Delineation of Regulatory Factors and Mechanisms that Govern the Differentiation of Mesenchymal Cells to the Osteoblastic Lineage

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Abstract

The commitment and differentiation of pluripotent mesenchymal stem cells is governed by developmental factors, cytokines and transcription factors which act in concert to determine the terminal differentiated state. They can be induced to commit to a variety of lineages such as adipocytes, osteoblasts, chondrocytes, myoblasts and fibroblasts. Our studies focused initially on the mechanism of action of parathyroid hormone related protein (PTHrP) in the inhibition of adipogenesis. *In vitro* studies that employed the pre-adipocytic cell line 3T3-L1 demonstrated the ability of PTHrP to delay the terminal differentiation of adipocytes by a cyclic AMP-protein kinase A dependent pathway that ultimately led to the phosphorylation and downregulation of the adipocyte differentiation factor Peroxisome Proliferator Activator Receptor Gamma (PPARγ). This inhibitory effect of PTHrP on adipogenesis was examined further in the earlier uncommitted pluripotent mesenchymal C3H 10T½ cell line. These cells can be induced to undergo adipogenesis by induction with bone morphogenetic protein 2 (BMP2). However, PTHrP treatment reduced the capacity of BMP2 to direct differentiation along the adipogenic lineage. In contrast to the inhibitory effect that PTHrP had on adipogenesis, PTHrP was observed to enhance osteogenesis. Both PTHrP effects appeared to be the result of its ability to sensitize C3H 10T½ cells to the agonist BMP2. The increased efficacy of BMP2 was determined to be a result of increased bone morphogenetic protein 1A receptor expression which was a consequence of the capacity of PTHrP to stimulate protein kinase C activity. Efforts to identify intracellular partners involved with the intracrine function of PTHrP resulted in the identification of the nuclear orphan receptor Rev ErbA as a candidate nuclear partner. Overexpression studies

involving both a nuclear form of PTHrP and Rev ErbA resulted in enhanced osteoblastic differentiation of C3H 10T½ cells. We further analyzed the regulation of the commitment process of cells of mesenchymal origin to the osteoblastic lineage by performing genomic scans in both the human and mouse genomes for genes harboring a Core Binding Factor alpha 1 (CBFA1) consensus binding sequence within their proximal promoter regions. Genes that contained similar response elements at complementary positions from the mRNA transcription start site were scored as potential candidate genes. These findings can, in the future, be complemented with microarray analysis to determine the expression profile of C3H 10T½ cells following introduction of human and mouse CBFA1. Genes that are also scored in the genomic analysis and that are activated by the mouse and human CBFA1 can subsequently be examined for direct regulation by CBFA1. This therefore represents a novel approach to the search for direct gene targets of the osteoblast-regulatory factor, CBFA1 and should lead to an increased understanding of the regulation of osteoblast development.

Resume

L'engagement et la différenciation des cellules souches pluripotentes du mésenchyme sont dépendants de facteurs développementaux, de cytokines et de facteurs de transcription qui agissent ensemble pour déterminer le stade terminal de différenciation. Elles peuvent être induites à s'engager en plusieurs lignées telles que les adipocytes, les ostéoblastes, les chondrocytes, les myoblastes et les fibroblastes. Notre étude avait pour but initial le mécanisme d'action de la protéine apparentée à l'hormone parathyroide (PTHrP) dans l'inhibition de l'adipogénèse. Les études in vitro utilisant la lignée cellulaire préadipocytaire 3T3-L1 ont démontré que les PTHrPs retardent la différenciation terminale des adipocytes via une voie de signalisation impliquant la protéine kinase A, AMP cyclique dépendante, conduisant à la phosphorylation et à la régulation négative du facteur de différenciation adipocytaire Peroxisome Proliferator Activator Receptor Gamma (PPARγ). L'effet inhibiteur de la PTHrP sur l'adipogénèse a été examiné plus en détails dans la lignée cellulaire pluripotente indifférenciée du mésenchyme C3H 10T½. Ces cellules peuvent être induite en adipogénèse sous l'effet de la Bone Morphogenetic Protein 2 (BMP2). Cependant, un traitement à la PTHrP réduit la capacité de la BMP2 à amener ces cellules en différenciation vers une lignée adipocytaire. A l'inverse de l'inhibition de la PTHrP sur l'adipogénèse, un effet positif sur l'ostéogénèse est observé. Ces deux phénomènes de la PTHrP semblent être le résultat de sa capacité à sensibiliser les cellules C3H 10T½ à l'agoniste BMP2. Cette efficacité croissante de la BMP2 est conséquente de l'augmentation de l'expression du récepteur de la protéine morphogénétique de l'os 1A, résultant de la capacité de la PTHrP à stimuler l'activité de la protéine kinase C. Les efforts pour identifier les partenaires

intracellulaires participant à la fonction intracrine des PTHrPs a permis d'identifier le récepteur nucléaire orphelin Rev ErbA comme partenaire nucléaire potentiel. Les études de surexpression impliquant les formes nucléaires à la fois de PTHrP et de Rev ErbA ont montré une augmentation de la différenciation des cellules ostéoblastiques C3H 10T½. Afin d'analyser plus en détails le processus d'engagement des cellules d'origine du mésenchyme en lignée ostéoblastique, nous avons réalisé un criblage génomique des gènes portant une séquence consensus de liaison en dedans de leurs régions promoteurs proximaux réspectifs, pour CBFA1 (Core Binding Factor alpha 1), chez l'homme et la souris. Les gènes contenant des éléments de réponse semblables sur les positions complémentaires à partir du site d'initiation de la transcription de l'ARN messager, ont été répertoriés comme gènes potentiels. Ces résultats seront ensuite complétés par une analyse à biopuce d'ADN afin de déterminer le profil d'expression des cellules C3H 10T½ après introduction de CBFA1 humain et murin. Les gènes criblés dans l'analyse génomique ainsi que ceux qui ont été activés par CBFA1 humain et murin seront examinés ultérieurement dans la régulation directe par CBFA1. Ceci représente une nouvelle approche d'identification des genes cibles modulant directement le facteur régulateur ostéoblastique, CBFA1 et devrait amener à une compréhension plus approfondie des mécanismes régulant le développement ostéoblastique.

Contributions of Authors

The manuscripts presented in this thesis are published or will be submitted for publication. The contributions of each author per manuscript are as follows:

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Chapter 5: **Chan GK**, Nagai Y, Karaplis AC, Goltzman D. **Chapter 5: Genomic Scanning for CBFA1 Responsive Genes in the Mouse and Human Genome.**

Chapter 5: George K. Chan was responsible for all work presented in the chapter. Yoshihiko Nagai performed all programming functions and provided scientific discussions.

All work presented in this thesis was conducted under the supervision of David Goltzman and Andrew Karaplis.

Other manuscripts to which the candidate has contributed to during his Doctoral studies:

1. Miao D, Tong XK, **Chan GK**, Panda D, McPherson PS, Goltzman D. Parathyroid hormone-related peptide stimulates osteogenic cell proliferation through protein kinase C activation of the Ras/mitogen-activated protein kinase signaling pathway. *J Biol Chem* 2001 Aug 24;276(34):32204-13
2. **Chan GK** and Duque G. Age-related bone loss: old bone, new facts. *Gerontology* 2002 Mar-Apr;48(2):62-71
3. Deckelbaum RA, **Chan G**, Miao D, Goltzman D, Karaplis AC. Ihh enhances differentiation of CFK-2 chondrocytic cells and antagonizes PTHrP-mediated activation of PKA. J Cell Sci. 2002 Jul 15;115(Pt 14):3015-25.

"For fear – is the exception with us. Courage, however, and adventure and joy in the unknown, the unattempted –courage seems to me the whole pre-history of man.

He has envied the wildest, most courageous animals all their virtues and robbed them: only thus did he become – man.

This courage, at length grown subtle, spiritual, this human courage with eagle's wings and serpent's wisdom: this, it seems to me, is today called – " *

Friedrich Nietzsche, 1891

From, "Of Science, Thus Spoke Zarathustra"

** The conclusion is "- science."*

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Rationale and Objectives

The first evidence that PTHrP might play a role in the differentiation process of cells of the mesenchymal lineage is based on the phenotype of the PTHrP heterozygous null mouse. These animals develop a premature form of osteoporosis that is characterized by reduced bone density and increased bone marrow adiposity due to lower levels of PTHrP.

Our initial objectives were therefore to determine whether PTHrP might affect the differentiation of adipocytes. Should PTHrP have a role in this differentiation process, we would then evaluate which specific PTHrP signaling pathway might be associated with this effect and whether PPARγ is affected by PTHrP signaling.

Because the PTHrP heterozygous null animals demonstrated not only an increase in bone marrow adiposity but also a decrease in total bone volume, we hypothesized that PTHrP might have a dual role in affecting the differentiation of both adipocytes and osteoblast. To investigate this phenomenon we employed a pluripotent mesenchymal cell line which can differentiate along both the adipocytic and osteoblastic lineages. In these studies, we made use of recombinant Bone Morphogenetic Protein 2 (BMP2) which can induce both adipogenesis and osteogenesis of clonal pluripotent mesenchymal cells and determined the effect of PTHrP on this process. We also wished to determine which specific downstream signaling events induced by PTHrP might be involved in these actions and whether PTHrP affects the signaling pathway of BMP2.

To investigate a potential role for intracrine PTHrP in regulation of mesenchymal stem differentiation, we will employ the yeast two-hybrid system to identify potential

nuclear partners for PTHrP. Positive clones will be identified and proteins that have demonstrated a role in mesenchymal cell differentiation will be studied further. Mapping of the protein interaction will be performed with deletion mutants of PTHrP to identify critical domains for the interaction. A nuclear form of PTHrP will also be employed with candidate proteins to investigate their combined roles in the differentiation process of mesenchymal stem cells.

Lastly, we focused on the regulatory events associated with the earliest stages of osteoblast development. In view of the fact that CBFA1 is the earliest known marker of the osteoblastic lineage we examined both the mouse and human genomes for all genes harboring CBFA1 responsive elements in their proximal promoter regions. The genes identified should provide a detailed schematic as to every gene that may be directly regulated by CBFA1, thus providing potential target genes associated with early osteoblast differentiation.

