in response to PTHrP was abolished by the R150C PTHR1 mutant (Fig. 1b). Cyclic AMP and IP3 accumulation were both augmented in a dose-dependent fashion when increasing amounts of PTCH1 cDNA was cotransfected with WT PTHRL (Fig. 1a, b).

Cotransfection of PTCH1 with R150C PTHR1, however, was not associated with augmented accumulation of cAMP nor of IP3 (Fig. 1a, b). Hedgehog signal transduction was measured in HeLa cells by cotransfection of a Hedgehog-responsive Gli2-Luciferase reporter construct. Luciferase activity was increased in cells transfected with R150C PTHR1 compared to cells transfected with WT PTHRL, when PTCH1 and SMO were also transfected (Fig. 1c).

PTCH1 and PTHR1 form a complex

Given that PTHR1 is a seven-pass transmembrane protein similar to SMO, a protein known to bind PTCH1, and that PTHR1 is coexpressed with PTCH1 in the
proliferative zone of the growth plate, it is possible that PTHR1 and PTCH1 form a complex in that zone. To test for this possibility, c-Myc – tagged PTHR1 and haemophilus influenza hemaglutinin (HA) – tagged PTCH1 cDNA constructs were cotransfected into COS-7 cells, and immunoprecipitation performed with anti-c-Myc monoclonal antibody two days following transfection. Western blot analysis of the immunoprecipitate was performed using anti-HA monoclonal antibody. An approximately 180kD band was consistently seen in lanes representing PTHR1-PTCH1 cotransfected cells, but not in singly transfected controls (Fig. 2a). This 180kD band was also seen when protein from PTCH1 – transfected cells was immunoprecipitated using anti-PTCH1 antibody and probed on Western blot with anti-HA antibody (Fig. 2a).

Reverse order coimmunoprecipitation using anti-HA monoclonal antibody followed by Western blot with anti-c-Myc polyclonal antibody was weakly positive in PTHR1-PTCH1 cotransfected cells (Fig. 2b).

Embryonic stem (ES) cells lacking both copies of the native Pthrl gene were used to test whether endogenous Ptch1 could be coimmunoprecipitated with exogenous PTHR1. Immunoprecipitation performed using anti-c-Myc monoclonal antibody followed by Western blot using anti-Ptch1 polyclonal antibody again revealed a unique ~180kD band in lanes from PTHR1 transfected cells (Fig. 2c).

Western blot band intensity of coimmunoprecipitates from either cell type was not affected by treatment of cultures with 10⁶M PTHrP (1-34) for 15 minutes prior to protein harvest (Fig. 2a, c). Band intensity was diminished, however, when COS-7 cells were treated with 5μg/ml Shh-N protein for 15 minutes prior to harvest (Fig. 2a).
Coimmunoprecipitation did not occur in either cell type when R150C PTHR1 was transfected in place of WT PTHR1 (Fig. 2a, c).

**DISCUSSION**

The development of several organs, including skin, breast and growth plate, is dependent on both the Hedgehog and PTHrP signalling pathways\(^{163,206-209}\). The interaction between these signalling pathways has only been investigated in the growth plate. Ihh upregulates PTHrP expression in the periarticular perichondrium, probably by an indirect route since PTCH1 is not expressed in that region of the perichondrium\(^{80}\). PTHrP in turn downregulates Ihh, likely by both direct and indirect means. Delay of chondrocyte differentiation by PTHrP decreases the number of hypertrophic cells, and thereby may indirectly downregulate Ihh\(^{79}\). A direct mechanism is implied by the finding that inhibition of Ihh expression by PTHrP in vitro does not require protein synthesis\(^{210}\).

The potential intracellular interaction between the Hedgehog and PTHrP signalling pathways in tissues where PTCH1 and PTHR1 are both expressed, such as the proliferative zone of the growth plate, is addressed here.

**PTCH1-PTHR1 association is required for effective accumulation of cAMP and IP\(_3\)**

The signalling assays demonstrated that PTHR1 signal transduction, via the second messengers cAMP and IP\(_3\), was positively influenced by cotransfection of cells with PTCH1. This induction of cAMP and IP\(_3\) accumulation was associated with the ability of PTCH1 to complex with PTHR1, because R150C PTHR1, which did not coimmunoprecipitate with PTCH1, was not able to augment induction of these second messengers. Protein kinase A (PKA), which is activated by cAMP, is an inhibitor of
Hedgehog signal transduction. Therefore, the ability of PTCH1 to increase cAMP accumulation in association with PTHR1 is a role consistent with its inhibitory function with regard to Hedgehog signalling. When cotransfected with PTCH1 and SMO, R150C PTHR1 constitutively stimulated Gli2-Luciferase activity. The R150C PTHR1 mutant therefore tips the balance of signals in favour of Hedgehog signalling, likely by limiting PKA activation. A model of intracellular interaction between the Hedgehog and PTHrP signalling pathways is found in Figure 3. Promotion of differentiation by Gli2 was demonstrated in the previous chapter.