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LIST OF ABBREVIATIONS

AC	Adenylyl Cyclase
ALP	Alkaline Phosphatase
BMP	Bone Morphogenetic Protein
BMPR-IA	Bone Morphogenetic Protein Receptor I A
cAMP	Cyclic Adenosine Monophosphate
CBFA1	Core Binding Factor Alpha 1
DAG	Diacylglycerol
MAPK	Mitogen Activated Protein Kinase
MSC	Mesenchymal Stem Cell
NLS	Nuclear/Nucleolar Localization Sequence
PEBP2αA	Polyoma Virus Enhancer Binding Protein 2 Alpha A
PKA	Protein Kinase A
PKC	Protein Kinase C
PLC	Phospholipase C
PPARγ	Peroxisome Proliferator-Activated Receptor-Gamma
PTH	Parathyroid Hormone
PTHrP	Parathyroid Hormone Related Peptide
PTH-1R	Parathyroid/Parythyroid Hormone Related Peptide Receptor

Note: Other standard abbreviations are defined within the text.

Chapter 1

Literature Review

1. BONE PHYSIOLOGY

1.1 Bone Physiology: An overview

Bone in its simplest form, can be described as a composite of cells embedded in an organic and inorganic matrix. The inorganic components of bone represents approximately 65% of its dry weight and is made up primarily of calcium phosphate, organized mainly as crystalline hydroxyapatite with some amorphous calcium phosphate salts(36, 243). Organic compounds make up the remaining 35% of the dry weight of bone, which consists mainly of type I collagen and to a lesser degree non-collagenous proteins such as osteocalcin, osteopontin, bone sialoprotein and osteonectin. Within the organic matrix are also enzymes such as alkaline phosphatase and a variety of growth factors such as transforming growth factor beta (TGFβ) (36). Multiple cell types form specific interactions to regulate the four principle functions of bone: mechanical support, the protection of vital organs, the housing of bone marrow and as a reserve for metabolic ions such as calcium and phosphate which are essential for life.

1.2 Bone Turnover

Throughout its lifetime, the skeleton is subjected to different stresses and thus requires regular maintenance. One principle characteristic of bone is the remodeling process, which serves to maintain skeletal homeostasis and preservation of anatomical integrity. During this process, old matrix is removed by osteoclasts and new bone is deposited by juxtaposed osteoblasts. These packets of bone resorbing and bone forming cells comprise the so-called basic multicellular unit (BMU).

Osteoblasts resemble fibroblasts in appearance and are derived from mesenchymal stem cells. They are localized to trabecular and endosteal bone surfaces, to haversian systems and to periosteal surfaces(17, 244), and express high levels of the cell surface enzyme alkaline-phosphatase, which can be demonstrated histochemically. The specific function of alkaline phosphatase has yet to be determined, however, mice that lack functional alkaline phosphatase suffer from hypophosphatasia, characterized by impaired mineralization of cartilage and bone matrix(17).

Osteoclasts are of hematopoetic origin and originate from macrophage-like cells. Their ability to resorb bone, is attributed to their capacity to secrete hydrogen ions to dissolve the mineralized matrix, and proteolytic enzymes including serine proteases and metalloproteinases to digest the organic matrix(343). Once bone is resorbed by osteoclasts, osteoblasts migrate to the area of excavated bone and begin to deposit new, unmineralized bone matrix termed osteoid(36, 242, 343). Osteoid is later mineralized and this end product represents the newly formed bone. **(Figure 1)**

1.3 Osteoblastogenesis

Bone formation requires the commitment and differentiation of mesenchymal stem cells (MSCs) towards the osteoblastic lineage. The initial commitment process of MSCs results in a limitation of their pluripotency. Following this process, these cells are referred to as "osteoprogenitors". To date, only members of the bone morphogenetic protein family of proteins have clearly demonstrated this osteoinductive ability(127, 344, 376).

Figure 1. Bone Turnover

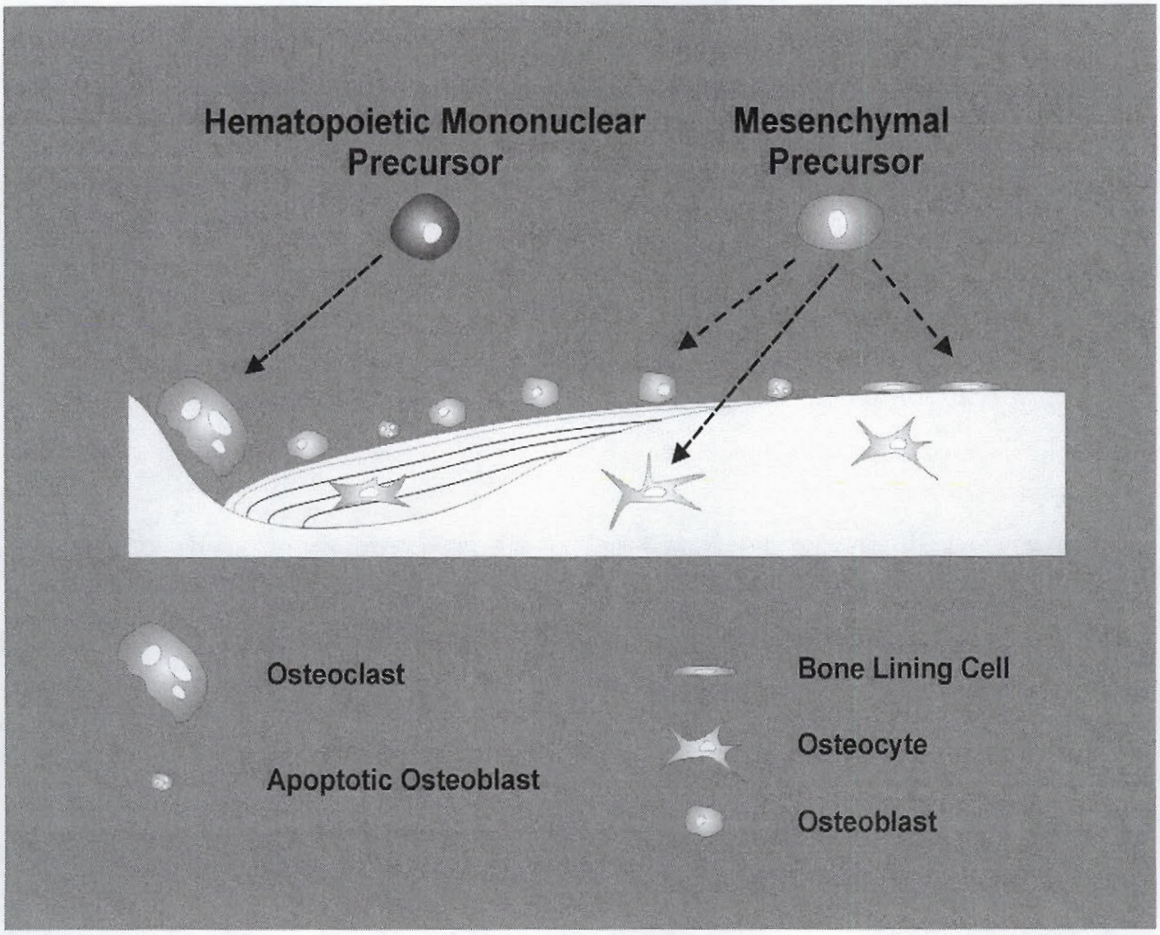


Figure 1. The adult skeleton is continuously remodeled, by the removal of old matrix and the deposition of new bone by a team of juxtaposed osteoclasts and osteoblasts, comprising the so-called basic multicellular unit (BMU). Osteoclasts are involved in the actual resorption of bone by secreting proteolytic enzymes and hydrogen ions that are required for removal of the deposited matrix. Once bone is resorbed by osteoclasts, osteoblasts migrate to the area of excavated bone and begin to deposit osteoid. Osteoid is later mineralized and this end product represents the newly formed bone.

At this stage of differentiation, the proliferation and differentiation of osteoprogenitors are regulated by a variety of factors. Fibroblast growth factor (FGF) (246, 270, 308), transforming growth factor β (TGFβ) (157, 276) and parathyroid hormone/parathyroid hormone related protein (PTH/PTHrP) (85) have been shown to stimulate proliferation of osteoprogenitor cells whereas leukemia inhibitory factor (LIF) (65, 66) and BMPs(224, 239) enhance the differentiation of these cell types. Several factors have demonstrated a dual role in enhancing both proliferation and differentiation such as, glucocorticoids(171, 252), prostaglandins(181), insulin-like growth factor (IGF) (24, 235), and PDGF(2, 49, 129, 259).

As osteoprogenitor cells differentiate further, they assume an osteoblast-like phenotype and can functionally deposit osteoid matrix. Growth factors that enhance osteoblast activity and consequently bone formation include IGFs (24, 235), BMPs(229), prostaglandins(135, 196), and LIF(65). Ultimately, osteoblast may become trapped in the newly synthesized osteoid tissue in pockets known as osteocytic lacunae. These embedded osteoblasts are called osteocytes and they are believed to represent terminally differentiated osteoblasts(274).

Gene targeting in conjunction with transgenic technology has provided researchers an opportunity to identify genes that appear essential for osteoblast differentiation. The osteogenic potential of Core Binding Factor Alpha 1 (CBFA1) was clearly demonstrated in mice in which the CBFA1 gene was ablated by homologous recombination(90, 266, 292). These mice lack both endochondral and intramembranous bone and have skeletons composed solely of chondrocytes that provide for a cartilaginous matrix. A second osteoblasts regulatory gene named Osterix (Osx), is a zinc finger-

containing transcription factor that is expressed in early osteoprogenitors(272). Osx-deficient mice also lack osteoblasts, which results in a complete lack of both endochondral and intramembranous bone. Osx deficient mice demonstrate CBFA1 expression in all developing skeletal elements; however, the reverse does not hold true. CBFA1 deficient mice reveal no Osx expression in the developing skeleton. These observations suggest that Osx acts downstream of CBFA1. Indian hedgehog (Ihh) has also been demonstrated to be essential for osteoblast differentiation. Ihh is a secreted morphogen that was initially identified as a regulator of chondrocyte differentiation(219, 373). Mice lacking Ihh have intramembranous, but no endochondral ossification. To determine whether Ihh is required for endochondral osteoblastogenesis, transgenic mice overexpressing the hedgehog interacting protein (HIP) in chondrocytes were generated(58). HIP effectively sequesters free Ihh thereby antagonizing Ihh signaling. Due to the tissue specific overexpression of HIP in chondrocytes, these transgenic animals lack Ihh signaling only in chondrocytes and develop similar distorted growth plates as the Ihh null animals. In contrast to the Ihh null animals, the HIP transgenic mice develop osteoblasts where endochondral bone formation takes place suggesting that Ihh is required for osteoblast differentiation during endochondral bone formation.

1.4 Adipogenesis within the Bone Marrow

Decreases in total bone volume may be accompanied by an increase in bone marrow adiposity(125). The reason for the increase in bone marrow adiposity is currently unknown, however many theories have been proposed for this increase in adipocyte numbers, and their role in bone marrow biology (125). Marrow adipocytes may serve a

passive role by occupying space that is no longer required for hematopoesis, or they might provide a local source of energy for emergency situations such as blood loss (hematopoesis) or fractures (osteogenesis) which occurs more frequently when trabecular bone mass is low. Marrow adipocytes may be involved in general lipid metabolism involving clearing and storing of circulating triglycerides. They might also share functions in the maturation of selected blood cell lineages or in the development of osteoblasts for bone formation. Adipocytes are a source of leptin that has been shown to be an angiogenic factor; therefore marrow adipocytes might be associated with vascularization within the bone marrow(325). Finally, bone marrow adipocytes may be a product of the inappropriate differentiation of pluripotent mesenchymal cells diverted from other lineages such as osteoblasts(175, 204).

2. MESENCHYMAL STEM CELLS

2.1 Discovery and Characterization

Primary marrow cell cultures are commonly used for the study of the conditions that govern the determination and differentiation of pluripotent mesenchymal cells. The basis for their usefulness in the study of bone biology can be attributed to Alexander Fridenshtein who first identified the conditions for culturing these marrow derived mesenchymal cells. He noted that these cells resembled fibroblasts in appearance and when cultured in the presence of fetal calf serum, they adhered to plastic and would in time form discrete colonies. Thus, Fridenshtein named these individually cultured cells as a "colony-forming unit-fibroblasts" (CFU-F). With a viable source of marrow progenitor cells and a method to expand their numbers, Fridenshtein sought to better characterize these cells and their differentiation potentials. By observing the phenotypes of different colonies derived from a single clone, he was able to discern phenotypic characteristics of either cartilage or bone(109). Fridenshtein, therefore concluded that osteoblasts and chondrocytes shared a common precursor and named this cell an osteogenic stem cell. The conclusions that Fridenshtein came to were in part correct; however, he was unaware that osteoblasts and chondrocytes represent only two potential lineages that could be derived from pluripotent mesenchymal stem cells.

Since his early descriptions, multipotential marrow cells have been isolated and cultured from rat, baboon, mouse and human marrow, and in each case, have demonstrated their capacities to differentiate into osteoblasts, chondroblasts, adipocytes, fibroblasts and skeletal muscle(61, 76, 132, 145, 237). Besides being pluripotent, bone marrow derived mesenchymal cells also demonstrate an innate ability for self renewal;

thus, each pluripotent mesenchymal cell prior to commitment can divide resulting in the generation of a sister cell with its pluripotency intact. It is not uncommon for cultures to exceed seventy doublings with little or no change in telomere length, or multilineage differentiation potential provided that the cells are maintained at a level of sub-confluence throughout the culture period.

Over time, the original name CFU-F has been gradually abandoned and replaced by various indistinct terms such as "marrow stromal cells" (299), "mesenchymal progenitor cells" (62) or "mesenchymal stem cells (MSC)" (47). Most of these terms reflect semantics rather than functional issues. In this introduction, we will use the generic term mesenchymal stem cells (MSC) to represent not only the stem cell *per se*, but also the various committed progenitors that comprise more than one mesenchymal differentiation potential(60, 76, 267, 414).

2.2 Master Genes

For a MSC to acquire a specific lineage, the expression or repression of master regulatory genes are required to initiate cellular responses for cell fate determination. Evidence for candidate master genes was first demonstrated by the discovery of MyoD, a muscle transcription factor capable of inducing the expression of a multitude of muscle-specific genes in a variety of committed cell types(382). This ability to direct a committed cell type towards another cell lineage is referred to as transdifferentiation and has since been adopted as a standard test for master genes. MyoD itself has been shown to be sufficient for initiating myogenic differentiation, however subsequent studies have demonstrated that myogenic differentiation is also regulated by other transcription factors

that includes Myf5, myogenin, and MRF4(42). Potential master genes have also been identified for other mesenchymal lineages, including Peroxisome Proliferator-Activated Receptor-Gamma (PPARγ) for adipocytes and Core Binding Factor Alpha 1(CBFA1) for osteoblasts.

PPARγ is a member of the nuclear family of transcription factors that was identified as being responsible for regulating the adipocyte specific expression of ap2(350). Forced expression of PPARγ in myoblasts and fibroblasts accompanied by ligand stimulation is sufficient to transdifferentiate both cell types towards the adipocytic lineage(164, 353). Although adipogenesis is dependent upon PPARγ, adipogenesis like myogenesis is also influenced by multiple transcription factors including, CCAAT-enhancer binding protein-alpha (C/EBPα) and ADD1/SREBP1, which have also been shown to induce terminal differentiation of adipocytes(57, 107, 108, 199, 355, 393, 402). Asides from being expressed in adipocytes, PPARγ expression has also been detected in the large intestine, the spleen, Peyer's patches located in the jejunum, skeletal muscle, liver, heart and bone marrow stromal cells(18, 37, 130, 202, 245, 264, 351). The observation that PPARγ might serve as a master gene for adipogenesis raises questions as to its role in non-adipocytic cells. Whether a master regulatory gene might serve more than one function as suggested by its expression in various tissue types is open to speculation, however, this issue is not limited to PPARγ.

A transcription factor that fulfills the requirements to be a master gene for osteogenesis is CBFA1. CBFA1 was shown to be necessary for bone development as was demonstrated by targeted disruption of the CBFA1 gene in mice. Absence of CBFA1 gene expression in these animals resulted in a lack of both endochondral and

intramembranous bone with skeletons composed solely of chondrocytes and a cartilaginous matrix(89, 266, 292). In addition to *in vivo* experimental evidence, CBFA1 can also induce expression of osteoblastic genes in nonosteogenic cells *in vitro*(87, 89) Yet, its function does not appear to be limited to osteoblast development. CBFA1 expression can also be detected in chondrocytic cells and has been shown to play a role in chondrocyte differentiation, albeit at a later stage of differentiation involving the prehypertrophic and hypertrophic chondrocytes(340).

In contrast to transcription factors that serve to positively define the cell lineage of MSCs, lineage repression also appears to be a common regulatory mechanism of mesenchymal lineage determination. One example of a regulatory factor that modulates differentiation via repression is nuclear factor of activated T cells (NFAT-p). In NFAT-p knockout mice, spontaneous expression of chondrogenesis was observed in extraarticular connective tissue (301). In that study, a detailed examination of NFAT-p expression in chondrocytes and MSCs showed that chondrogenic induction correlates with NFAT-p repression, and that in chondrocytic cells, NFAT-p overexpression can extinguish chondrogenesis. In another study highlighting the effects of repression on the commitment and differentiation of mesenchymal cells, stable transfection of the PPARγ2 gene was shown to inhibit CBFA1 expression in osteogenic cells and repress osteoblastic expression(225). Repressive nuclear factors have also been identified for adipogenesis as demonstrated by the repressive role of GATA-2 and GATA-3. Overexpression of either GATA-2 or GATA-3 results in an arrest of the adipocyte differentiation program at the pre-adipocyte stage(348).

2.3 Plasticity

Current models for depicting the mesenchymal differentiation process would suggest that no single master gene exists for a given mesenchymal cell lineage. Instead, MSC differentiation appears to be under the influence of multiple inductive and repressive factors that, often simultaneously, can lead to the expression of "end-stage" markers for more than one lineage. With this in mind, the initial commitment step of pluripotent MSCs does not represent a final decisive moment in the MSCs lifetime, nor are they necessarily bound to stay the course of differentiation. Examples of transdifferentiation have been demonstrated involving differentiated cells of mesenchymal origin adopting a new lineage; such as adipocytes undergoing conversion to osteoblasts and vice versa(27, 78, 225, 403). Although this phenomenon may represent itself as a lack of distinction amongst the early stages of commitment and differentiation, this characteristic of MSCs is commonly referred to as "plasticity". Thus, in addition to their ability to divide without limits and to give rise to distinctive cell types, adult stem cells are also remarkably malleable and exhibit a high degree of plasticity. **(Figure 2)**

2.4 Clinical Application of Mesenchymal Stem Cells

Uncommitted multipotent mesenchymal cells residing within the bone marrow may serve as a source of progenitors for distant mesenchymal tissues (47). By means of the systemic vasculature, MSCs are able to leave their original "tissue" niche and circulate in the blood stream (95, 101, 302). Upon homing in to an appropriate microenvironment, a circulating stem cell may then differentiate in a manner that is consistent with the context of its surroundings(381). This characteristic may be required

to support tissue growth and repair, and has been shown to occur *in vivo* when uncommitted progenitors were recruited from the bone marrow to assist in muscle repair(102).