**Cell autonomous model of Hedgehog-PTHrP interaction**

An alternate mechanism to that proposed by earlier authors of IHH-PTHrP signalling in the growth plate is possible with this model. The model in Figure 3 predicts that the appropriate proliferation and differentiation readout of a given chondrocyte will depend on its location in the growth plate. As a chondrocyte progresses through the growth plate, it will receive changing amounts of the IHH and PTHrP ligands, whose signal transduction pathways will compete intracellularly. This competition will determine the relative strength of the proliferation and differentiation - delay instructions. In the proximal proliferative zone, the concentration of PTHrP will be high (since PTHrP is expressed by the periarticular perichondrium) and that of IHH will be low (since IHH is expressed by the prehypertrophic and hypertrophic zones), therefore differentiation will be strongly delayed. In the distal proliferative zone, PTHrP concentration will be low and that of IHH will be high, allowing the chondrocytes to become hypertrophic. Of course, this is an overly simplistic model, and does not take into account the effect of the feedback loop between IHH and PTHrP at the transcriptional level.
The R150C PTHR1 enchondromatosis mutation alters the normal balance of signals by preventing association of PTHR1 with PTCH1. The mutant abolishes accumulation of IP3 and augments Hedgehog signal transduction in a manner that is likely cell autonomous and dependent upon dominant suppression of PKA. Together, these effects cause excessive chondrocyte differentiation delay and proliferation, which are conceivably necessary for the genesis of enchondromas.

METHODS

Cyclic AMP, IP3 and Hedgehog signalling assays

COS7 cells were plated at a density of 5 X 10^4 cells/ml in DMEM + 10% fetal bovine serum (FBS) and transfected the following day with the aid of a transfection reagent (Fugene 6, Roche). Cells were transfected with some combination of 1µg each of WT PTHR1, R150C PTHR1, PTCH1, or SMO cDNA, plus 0.4µg β galactosidase plus pcDNA3 to a total of 4.4µg. One day following transfection, cells from each 60mm plate were split to three wells of a six well plate. Two days following transfection, cells were serum starved for two hours and then treated with either PTHrP (1-34) in DMEM containing 0.1% FBS carrier, 5µg Shh-N in carrier or carrier alone for 15 minutes for cAMP assays and for 40 minutes for IP3 assays. Ice cold 65% ethanol was used to lyse COS-7 cells, and cAMP assays were performed using an “enzymeimmunoassay” kit (RPN 225, Amersham). For IP3 assays, the same transfection protocol was used in Pthr 1ES cells plated on 0.1% gelatin in DMEM containing 15% FBS and leukemic inhibitory factor (LIF). Lysates obtained by incubation of cells with ice cold 7.5% trichloroacetic acid were subjected to a tritiated IP3 assay (TRK 1000, Amersham).
Transfection efficiency was typically over 40% in COS-7 cells and 10%-20% in ES cells as assessed by cotransfection with green fluorescent protein cDNA in a separate set of experiments. For Hedgehog signalling assays, 0.6μg of a Hedgehog – responsive Gli2-Luciferase reporter gene was cotransfected into HeLa cells, and luciferase activity was determined from lysates. All assays were performed at least twice in duplicate, and results were controlled for transfection efficiency by assessment of β-Galactosidase activity using a colourimetric assay (Stratagene) two days following cotransfection of 0.4μg β-Galactosidase cDNA.

**Coimmunoprecipitation and Western blot**

COS-7 cells or Pthrl−/− ES cells were plated as above, this time in 100mm wells, and transfected with equal amounts of each cDNA of interest to a total of 6.0μg. PTHRII constructs were tagged with c-Myc and PTCH was tagged with HA. The ES cells were differentiated to early embryoid bodies (in which there is active Hedgehog signalling) by removing LIF from the medium and initiating suspension culture. Two days following transfection, cells were treated as above and lysed with 1ml extraction buffer (50mM Tris, pH7.5, 150mM NaCl, 1mM EDTA, 0.5% NP-40 containing protease inhibitor) on ice. Lysates were incubated with 2μg/ml of immunoprecipitation antibody (anti-HA monoclonal, anti-c-Myc monoclonal or anti-PTCH polyclonal antibody (Santa Cruz)) at 4°C overnight on a rotating apparatus. Samples were then incubated with 30μl Protein G Plus/Protein A Agarose Suspension (Oncogene) for one hour at 4°C on a rotating apparatus to bind proteins complexed with the antibody. The agarose suspension was washed three times with extraction buffer (containing only 0.1% NP-40), and the bound proteins were eluted with sample buffer containing reducing β-mercaptoethanol in
a boiling water bath. Samples were run on gradient polyacrylamide gels and transferred to nitrocellulose for Western blot. Blocking was carried out with 5% milk at 4°C overnight on a rocking apparatus. Blots were incubated with primary antibodies diluted 1/500 in 3% BSA/TBS-T at 4°C overnight on a rocking apparatus. Secondary IgG antibodies (Jackson Immuno) included anti-mouse (diluted 1/5000), anti-rabbit (1/10 000) or anti-goat (1/1000), and detection was carried out using a chemiluminescent horseradish peroxidase system (ECL).
Figure 1

(a) Graph showing (fm cAMP / 15 min.) / B Gal.