Besides being able to function external of the marrow cavity, marrow MSCs may also serve a more localized need within the bone marrow in addressing deficiencies in functional osteoblasts that are required for maintenance of the structural integrity of the bone that serves as housing for the marrow. A first glimpse of the therapeutic potential of stem cells in this regard was demonstrated by allogeneic bone marrow transplantation (considered as a common source of hematopoetic and mesenchymal progenitors) in children with osteogenesis imperfecta. Osteogenesis imperfecta is a genetic disorder of osteoblastic cells in which generalized osteopenia occurs and leads to excessively frail bones with fracturing, deformities and short stature. The underlying defect is a mutation in one of the two genes encoding type I collagen, the primary structural protein of bone. Following marrow transplantation, histological changes have been observed in trabecular bone indicative of new dense bone formation. In addition, increased growth rate and reduced frequencies of bone fracture were also seen(160). These changes detected 3 months after marrow transplantation, were associated with the engraftment of functional mesenchymal progenitors from the transplanted marrow (121).

Figure 2. Plasticity

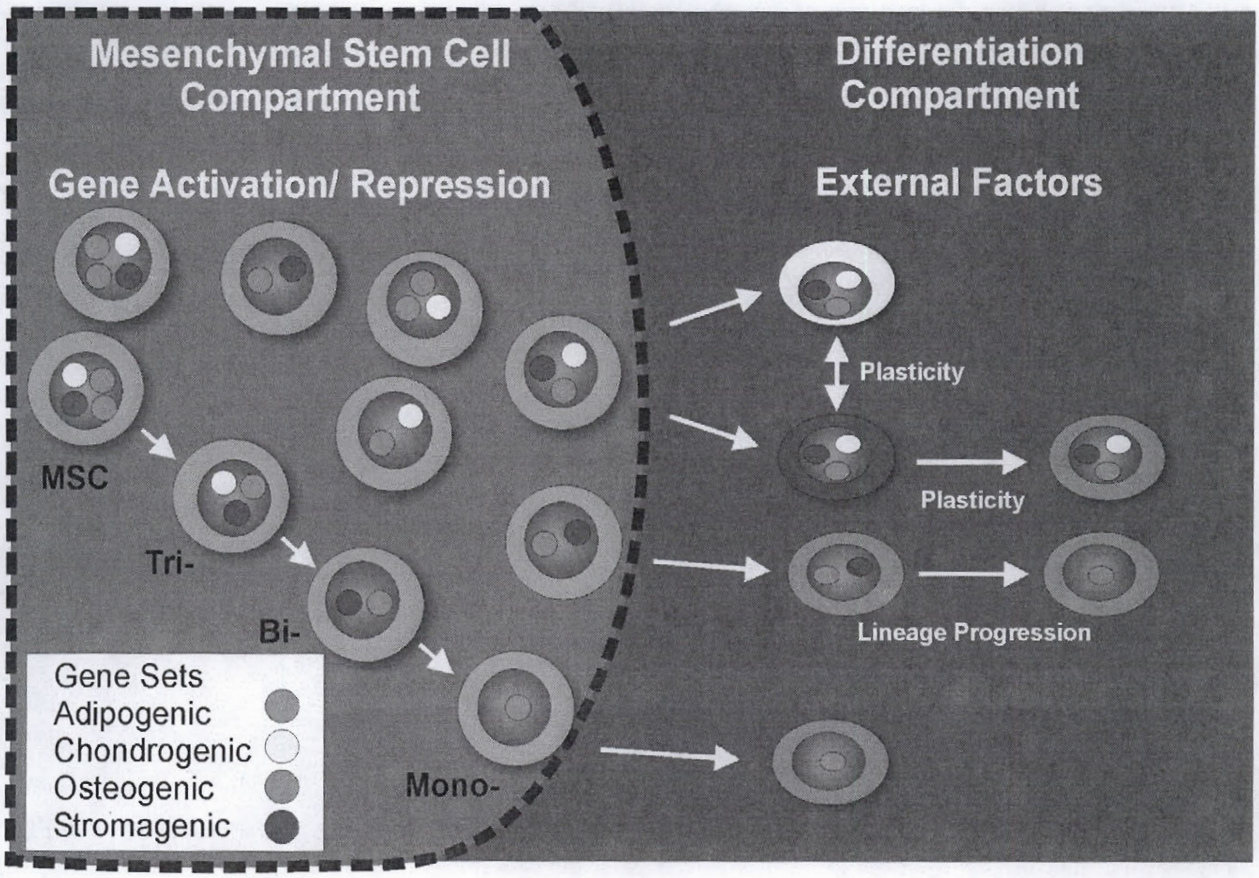


Figure 2. Mesenchymal stem cell (MSC) differentiation appears to be under the influence of multiple inductive and repressive factors that, often simultaneously, can lead to the expression of "end-stage" markers for more than one lineage. Examples of transdifferentiation have also been demonstrated involving differentiated cells of mesenchymal origin adopting a new lineage; such as adipocytes undergoing conversion to osteoblasts and vice versa. This characteristic of MSCs is commonly referred to as "plasticity".

2.5 Mesenchymal Stem Cell Microenvironment

The data from marrow transplantation of patients with osteogenesis imperfecta suggests that bone forming potential may in some cases be attributed to the genotype of the MSC. In other situations, the MSC microenvironment might contribute to bone forming potential in view of the fact that the microenvironment may provide a composite of inducers and repressors for MSC fate determination. Thus, it is conceivable that by manipulating the conditions in the microenvironment, for example by the introduction of commitment and differentiation factors, we can potentiate the capacity of the microenvironment to favor one lineage over another. Conversely, changes to the microenvironment might be induced to occur, or might occur endogenously that could compromise the desired commitment process leading to a cell lineage which would be ineffective in addressing the needs of a given system. This may prove to be the case with age-related osteoporosis. Age-related osteoporosis is characterized by a reduction in bone mass and microarchitectural deterioration of bone. As bone mass is compromised, an increase in bone forming osteoblast numbers or osteoblast activity would be expected to address the need to increase bone mass. Instead, this decrease in total bone volume is accompanied by a reciprocal increase in bone marrow adiposity. Because osteoblasts and adipocytes are both derived from a common progenitor cell, it has been suggested that progenitor cells that were intended to differentiate along the osteoblastic lineage is instead, diverted to the adipocytic lineage accounting for the substantial increase in adipocytic numbers. This increase in bone marrow adiposity in cases of age related osteopenia is known to be an active process and in a more global sense, may be associated with other forms of bone loss(125, 256).

Potential MSCs have also been identified in extra-marrow mesenchymal tissues including subcutaneous fat and muscle. To better characterize MSCs, a more detailed description of the bone marrow microenvironment, as well as that of other "local" mesenchymal tissue microenvironments may be useful in determining the requirements for maintaining an uncommitted progenitor cell in a state of pluripotency. Conversely, the molecular and cellular analysis of microenvironments conducive for commitment and differentiation may also help to identify the determining factors for mesenchymal progenitor commitment and differentiation(161, 195, 206, 208, 406).

Transgenic and knockout animal models may be useful in defining the effects of subtle changes to these microenvironments such as in the case of heterozygous null mutants of parathyroid hormone related protein (PTHrP). These mice demonstrate a change in the normal MSC commitment process within the bone marrow as a result of lower PTHrP levels. Besides looking at perturbations in the MSC microenvironment, it may also be meaningful to distinguish between a physiologically ongoing versus an injury-derived microenvironment where MSCs may need to undergo differentiation to address and counter the damage incurred. This distinction may be important in terms of specification of a cell phenotype(102).

3. PARATHYROID HORMONE RELATED PEPTIDE

3.1 Discovery and Characterization

In 1941, a patient with renal carcinoma with signs of hypercalcemia and hypophosphatemia, a condition subsequently known as humoral hypercalcemia of malignancy (HHM), was presented to Fuller Albright. Because parathyroid hormone (PTH) was known at the time to increase serum calcium levels and enhance urinary phosphate excretion, Albright reasoned that the tumor was most likely producing a systemic factor that was either PTH or a protein that was very similar in nature to PTH. Upon isolating the protein factor responsible for HHM, the protein was found to share amino acid sequence homology with PTH such that 8 of the first 13 NH_2-terminal amino acids were identical. This limited homology of their NH_2-terminal regions was nevertheless sufficient to impart the tumor factor with similar actions to PTH allowing it to bind to a common receptor termed the PTH receptor type I **(Figure 3)**. The gene for the tumor factor was cloned and was found to be similar in structure to that of PTH. Based on this observation, it was suggested that PTH and the tumor factor share a common genetic origin, and accordingly, the tumor factor was named parathyroid hormone related protein (PTHrP) (240, 336, 401).

The PTHrP gene is slightly more complex then the PTH gene, such that the PTH gene spans 3 exons and the PTHrP gene spans five exons in mice and eight in humans. The protein products of the mouse gene encodes a 141 peptide, whereas the human gene can produce three peptides: PTHrP(1–139), PTHrP(1–141), and PTHrP(1–173) through alternative splicing that are identical from amino acids 1–139 and vary in the latter