(b) Graph showing (pM IP3 / 40 min.) / B Gal.
 Intracellular cAMP and b, IP₃ accumulation was augmented in a dose-dependent manner when increasing amounts of PTCH₁ cDNA were cotransfected with WT, but not R150C, PTHR₁. c, R150C PTHR₁ constitutively activated a Hedgehog-responsive Gli2-Luciferase reporter in COS-7 cells when cotransfected with Hedgehog receptor-complex proteins PTCH₁ and SMO.
Figure 2

a) COS-7 cells

Western αHA

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<tr>
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peDNA1 lysis

PTCH1 (αPTCH1 IP)

PTCH1

WT PTHR1 + PTCH

R150C PTHR1 + PTCH

IP αc-Myc

b) COS-7 cells

Western αC-Myc

<table>
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<td>120 kD</td>
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peDNA1 (αHA)

WT PTHR1 lysis

R150C PTHR1 + PTCH (αC-Myc)

WT PTHR1 + PTCH (αHA)
c

\[ \text{Pthr1}^{+} \text{ ES cells} \]

\[ \text{IP } \alpha_{-}\text{Myc, Western } \alpha_{\text{PTCH1}} \]

\[ \text{PTHrP} \quad - \quad + \quad - \quad + \quad - \quad + \]

\[ 205 \text{ kD} \]

\[ \text{pcDNA1} \quad \text{WT} \quad \text{R150C} \quad \text{PTH1} \quad \text{PTHR1} \]

a. Immunoprecipitation from COS-7 cell lysate of HA-tagged PTCH1 with anti-PTCH1 polyclonal antibody was detected on Western blot using anti-HA monoclonal antibody as an ~180kD band. HA-tagged PTCH1 coimmunoprecipitated with c-Myc - tagged WT PTHR1 using anti-c-Myc monoclonal antibody, and was detected on Western blot probed with anti-HA antibody as a band of the same size as that obtained specifically for PTCH1. Treatment of COS-7 cells with 10^{-6} M PTHR1 did not alter coimmunoprecipitation of WT PTHR1 with PTCH1 compared with treatment with 0.1% FBS carrier alone. Treatment of cultures with 5\mu g/ml Shh-N, however, diminished WT PTHR1 - PTCH1 complex formation. Coimmunoprecipitation did not occur between R150C PTHR1 and PTCH1.

b. Reverser order immunoprecipitation of c-Myc - tagged WT PTHR1 with HA - tagged PTCH1 using anti-HA antibody followed by Western blot with anti-c-Myc antibody revealed a faint band the same size as a c-Myc - tagged WT PTHR1 lysis - specific band.

c. Endogenous PTCH1 was coimmunoprecipitated with exogenous WT, but not R150C. PTHR1 in embryoid bodies derived from Pthr1^{+} ES cells.
Model of cell-autonomous Hedgehog - PTHrP signalling. The interrupted arrows are novel propositions based on the data in chapters three (arrows from Gli2) and four (membrane arrow).
CHAPTER 5

Conclusions and Future Directions
The data were discussed in detail in the proceeding chapters. I had asked whether cartilaginous neoplasia is due, at least in part, to dysregulation of the IHH-PTHrP pathway. I found that dysregulation of that pathway is common in enchondromas, and that it can be causal. The following section is intended to integrate the relevance of all the findings.

1) **Genes that regulate skeletal development are expressed in skeletal tumours.**

In both cartilage and osteoid – forming tumours (Appendix), genes that regulate the differentiation of normal cells that produce the same matrix are expressed. This expression, however, is not universal, nor is it necessarily predictive with regard to tumour phenotype. In osteoid – forming tumours, the level of expression of markers of normal osteoblast differentiation did not necessarily predict the neoplastic cellular phenotype (Appendix). Although expression of the IHH/PTHrP pathway members studied was most complete and consistent in tumours that are microscopically most similar to normal cartilage, namely enchondromas and chondrosarcomas, a distinction between these two tumour types was not evident despite the difference in invasive and metastatic potential between them. Function of the IHH and PTHrP pathways in these two tumour types was also similar. Given the frequent and varied cytogenetic changes described in chondrosarcoma, it is likely that other pathways operate independent of IHH and PTHrP signalling to give chondrosarcoma its malignant properties.

2) **Dysregulation of developmental signalling pathways can initiate skeletal neoplasia.**
I’ve shown that a developmentally important protein may initiate neoplasia if it operates out of context with its co-regulators. Obviously, an imbalance in the function of an entire pathway, and not of any one particular signalling member, is the key to pathogenesis. This concept is supported by the finding that overexpression of Gli2, which has biochemical and functional similarities with the effects of the enchondromatosis R150C PTHRI mutant, was demonstrated in human cartilage tumours, and caused the same pathological condition as R150C PTHRI in mice.

3) Knowledge of how signals are altered in pathological processes can contribute to the understanding of normal development.

Studying Hedgehog signalling in cartilage tumour explants led to a prediction regarding normal growth plate function. It was felt from early studies in the chick that Ihh was responsible for both chondrocyte proliferation and differentiation delay. In the tumour explant cultures described in Chapter Two, under circumstances in which the feedback loop between IHH and PTHrP was not readily demonstrable, it was clear that Hedgehog stimulation increased proliferation, but did not affect differentiation. In later murine studies that isolated the role Ihh from that of PTHrP, it was shown that indeed Ihh is exclusively responsible for inducing chondrocyte proliferation, and that its effects on differentiation are secondary to the induction of PTHrP expression.

Further insight into the regulation of chondrocyte behaviour was made possible by investigation of the mechanism of pathogenesis initiated by the R150C PTHR1 mutant. Demonstration of how PTCH1 and PTHR1 might cooperatively regulate proliferative zone chondrocyte proliferation and differentiation was aided by the availability of a
ETHEU variant, namely the R150C mutant, that did not associate with PTCH1.

Understanding pathologic mechanisms, therefore, can facilitate understanding normal function.

FUTURE DIRECTIONS

A number of experiments can be proposed to further understand skeletal pathology and development by building on the data presented in the proceeding chapters. Even the partial understanding of cartilage tumour pathogenesis gleaned by the proceeding chapters may be useful in devising therapy. Delivery of pharmacologic agents that block Hedgehog signalling (cyclopamine, triparanol\textsuperscript{184,213} and/or PTHrP signalling (PTH (7-34))\textsuperscript{214}, might be a practical approach to attempting reduction in cartilage tumour proliferation and to overcoming blocks to completion of differentiation. In the skeletally mature person with enchondromatosis for example, systemic treatment would ideally cause differentiation to normal bone of all enchondromas, and therefore prevent the appearance of chondrosarcoma. In a skeletally immature person, systemic delivery of agents that interfere with chondrocyte behaviour might stunt growth, therefore local delivery of these agents to sites where deformity from an enchondroma is likely might be more appropriate.

The animal models of enchondromatosis and of synovial chondromatosis can be of use in testing potentially therapeutic agents. The endpoint of such tests might be more definitive with animals that exhibit a more severe phenotype than the ones described in Chapters Two and Three. Crossing \textit{ColII-R150C PTHR1} mice with \textit{ColII-Gli2} mice, or with mice that lack known tumour suppressors such as \textit{Ptc1}, might result in a more
severe phenotype that would aid the assessment of therapeutic trials. Another way of testing the therapeutic value of agents such as cyclopamine is to treat mice implanted with human cartilage tumour xenografts.

In addition to the \textit{R150C PTHR1} variant, it’s likely that there are other genetic alterations that cause enchondromatosis. It is possible that these other alterations disrupt IHH-PTHrP signalling in a manner similar to \textit{R150C PTHR1}. Identification of these mutations would be useful in more precisely defining the pathogenesis of cartilage tumours by establishing genotype-phenotype correlations, in devising therapy, and possibly in better understanding normal growth plate regulation.

Synovial chondromatosis in \textit{Gli3} mice is the only naturally occurring murine cartilaginous neoplasia I’m aware of. It may be worthwhile determining whether there is loss of heterozygosity of \textit{Gli3} in those lesions, and whether the same phenomenon takes place in human synovial chondromatosis.