Figure 3. PTH and PTHrP Homology

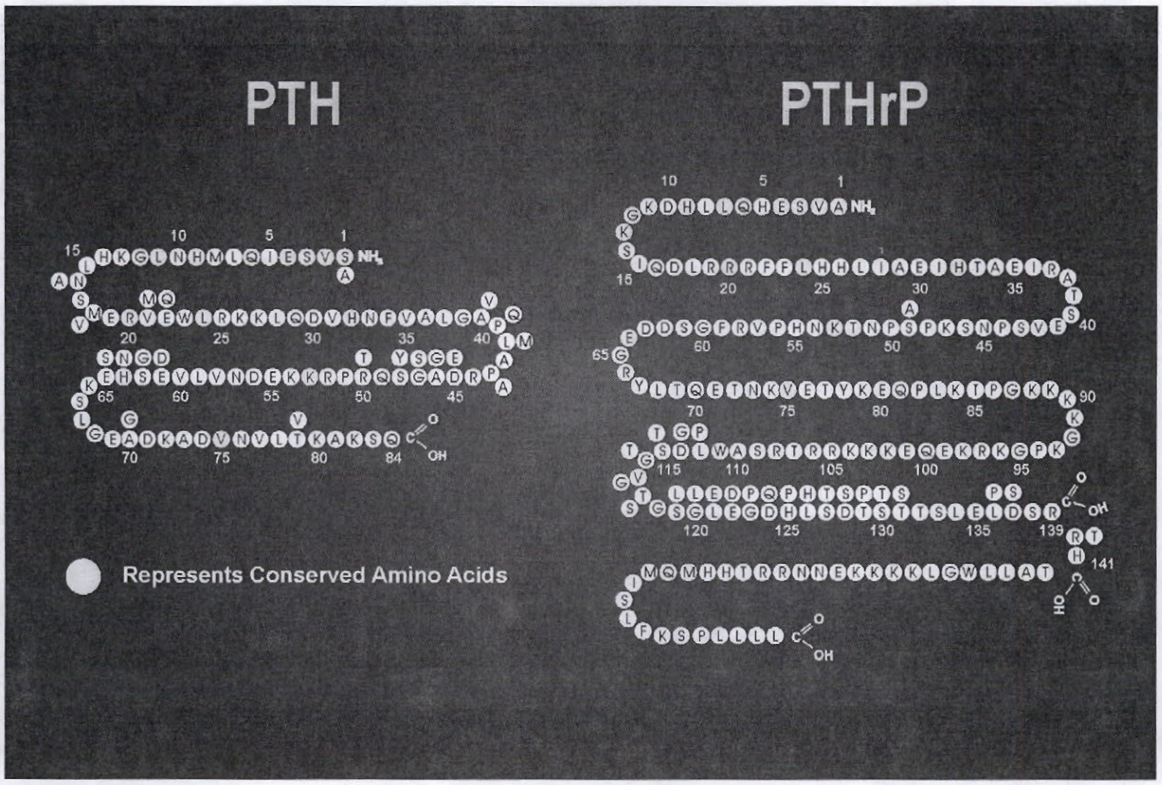


Figure 3. Amino acid sequence of parathyroid hormone (PTH) and parathyroid hormone related peptide (PTHrP). PTHrP shares amino acid sequence homology with PTH such that 8 of the first 13 NH_2-terminal amino acids are identical. This limited NH_2-terminal homology is sufficient to allow PTH and PTHrP to bind to a common receptor termed the PTH receptor type I (PTH-1R).

portion of their amino acid content **(Figure 4)** (399).

These PTHrP translation products may undergo further posttranslational processing, resulting in protein segments of PTHrP that can act via their own receptors with their own distinct physiological functions independent of the amino terminus **(Figure 5)**. Processing of the full length PTHrP generates an amino-terminal secretory form of PTHrP believed to be comprised of amino acids 1-36. This amino-terminal fragment can act through the PTH-1R thereby exerting its effects on bone turnover, smooth muscle relaxation, vasorelaxation and a variety of other processes(47, 61, 294). Processing of PTHrP may also generate three different mid region secretory forms, PTHrP(38–94), PTHrP(38–95), and PTHrP(38–101) that have been postulated to act through an unidentified receptor resulting in stimulation of the phosphatidylinositol/protein kinase C pathway which may play a role in placental calcium transport(207, 290, 326, 334, 392). A carboxy-terminal region of the PTHrP peptide, PTHrP(107–139) may also be generated via proteolytic processing. Contradicting reports exist regarding whether this carboxy-terminal PTHrP fragment inhibits or stimulates osteoclastic bone resorption(100, 269).

In addition to acting in an autocrine or paracrine manner by stimulating cell surface receptors, PTHrP may be capable of functioning in an intracrine manner within the nucleus through a nonclassical nuclear/nucleolar localization sequence(NLS) found within the PTHrP molecule at amino acids 88-106 **(Figure 6)**. Transport from the cytoplasm to the nucleus appears to be mediated by the nuclear transport protein importin β(215) and by a phosphorylation event involving $p34^{cdc2}$ kinase that recognizes a consensus $p34^{cdc2}$ kinase site adjacent to the NLS of PTHrP at Thr^{85}(216). In certain

Figure 4. PTHrP Isoforms

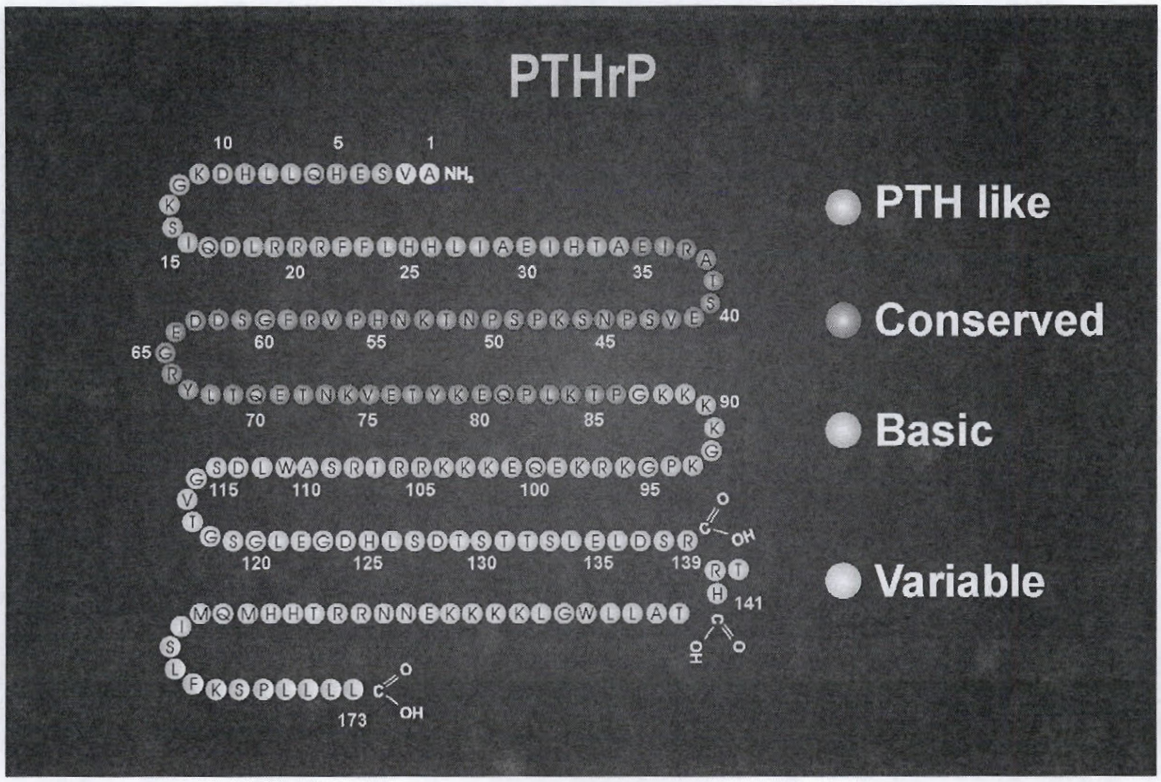


Figure 4. The protein products of the human gene can produce three peptide: PTHrP (1–139), PTHrP(1–141), and PTHrP(1–173) through alternative splicing, that are identical from amino acids 1–139 and vary in the latter portion of their amino acid content

Figure 5. PTHrP Processing

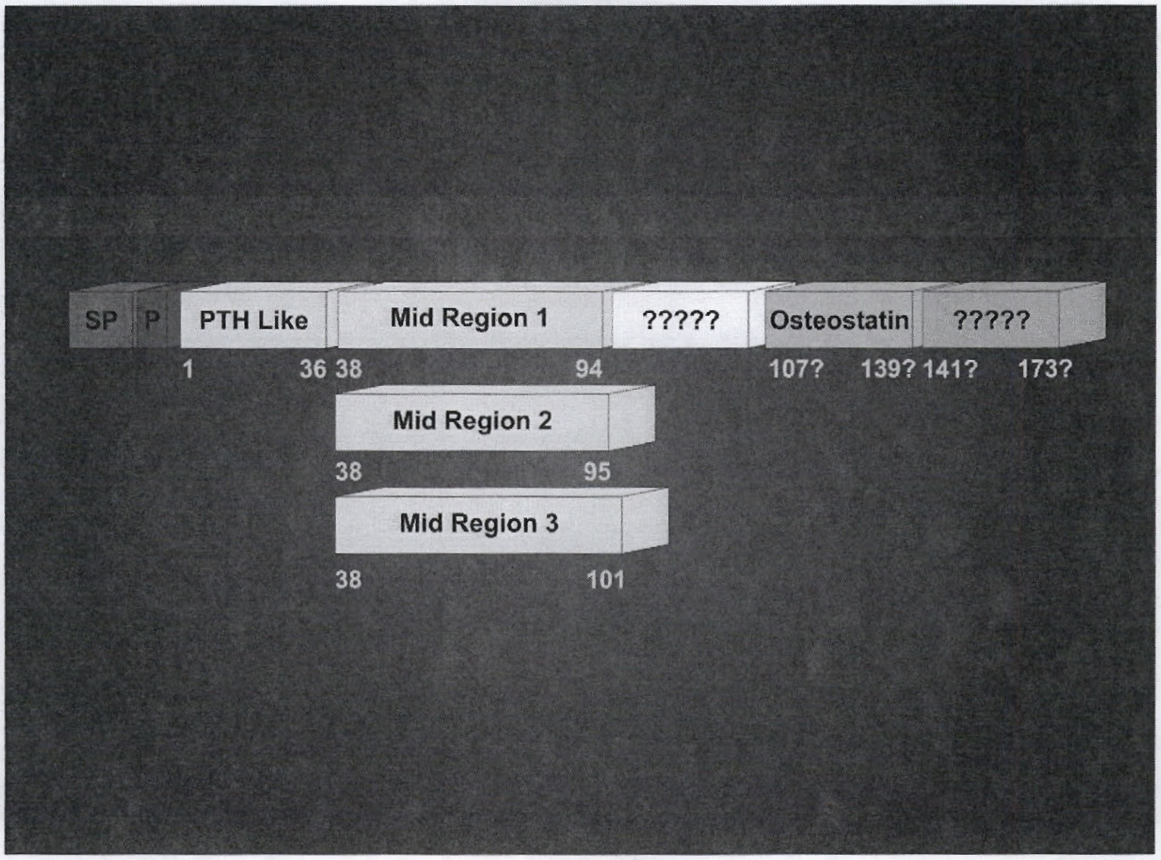


Figure 5. These PTHrP translation products may undergo further extensive posttranslational processing resulting in protein segments of PTHrP with distinct physiological functions independent of the amino terminus. Processing of the full length PTHrP generates an amino-terminal secretory form of PTHrP believed to be comprised of amino acids 1-36. This amino-terminal fragment can act through the PTH-1R. Processing of PTHrP may also generate three different mid region secretory forms, PTHrP(38–94), PTHrP(38–95), and PTHrP(38–101) that have been postulated to act through an unidentified receptor resulting in stimulation of the phosphatidylinositol/protein kinase C. A carboxy-terminal region of the PTHrP peptide, PTHrP(107–139) may also be generated via proteolytic processing. Contradicting reports exist regarding whether this carboxy-terminal PTHrP fragment inhibits or stimulates osteoclastic bone resorption.