The possibility that \textit{PTCH1} forms a complex with \textit{PTHR1} that alters intracellular signalling has profound implications with regard to the regulation of development of the early embryo as well as of a number of tissues. The evidence presented in Chapter Four is preliminary, and requires demonstration of \textit{PTCH1-PTHR1} association at endogenous levels of expression of both proteins. Currently, a good \textit{PTHR1} antibody is not available to carry this out. There are other methods of determining whether a physical interaction between the two proteins exists. Stable expression to near physiologic levels of fluorophore conjugated \textit{PTCH1} and \textit{PTHR1} can be followed by analysis by fluorescent resonance energy transfer (FRET) analysis. Excitation of the fluorophore with the higher wavelength by transfer of energy from the fluorophore with the lower, but overlapping
wavelength would imply the proteins are within 100 Angstroms of one another, and are therefore bound to each other. A less definitive alternative is to express GST-fusion proteins, purify GST-bound proteins, and identify them by Western blot. The argument that effective accumulation of cAMP requires association of PTHR1 with PTCH1 might be strengthened by demonstration of a greater affinity between PTCH1 and activating PTHR1 mutants, namely H223R and T410P. The physiologic significance of PTCH1-PTHR1 association was partially assessed at a biochemical level in Chapter Four, but needs to be determined in organs in vivo. The best way of doing this is to generate mice in which PTCH1 and PTHR1 do not form a complex and compare the development of various organs with those of mice in which PTCH1 does associate with PTHR1. To generate such mice, the R150C mutation can be knocked into wild type ES cells, assuming of course that this mutation truly disrupts Pthrl’s ability to complex with PTCH1. Alternatively, ES cells that lack both copies of endogenous Pthrl can be electroporated with either R150C PTHR1 or WT PTHR1, and aggregated with wild type morulas. Another way of assessing the functional interaction of PTHR1 with PTCH is to express PTHR1 in Drosophila, which don’t have endogenous PTHR1. The model of Chapter 4 (Fig. 3) predicts that Hedgehog signalling will be attenuated in areas where PTHR1 is activated. What’s more, it can be determined whether there is cell-autonomous interaction in cells expressing the PTHR1 transgene that alters subcellular localisation of smo and ptc proteins.

The concept that normal developmental signals may be dysregulated to cause neoplasia can be further exploited to investigate the pathogenesis of other skeletal tumours. For example, given the expression of neural markers in Ewing’s sarcoma and
peripheral neuroectodermal tumours\textsuperscript{215}, and the importance of Notch signalling in lateral inhibition of identical differentiation of neurons\textsuperscript{216}, a lack of Notch signalling that allows expansion of a proliferating, stem cell – like population of neuroectodermal cells may be important in the genesis of those tumours. Evidence of the importance of Notch signalling in Ewing’s sarcoma is already evident, since the EWS-FLI fusion transcription factor found in Ewing’s sarcoma has been shown to dysregulate \textit{Manic fringe}, a Notch signal modifier\textsuperscript{217}. Osteosarcoma consists of a population of proliferating mesenchymal cells\textsuperscript{30}, which are possibly analogous to cells in embryonic paraxial and lateral plate mesoderm and in the limb bud progress zone which are capable of producing cartilage and bone under the influence of certain signals. Proliferation of these embryonic cells is dependent, in part, upon Fibroblast growth factor and Shh signals\textsuperscript{218,219}. It might be worthwhile investigating whether signals that regulate development of embryonic mesoderm are reactivated in osteosarcoma. Giant cell tumour, sometimes referred to as osteoclastoma\textsuperscript{30}, might be caused by overactivation of signalling pathways that induce monocytes to form osteoclasts. These include the PTHrP\textsuperscript{220} and Osteoprotegerin\textsuperscript{221} pathways. A somatic activating mutation of PTHR1, for example, could conceivably give rise to giant cell tumour. Vascular tumours, such as hemangioma, perihemangiocytoma, angiosarcoma, aneurysmal bone cyst and telangiectatic osteosarcoma might be caused in part by an excess of signals that regulate angiogenesis, such as Vascular endothelial growth factor-A, which requires tight regulation of expression level for normal function\textsuperscript{222}. Chordoma, a tumour felt to be derived from remnant notochordal tissue\textsuperscript{30}, might be maintained by signals elaborated by the embryonic notochord. Given the partial chondroid differentiation of this tumour,
notochordal signals that induce paraxial mesoderm to differentiate into cartilaginous sclerotome, namely SHH and PAX1/PAX9\textsuperscript{223,224}, may be active in chordoma.

Dysregulation of developmental signals may be a relatively common mechanism of pathogenesis of skeletal tumours, especially those that arise in children.
APPENDIX

Expression of osteocalcin and its transcriptional regulators CBFA1 and MSX2 in osteoid forming tumours


*Performed all procedures other than SSCP and sequencing.
**SUMMARY**

Osteosarcoma, fibrous dysplasia and myositis ossificans contain osteoid producing cells that are not morphologically typical osteoblasts. Nevertheless, these pathologic cells may share differentiation steps with osteoblasts at the molecular level.

Osteocalcin (OC), a bone – specific extracellular matrix protein, is a marker of mature osteoblasts. OC is upregulated by the transcription factor CBFA1, which is responsible for commitment to the osteoblastic lineage, and downregulated by MSX2, a Homeobox containing transcription factor expressed during the early proliferative phase of osteoblast differentiation.