cases, phosphorylation by $p34^{cdc2}$ kinase is a prerequisite for nuclear transport as is the case with nucleoplasmin (365). However, PTHrP like Simian virus-40 T-antigen (179) and the transcriptional co-repressors of the Groucho/transducin-like Enhancer of split (Gro/TLE) family, demonstrate cytoplasmic accumulation when phosphorylated by $p34^{cdc2}$ kinase(216). This can be visualized at the onset of S phase, when $p34^{cdc2}$ kinase activity begins, until the G_2/M phase of the cell cycle when $p34^{cdc2}$ kinase activity is at its highest.

How a secreted protein such as PTHrP might gain access to the cytoplasm for nuclear transport is still uncertain, however several mechanisms have been reported. As a secreted protein, PTHrP has been shown to undergo endocytosis in a cell type specific fashion. Internalization of PTHrP has been demonstrated in a variety of cell lines including the chondrocytic cell lines CFK2 and 27m21 and the osteoblastic cell line UMR 106.01 cells(1, 216). As an alternative explanation, PTHrP translation may employ an alternative translational start site involving a CUG codon downstream of the authentic AUG start codon(7, 273). This would allow PTHrP to bypass the secretion step and remain in the cytoplasm.

The intracrine function of PTHrP appears to serve multiple functions and varies from cell-type to cell-type. In the chondrocytic CFK-2 cell line and the MCF-7 breast cancer cell line, overexpression of PTHrP with an intact NLS results in reduced apoptosis and enhanced survival under conditions of serum deprivation(149, 357). In vascular smooth muscle cells (VSMC), the intracrine effect of PTHrP appears to be mitogenic as VSMCs of PTHrP deficient animals demonstrate reduced proliferation (74, 247). Intracrine PTHrP has also been shown to promote proliferation of the prostate cell line

PC-3 albeit through an interleukin-8 (IL-8) dependent mechanism. Cells transfected with PTHrP1-87 and 1-173 also showed increased cell proliferation and the COOH-terminal truncation mutant PTHrP1-87 induced a 5-fold simulation of IL-8 and a 3-fold increase in IL-8 mRNA. Exogenous PTHrP1-34 and 1-86 peptides did not significantly affect IL-8 production nor did they affect proliferation(133).

3.2 Parathyroid Hormone Receptor Family

The PTH-1R is a member of the seven transmembrane-domain G protein-coupled receptor superfamily that is comprised of seven transmembrane α-helical domains flanked by an NH_2-terminal extracellular domain and a COOH-terminal intracellular domain. Although first identified as the receptor responsible for eliciting the effects of PTH (1-34) in maintaining calcium and phosphate homeostasis, it was later shown to be responsive to PTHrP (1-34) and PTHrP (1-36) as well. Signaling by the PTH-1R is potentiated by two different signaling Gα-proteins: $G_\alpha s$ stimulates the adenylyl cyclase (AC)-protein kinase A (PKA) signaling pathway, and $G_\alpha q$ initiates the phospholipase C (PLC)-protein kinase C (PKC) pathway **(Figure 6)**.

The identification of a second member of the PTH/PTHrP receptor family named the type 2 PTH receptor (PTH-2R) subtype was initially identified through hybridization cloning methods from a human brain cDNA library **(Figure 7)**. In comparing the amino acid sequence of the human PTH-2R with that of the human PTH-1R, it was determined that the two receptors were 51% homologous in amino acid sequence(362). This difference in homology appears sufficient for ligand specificity such that the human PTH-2R binds to and responds to PTH, but not to PTHrP. Specificity for PTH is believed

to be a result of two substitutions in PTHrP, where Ile5 and Trp23 in PTH is replaced by residues His5 and Phe23 in PTHrP. Based on results from the human PTH-2R, it was expected that the rat homologue of the PTH-2R would also be specific for PTH, however this proved not to be the case. Instead, the rat PTH-2R was responsive to neither PTH nor PTHrP (155). A search for a rat peptide ligand that could stimulate the PTH-2R, led to the discovery of a previously unidentified peptide of 39 amino acids that was able to specifically stimulate both the rat and the human PTH-2R subtypes without activating the PTH-1R **(Figure 7)**(363). This peptide was named TIP39 for "tubero-infundibular peptide of 39 amino acids". In comparing the amino acid sequence of TIP 39 with that of PTH and PTHrP, little homology was noted.

The PTH/PTHrP receptor family has since grown to include another receptor family member that was identified in zebrafish. This third receptor demonstrated moderate homology with the zebrafish PTH-1R(61%) and PTH-2R(48%) and is responsive to both PTH and PTHrP, but not to TIP39. This homologue has not been shown to exist in higher vertebrates, nor has a physiological role been determined for this receptor in zebrafish (156, 314, 366).

3.3 PTH/PTHrP type 1 Receptor Signaling

The cumulative data regarding receptor binding of PTH (1-34) and of PTHrP (1-34) or (1-36), suggests that the mechanism of interaction of these peptides with PTH1-R is based upon two principal components. One involves an interaction between the COOH-terminal domain of these ligands and the NH_2-terminal domain of the receptor, which contributes predominantly to binding affinity. The second component involves an

Figure 6. PTH/PTHrP Receptor Signaling

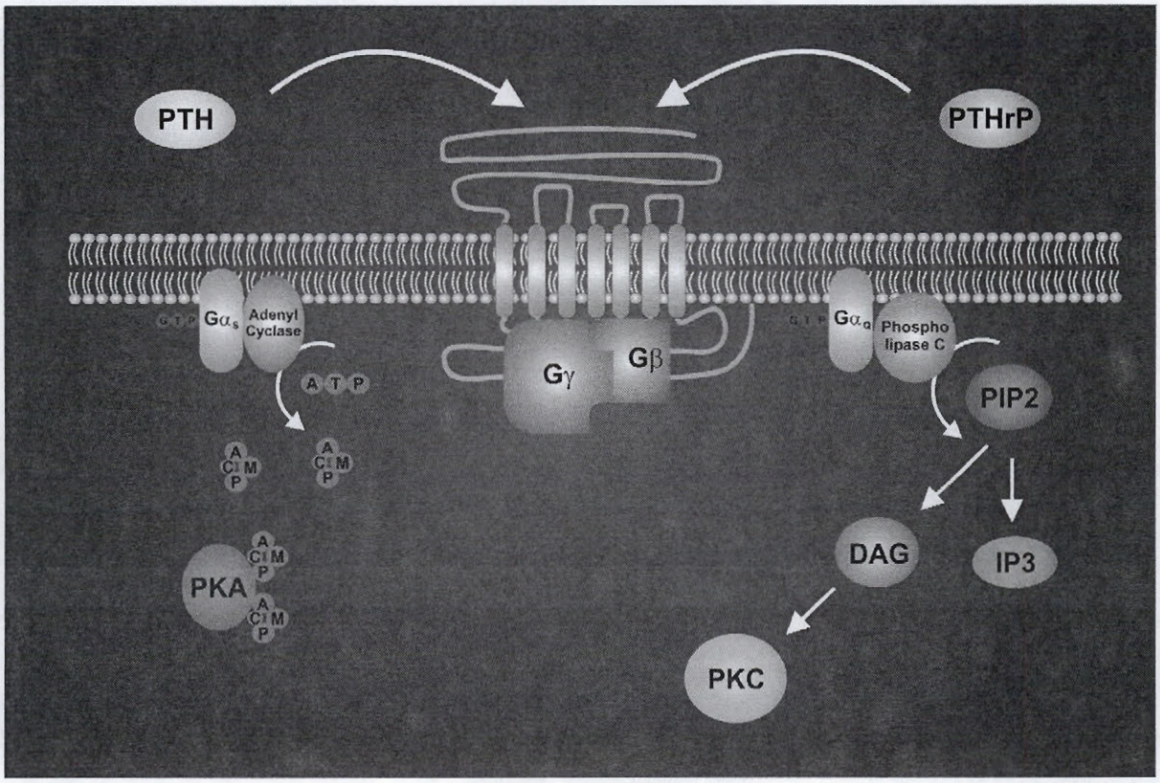


Figure 6. The parathyroid hormone type 1 receptor (PTH-1R) is a member of the seven transmembrane-domain G protein-coupled receptor superfamily, which is comprised of seven transmembrane α-helical domains flanked by an NH_2-terminal extracellular domain and a COOH-terminal intracellular domain. The PTH-1R is responsive to both PTH and PTHrP. Signaling by the PTH-1R is potentiated by two different signaling Gα-proteins: $G_{\alpha}s$ stimulates the adenylyl cyclase (AC)-protein kinase A (PKA) signaling pathway, and $G_{\alpha}q$ initiates the phospholipase C (PLC)-protein kinase C (PKC) pathway