Semi-quantitative RT-PCR analysis was used to compare expression levels of OC, CBFA1 and MSX2 in 34 osteosarcoma, 5 fibrous dysplasia and 5 myositis ossificans specimens and 7 normal cortical bone samples. Despite normal or elevated levels of CBFA1 expression in most specimens, osteocalcin expression was low or undetectable in most osteosarcomas (25/34) and myositis ossificans (4/5) cases. No CBFA1 DNA binding domain mutations were identified by single strand conformation polymorphism and sequencing to account for this observation. However, a high level of MSX2 expression was demonstrated in these lesions, which may be inhibitory to OC transcription. The presence of moderate levels of OC found in fibrous dysplasia may contribute to the characteristic disconnected appearance of trabeculae in that entity, since OC is a negative regulator of bone formation.
INTRODUCTION

Osteoid – forming tumours

Osteoid is the extracellular matrix of bone that is normally produced by mature osteoblasts. Cells found in some pathologic processes such as osteosarcoma, fibrous dysplasia and myositis ossificans also produce osteoid. Osteosarcoma is a skeletal malignancy in which, by definition, osteoid is produced directly by malignant spindle cells. Some degree of morphologic osteoblastic differentiation can be seen in cases of osteosarcoma, but pathologists have noted the cellular pleomorphism of this tumour. Although it might be naturally assumed that osteosarcoma cells are derived from osteoblast progenitors, this notion has not been explicitly demonstrated in the literature by a systematic effort to better define the origin and lineage of these cells from human tumour specimens. Expression of some osteoblast-associated markers such as alkaline phosphatase, osteonectin and bone morphogenic proteins has been demonstrated, primarily in cell lines. Fibrous dysplasia is a benign skeletal lesion characterized by the production of disconnected trabeculae of woven bone directly from spindle cells in a fibrous stroma background. The term fibro-osseous metaplasia has been used to refer to the bone production in fibrous dysplasia because of the absence of histologically typical, cuboidal osteoblasts on the surface of the osteoid. Myositis ossificans is a benign, usually self-limited ossifying lesion of muscle, often occurring secondary to trauma. Proliferating undifferentiated mesenchymal cells infiltrate skeletal muscle in the acute phase. This lesion exhibits a clear zonal architecture within four weeks, in which there is progressive osteoblastic differentiation accompanied by decreasing proliferation and increasing bone formation from the center toward the
periphery seen in cross-section\textsuperscript{225}. The peripheral cells thus mature first, and ossification progresses from the periphery towards the center over time. In osteosarcoma and fibrous dysplasia in particular, osteoid is formed from cells that do not have the typical histologic appearance of mature osteoblasts. Genes specific to normal osteoblastic differentiation are likely expressed by these pathologic cells.

**Osteoblast differentiation**

Osteocalcin (OC) is a bone-specific extracellular matrix protein, whose expression follows the proliferative phase of osteoblastic differentiation\textsuperscript{232}. It is considered a marker of mature osteoblasts\textsuperscript{233}. Among the factors that regulate OC, the transcription factors CBFA\textsubscript{1}\textsuperscript{234} and MSX\textsubscript{2}\textsuperscript{235,236} are known to upregulate and downregulate its expression, respectively.

CBFA\textsubscript{1} (Core binding factor alpha 1) is a molecular signal that has recently been shown to commit mesenchymal cells to the osteoblastic lineage. CBFA\textsubscript{1} is a runt-domain containing gene which is expressed in cells of the mesenchymal anlage of the developing skeleton and continues to be expressed exclusively in those cells that differentiate along the osteoblastic lineage\textsuperscript{234}. CBFA\textsubscript{1} homozygous null mutant mice display a complete lack of endochondral and intramembranous ossification, demonstrating that CBFA\textsubscript{1} is necessary for osteogenesis\textsuperscript{237}. Forced expression of CBFA\textsubscript{1} in nonosteoblastic cells demonstrates that CBFA\textsubscript{1} is sufficient for transcription of osteoblast-associated genes such as Bone sialoprotein, Type I collagen and OC in vitro\textsuperscript{234}. Heterozygous loss of function mutations of this gene are present in cleidocranial dysplasia\textsuperscript{227,238}, and CBFA\textsubscript{1} may potentially play an oncogenic role by collaborating with
the MYC oncogene\textsuperscript{239}, which has been found to be sporadically amplified in osteosarcomas\textsuperscript{240}. CBFA1 binds the OC promoter and upregulates its transcription\textsuperscript{234}.

In comparison, the product of the homeobox containing gene MSX2, which also binds the OC promoter, strongly suppresses OC transcription\textsuperscript{235,236}. MSX2 is expressed during the early proliferative phase of osteoblastic differentiation, and is downregulated prior to osteoblast maturation\textsuperscript{241}. Mutations of this gene are found in individuals with craniosynostosis\textsuperscript{242}.

Cells of pathologic processes that form osteoid may be at least partially committed to the osteoblastic lineage, but stalled at a stage of differentiation consistent with their clinical behaviour, with more aggressive lesions at a less differentiated stage. In this report, I investigate whether this commitment might be reflected in the pattern of transcription of these osteoblast lineage-specific genes.