Figure 7. PTH/PTHrP Gene Family and PTH/PTHrP Receptor Gene Family

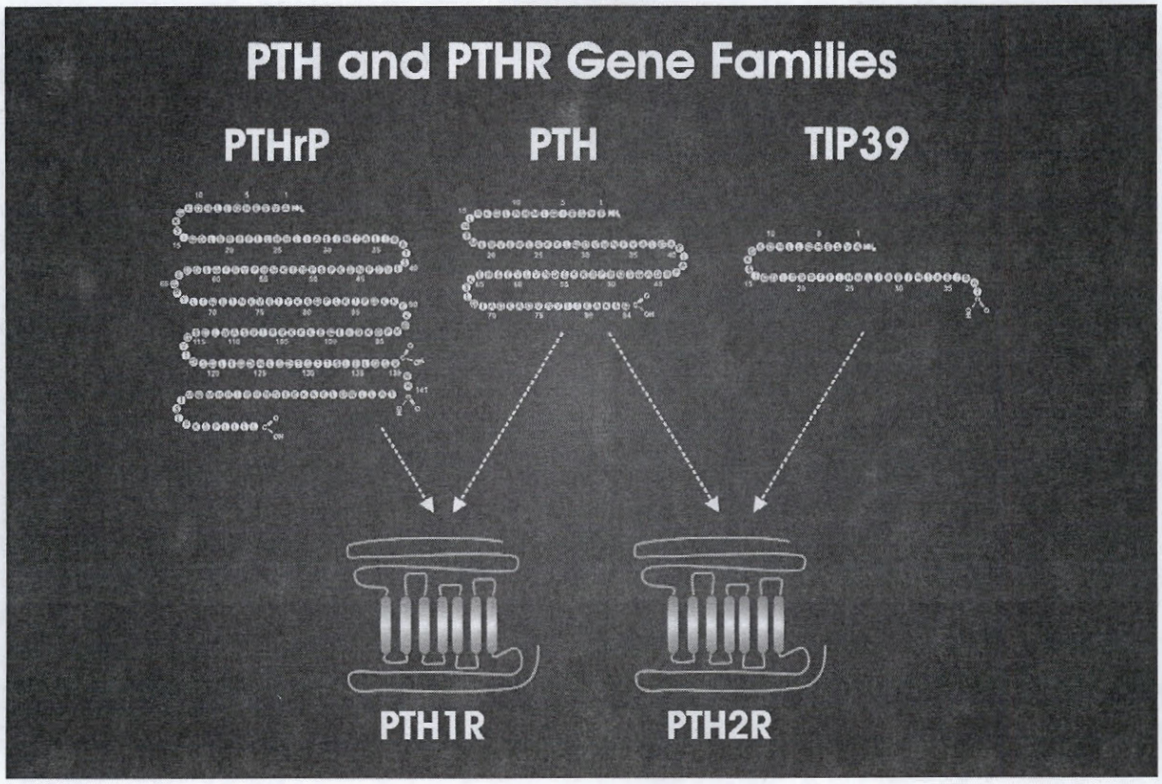


Figure 7. The PTH/PTHrP receptor family is comprised of two family members that are responsive to members of the PTH/PTHrP gene. In humans PTH-1R is responsive to both PTH and PTHrP and the PTH-2R is responsive to PTH and TIP39. In rats, the murine homologue of PTH-1R is responsive to both PTH and PTHrP, however, the PTH-2R is responsive to only TIP39.

interaction between the NH_2-terminal portion of these ligands and the juxtamembrane region of the receptor, which contributes to signaling.

The PTH-1R is coupled to two distinct signaling pathways involving the $G_s\alpha$ and $G_q\alpha$ guanine nucleotide binding proteins **(Figure 6)**. In their inactive states, the $G_s\alpha$ and $G_q\alpha$ proteins interact with Gβ and Gγ subunits as a heterotrimer and are non-covalently bound to GDP. Upon agonist binding to the receptor, the Gα subunit releases GDP which is subsequently replaced by GTP resulting in a conformational change favoring the dissociation of the Gα subunit from the Gβ and Gγ subunits and thus allowing the free Gα subunit to stimulate its respective signaling pathway.

$G_s\alpha$ when released from the receptor G-protein complex is able to couple to adenylyl cyclase (AC) which converts adenosine monophosphate (AMP) to its cyclic form. The secondary messenger cAMP can then bind to the regulatory subunits of protein kinase A (PKA), thereby promoting the release and activation of the catalytic PKA subunits. Release of $G_q\alpha$ from the G-protein complex promotes its association with phopholipases which in turn enhances the hydrolysis of membrane bound phosphatidyl-inositol 4,5 bisphosphonate (PIP_2) into soluble inositol (1,4,5)-triphosphate (IP_3) and diacylglycerol (DAG) which remains on the cytosolic face of the cell membrane. IP_3 can diffuse throughout the cytoplasm to act upon specific receptors found on the on the endoplasmic reticulum which allows for the opening of specific calcium ion channels causing Ca^{2+} to exit from the ER into the cytosol, resulting in a net increase in cytoplasmic Ca^{2+} levels. The influx of calcium into the cytoplasm facilitates another branch of $G_q\alpha$ signaling which involves activation of PKC signaling. Normally, PKC in

its resting state is a soluble cytosolic protein, however Ca^{2+} ions promotes recruitment of PKC to the cell membrane where it can then be activated by membrane bound DAG.

Following receptor activation of PTH-1R, receptor activity is markedly reduced within minutes of initial exposure to PTH or PTHrP(31, 114). Downregulation of PTH-1R signaling occurs following conversion of GTP-Gα to GDP-Gα, which promotes the reconstitution of the heterotrimeric Gα-Gβ-Gγ receptor associated complex. This process can occur via intrinsic GTPase activity of Gα(329). This desensitization is accompanied by rapid internalization of the PTH–PTH-1R complex (103, 167, 238). Phosphorylation of the COOH-terminal tail of PTH-1R might play a role in internalization as evidenced by the inhibitory effect of staurosporine, a broad-spectrum kinase inhibitor on PTH-1R phosphorylation (300) and internalization (103). However, questions remain regarding the necessity of the phosphorylation event as a phosphorylation deficient PTH-1R mutant expressed at high levels in HEK-293 cells was internalized upon agonist binding just as efficiently as was the wild-type receptor (238). Furthermore, a PTH-1R lacking its COOH terminus and consequently its phosphorylation site was internalized upon agonist binding just as efficiently as the intact receptor (104).

Naturally occurring ligands for the PTH-1R include the amino-terminal domains of PTHrP and PTH, which bind to, and activate the PTH-1R with affinities and potencies in the low nanomolar range. Synthetic PTH and PTHrP peptides have been useful in clarifying key amino acid residues necessary for PTH-1R mediated activation of AC-PKA signaling. It appears that residues close to the amino-termini of PTH and PTHrP are required as PTH(3–34), PTH(7–34) and [Asn10,Leu11]-PTHrP(7–34) ligands can bind to PTH-1R with high affinity, without stimulating cAMP accumulation. As a result of these

findings, these fragments have been used as competitive antagonists for PTH and PTHrP mediated PKA signaling(278). The amino acid residues that are essential for PTH and PTHrP for PTH-1R mediated activation of PLC-PKC signaling remains unclear. Two studies have indicated that residues PTH(29-32) are sufficient to activate PKC in ROS 17/2 rat osteosarcoma cells and in Chinese hamster ovary cells transfected with rat PTH-1R (19). These findings would suggest that the amino-terminus of PTH is not required for stimulation of the PLC-PKC signaling pathway. However, this cannot be said of PTHrP, as only 3 amino acids are homologous between PTHrP and PTH through amino acid residues 15-34. As a result, this effectively limits the extent to which one can extrapolate the minimal amino acid requirements for PTHrP activation of PKC from this study employing PTH.. In contrast to these findings, more recent data would implicate the amino-terminus of PTH as being important for PLC activation as demonstrated by substitution or deletion of amino-terminal residues of PTH. [Gly1]PTH(1-34), PTH(2-34) and PTH(3-34) peptides have a reduced capacity to stimulate inositol phosphate (IP) production via PLC in porcine kidney LLC-PK1 cells transfected with human PTH-1R versus wild type PTH(1-34) (338).

One possible explanation for these different findings may be that residues 29-32 of PTH may mediate PKC activation via a phospholipase other than PLC. By studying PTH-1R function in kidneys, it was shown that PTH-1R is able to couple to more than one phospholipase. In the distal tubule cells of the kidney, PTH-1R couples to phospholipase D (PLD), whereas in the proximal tubule cells the PTH-1R couples to PLC. Furthermore, PTH-1R activation requirements differ from the distal tubule to the proximal tubule with PTH-(1-31) increasing [Ca2+] in distal tubule, but not in proximal

tubule cells and PTH-(3-34) causing a partial increase in [Ca2+] in proximal cells, with no effect in distal cells (110). Taken together, this would suggest that the amino terminus of PTH is required for PTH-1R mediated activation of PLD, but not for receptor mediated activation of PLC.

3.4 PTHrP and Development

In spite of their similar agonistic actions on the PTH-1R, PTH and PTHrP differ extensively in their distribution. Where PTH expression is limited to the parathyroid glands, the thymus(297) and the hypothalamus(134). PTHrP is expressed in many tissues, both fetal and adult, including epithelia, mesenchymal tissues, and the central nervous system. This widespread distribution would suggest multiple roles for PTHrP, which have been confirmed by genetic analysis(40).

The first evidence for a physiological role for PTHrP was demonstrated by gene-knockout experiments in which mice homozygous for a null mutation of the PTHrP gene were found to die in the neonatal period(191). Absence of PTHrP in these animals during development resulted in severe defects in the development of growth plate cartilage of long bones causing severe *chondrodysplasia*. Prior to skeletal development, chondrocytes condense in the mesenchyme, delineate the skeletal elements and provide a cartilaginous scaffold upon which osteoblasts can subsequently lay down bone for long bone development **(Figure 8)**. This process termed endochondral bone formation is an orderly process giving rise to the proper shape of the skeletal elements. The first phase involves chondrocyte proliferation and the deposition of a specialized extracellular matrix containing type II collagen, which will serve to define the length of the future skeletal

element. Following proliferation, the chondrocytes progress to a terminally differentiated state in which they first become hypertrophied and change their program of matrix synthesis to type X collagen. Hypertrophic chondrocytes then mineralize their matrix, and finally undergo programmed cell death, or *apoptosis*. The cartilage matrix then undergoes vascular invasion, which directs chondroclasts to resorb the cartilage matrix. Osteoprogenitor precursors can then invade the excavated cavity and differentiate into osteoblasts allowing for the synthesis of a bone matrix **(Figure 8)**.