**RESULTS**

Analysis of samples by semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) revealed that similar levels (within 4X of each other) of OC, CBFA1 and MSX2, were expressed by most normal cortical bone samples. The bone sample (B0) chosen to compare relative pathologic specimen expression levels was representative of this group (Tables 2 and 3). The osteoblast cells from short term culture expressed OC and MSX2 at a lower level compared to the cortical samples (Table 3).

CBFA1 was expressed by the osteosarcoma specimens at levels similar to cortical bone in 28 of 34 cases (82\%) (Table 3). OC expression was decreased in 25 of 34 (74\%) osteosarcomas compared to normal bone (Table 3, Figure 1). In nineteen of these
samples, OC transcripts were undetectable. In order to determine whether the lack of OC expression was due to disruption of CBFA1 transcriptional activity in osteosarcoma, mutational analysis of the runt DNA binding domain of CBFA1 was carried out. No mutations were found in the osteosarcoma specimens screened by single strand conformation polymorphism analysis (SSCP) and sequencing. MSX2 expression levels in the osteosarcoma specimens were mostly comparable to that of normal bone (29/34 or 85%) (Table 3, Figure 1). The level of expression did not correlate with a specific histologic subtype or grade of osteosarcoma for any gene studied (data not shown).

In the fibrous dysplasia and myositis ossificans specimens, expression levels of CBFA1 and MSX2 both were comparable to those of normal bone in 3/5 cases, and low in 2/5 cases. Expression levels of OC in myositis ossificans, like osteosarcoma, were nil in most (4/5) cases. OC levels were never zero in fibrous dysplasia (Table 3, Figure 1). Fibrous and ossified areas of one case of myositis ossificans (MO5a and MO5b, respectively) demonstrated similar levels of expression of all 3 genes studied.

DISCUSSION

The osteoid forming lesions I studied expressed osteoblast lineage specific genes despite the frequent presence of less than typical osteoblasts. CBFA1 expression was seen in all entities at levels comparable to normal bone, suggesting that they are at least partially committed to the osteoblastic lineage. The overall lack of gross variation in CBFA1 transcription levels between specimen types of differing histologic appearance and clinical behaviour is compatible with evidence from murine in situ hybridization
studies demonstrating that CBFAI is transcribed in mesenchymal osteoblast precursors and throughout osteoblastic differentiation.\textsuperscript{234}

In contrast, expression of osteocalcin was variable between specimen types. Fibrous dysplasia specimens expressed OC in all specimens, and did so at levels comparable to normal bone in 3/5 (60\%) of cases, suggesting that fibrous dysplasia cells are fairly differentiated along the osteoblastic lineage at the transcriptional level, despite the lack of phenotypically recognizable osteoblasts.\textsuperscript{243} These cells may represent hybrid mesenchymal cells that differentiate partially along both the fibroblastic and osteoblastic lineages.

The absence of OC transcripts in most cases of osteosarcoma and myositis ossificans suggests that ossification in these lesions does not require OC. This finding is consistent with the phenotype of the OC deficient mice generated by Ducy et al\textsuperscript{244} which develop abundant bone. The lack of OC is associated with an increase in bone of improved functional quality without affecting the number of osteoblasts, bone resorption or mineralization, suggesting that OC is a negative regulator of bone formation. OC expression in fibrous dysplasia, which on the whole was higher than in the other lesion types (Table 3), may therefore contribute to inhibition of bone formation in that entity.\textsuperscript{243}

In osteosarcoma, CBFAI was apparently unable to upregulate transcription of osteocalcin. There may be several reasons for this observation. It might be explained by the relative immaturity of osteosarcoma cells. That is, there may be other factors expressed by these cells at their stage of differentiation which inhibit OC transcription. Induction of osteocalcin expression in an osteosarcoma cell line has been found by Chandar et al\textsuperscript{245} to be dependent on the presence of wild-type p53, which is mutated in
a subset of osteosarcomas. Lack of OC transcription may be due to the presence of CBFA1 dysfunction in osteosarcoma. However, no mutations of the DNA binding runt domain were identified. To further investigate why OC transcription is inhibited in osteosarcomas, expression of an inhibitor of OC transcription, MSX2, was analyzed.

MSX2 transcription is a marker of the early, proliferative phase of osteoblast differentiation, and therefore would be expected to be expressed at a high level in relatively undifferentiated cells, such as those of osteosarcoma, and at a lower level in more differentiated cell types. However, such a straightforward correlation was not observed. Although the level of MSX2 transcripts was robust in most osteosarcoma specimens, comparable levels were also observed in the fibrous dysplasia and myositis ossificans cases analyzed (Table 3), possibly reflecting the actively proliferating subset of cells in these lesions. Unexpectedly, a similar level of MSX2 expression was observed in cortical bone, where the cells presumably are mature. This finding may be partially due to residual microscopic periosteum left behind after dissection, or may be evidence of a proliferating, less mature subpopulation of osteoblasts within cortical bone. Alternatively, MSX2 may indeed be expressed by mature osteoblasts, contrary to in vitro studies.

The results suggest that osteoid-forming pathologic cells are at least partially committed to the osteoblastic lineage at the genetic level despite their lack of characteristic histologic features of osteoblasts. However, the data also indicate that clinical behaviour cannot be predicted on the basis of differential expression of transcription factors (CBFA1 and MSX2) that induce phenotypic hallmarks.
METHODS

Specimens

Cryopreserved pathologic specimens were obtained from the Sarcoma Tissue Bank at Mount Sinai Hospital, Toronto. Specimens in this tissue bank are cut from within the substance of a tumour, and confirmed histologically as being entirely lesional tissue following open biopsy or resection by a pathologist experienced in musculoskeletal neoplasia prior to being flash frozen in liquid nitrogen and stored at -70°C. I feel the specimens are reasonably representative samples of each tumour, given the similar levels of expression of at least one gene (MDRI) from different locations within osteosarcomas demonstrated earlier\textsuperscript{247}. I studied 34 osteosarcoma, 5 fibrous dysplasia and 5 posttraumatic, intramuscular myositis ossificans cases. The osteosarcoma specimens were all high grade central lesions. For one case of myositis ossificans, both the fibrous and ossified portions of the same lesion were available for study.

Cortical bone samples were obtained intraoperatively from individuals ranging in age from 10 to 72 years. Normal bone from fresh osteochondral allograft specimens, residual fresh autograft and fracture cases were compiled as a panel of controls. The lower extremity, pelvis and spine were the anatomical sources of the specimens. Periosteum and endosteum were completely stripped leaving only cortical bone. The samples were then processed in the same manner as the tumour samples.

A short term osteoblast culture was established from washed trabecular bone obtained intraoperatively. The osteoblast population was passaged once prior to being harvested and appropriate expression of the osteoblastic markers alkaline phosphatase, OC and CBFA1 demonstrated.
Semi-quantitative RT-PCR

RNA extraction RT-PCR was carried out as described in Chapter 2. PCR reactions were carried out in a DNA thermal cycler at an annealing temperature of 58°C for 21, 23 and 25 cycles for \( CBFA1/\beta_2M \) and \( OC/\beta_2M \) primer pairs and for 24, 26 and 28 cycles for the \( MSX2/AS \) primer pair to ensure comparison within the linear range of amplification. PCR products were electrophoresed on 12% (\( CBFA1/\beta_2M \) and \( MSX2/AS \)) and 16% (\( OC/\beta_2M \)) polyacrylamide gels and stained with ethidium bromide. Positive and negative controls for each primer pair, including a no-template water control carried over from the RT step, were run on each gel. A negative control lymphoblastic cell line was included for each pair of genes. Relative levels of band intensity of the gene of interest were compared to the internal control house keeping gene and normalized to a positive control normal bone (B0) sample on each gel. Band intensities over 4X or under 4X that of B0 were arbitrarily designated high and low expression, respectively. Intensities within 4X that of B0 were designated as comparable levels of expression.

Mutational analysis

Single strand conformation polymorphism (SSCP) analysis was used to screen for mutations in the \textit{run} DNA binding domain of the \( CBFA1 \) gene in the osteosarcoma specimens. Genomic DNA was used as template for PCR amplification of fragments containing an exon and its adjacent intronic boundaries using primers given in Table 1b. The \(^{32}\text{P}-\text{ATP} \) incorporated PCR product was denatured and electrophoresed on a native polyacrylamide gel containing 10% glycerol. Band shifts were further analyzed using a sequencing kit (Thermo Sequenase Sequencing Kit, Amersham Life Science, Cleveland, Ohio).
### TABLE 1a: PCR primer sequences (5' - 3')

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### TABLE 1b: CBFA1 RUNT domain SSCP primer sequences (5' - 3')

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**TABLE 2: Expression ratios: Lesion of interest/ B0**

Ratio of the expression level of a given gene in the lesion of interest over the reference bone standard, B0. OSA = Osteosarcoma. FD = Fibrous Dysplasia. MO = Myositis Ossificans. OB = Osteoblast culture.

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TABLE 3: Categorisation of Expression Levels

Number of specimens of each type that have a level of expression of the gene of interest significantly above, comparable to, or below that of the reference bone standard, B0. OSA = Osteosarcoma, FD = Fibrous Dysplasia, MO = Myositis Ossificans, OB = Osteoblast culture.

NUMBER OF SPECIMENS:

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Figure 1

Examples of semiquantitative RT-PCR gels representative of each tissue type: FD = Fibrous Dysplasia, MO = Myositis Ossificans, OSA = Osteosarcoma, BO = reference bone standard, OB = Osteoblast culture.
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