In the absence of PTHrP, chondrocytes proliferation is diminished and chondrocytes proceed through the differentiation process at an accelerated pace thereby resulting in fewer chondrocytes and shortened bones. Accelerated differentiation also leads to an extracellular matrix that is mineralized prematurely, and to the early apoptosis of chondrocytes(8, 11, 191). The findings from mice with targeted PTHrP deletion were complemented by findings from the study of mice with targeted overexpression of PTHrP in chondrocytes using the cartilage-specific type II collagen promoter. These mice suffer from a delay in chondrocyte differentiation and a suspension of chondrocyte apoptosis, which leads to pockets of chondrocytes within mature bone(9). In examining both the loss of function and gain of function of PTHrP in cartilage differentiation, it would appear that PTHrP enhances proliferation and inhibits the terminal differentiation of chondrocytes.

The pleiotropic effects of PTHrP on chondrocyte biology are believed to occur through the PTH-1R, as PTH-1R signaling has also been described as being both a component and necessary for proper development of the skeleton(218). This was demonstrated in mice with targeted ablation of the PTH-1R, which have a similar, but

more exacerbated phenotype than the PTHrP knockout mice. The reason for the more severe phenotype may be attributed to PTH. PTH-deficient mice demonstrate a defect in cartilage matrix mineralization and osseous abnormalities(257) which indicates that circulating systemic PTH serves a function during the developmental phase of the animals skeleton and may explain why PTH-1R animals demonstrate a more severe chondrodysplasia than PTHrP deficient mice. In line with these findings, addition of either PTHrP or PTH to cartilage explants from mice with a homozygous deletion for the PTHrP gene ligands reverses the proliferative defect observed in the growth plate of PTHrP knockout mice(218). Because PTH and PTHrP act through the same G-coupled receptor, the observation that PTH mimics the proliferative effect of PTHrP on these explants suggests that the PTH-1R is important in this process.

Although mice that lack functional PTHrP die prematurely, PTHrP heterozygous null animals remain viable, and therefore, provide a useful model for assessing gene dosage of PTHrP in relationship to development. Mice with one functional allele for PTHrP suffer from haplotype insufficiency by 3 months of age and develop a premature form of osteoporosis as characterized by reduced trabecular bone volume and an increase increase in bone marrow adiposity (9). Because the PTH-1R is expressed by both osteoblasts and pre-osteoblasts, it was proposed that locally produced PTHrP serves an autocrine/paracrine function in directing osteoblast differentiation and that increased bone marrow adiposity was a consequence of the inappropriate differentiation of osteoblasts due to lower levels of PTHrP.

Figure 8. Endochondral Bone Formation

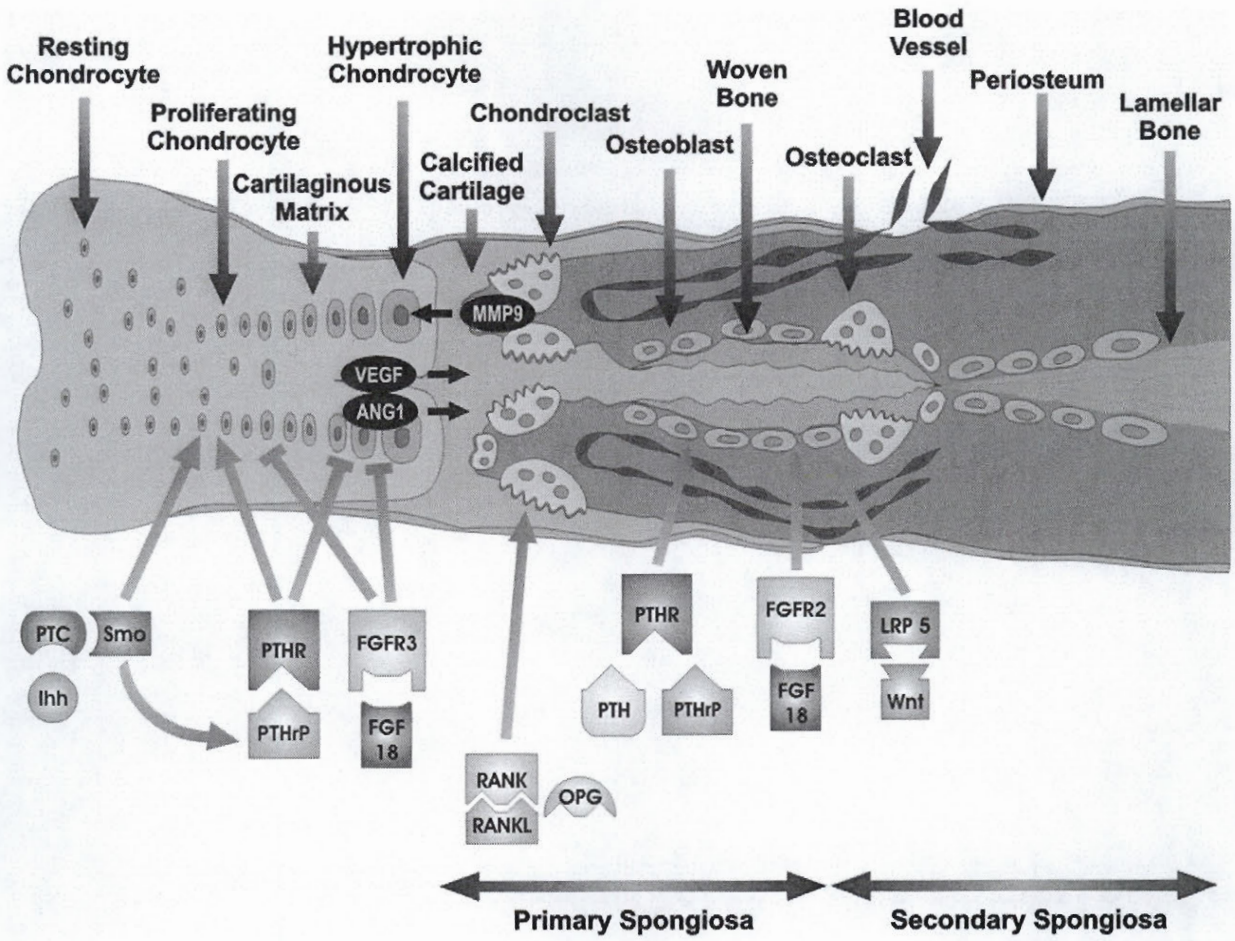


Figure 8. Endochondral bone formation defines the proper shape of the skeletal elements. The first phase involves chondrocyte proliferation and their deposition of type II collagen which will serve to define the length of the future skeletal element. Following proliferation, the chondrocytes progress to a terminally differentiated state in which they first become hypertrophied and change their program of matrix synthesis to type X collagen. Hypertrophic chondrocytes then mineralize their matrix, and finally undergo programmed cell death (*apoptosis*). The cartilage matrix then undergoes vascular invasion which directs chondroclasts to resorb the cartilage matrix. Osteoprogenitor precursors can then invade the excavated cavity and differentiate into osteoblasts allowing for the synthesis of a bone matrix.

3.5 PTHrP and Osteoblast Biology

Despite the catabolic effects of continuous exposure to the NH_2 -terminal domain of PTH, intermittent administration of the NH_2 -terminal domain of PTH has been known to have an anabolic action(23). The NH_2 -terminal region of PTHrP like PTH, has also been shown to have positive effects on bone formation(331). In addition to increasing trabecular bone density, administration increases bone strength and reduces fracture rates despite occasional increases in cortical porosity. The mechanism by which PTH (or PTHrP) enhances bone formation appears to be through the PTH-1R and most likely involves activation of adenylyl cyclase or PLD or both as demonstrated by the requirements of residue 1 and 2 from the PTH peptide(154). As a result, there is much interest in developing agonists for the PTH-1R as a potential therapy for osteoporosis(315).

Several mechanisms have been proposed to explain how PTH and PTHrP are able to exert both a catabolic and anabolic action on bone. Continuous PTH administration has been shown to stimulate production of RANKL (receptor activator of nuclear factor-κB ligand) by committed pre-osteoblasts or osteoblastic stromal cells. Osteoblast bound-RANKL then bind to and activate the RANK receptor found on osteoclasts and pre-osteoclasts leading to enhanced osteoclast differentiation, activity, and consequently bone resorption(234). Sustained PTH levels has also been shown to decrease expression of osteoprotegrin, a soluble decoy receptor for RANKL which acts by sequestering free RANKL thereby limiting its ability to stimulate RANK(289). In contrast to these findings, when PTH is administered in an intermittent fashion, transient or no changes

can be detected for RANKL and osteoprotegrin expression leading to a milieu that favors bone formation by committed pre-osteoblastic cells(234).

Asides from affecting osteoclastic resorption of bone, PTH has been shown to act in an anabolic manner by increasing both osteoblast and osteoprogenitor numbers. Intermittent treatment of normal and osteopenic mice with PTH was also shown to prolong the life-span of mature osteoblasts by preventing their apoptosis(185), leading to an increase in the osteoblast population, and an increase in bone forming potential. PTH has also been shown to enhance the transcriptional activity of Core Binding Factor Alpha 1 (CBFA1), a transcription factor essential for bone formation *in vivo* by a PKA dependent phosphorylation event(321). As CBFA1 has been shown to be directly related to osteoblast differentiation, increased CBFA1 activity might enhance osteoblast differentiation. Gene expression profiling of osteoblasts has demonstrated that treatment with PTH results in the induction of IGF-1 in osteoblast-enriched cultures isolated from fetal rat parietal bones(250). As IGF-1 is a potent inducer of osteoblast differentiation, this may explain at least in part, the mechanism by which PTH increases osteoblast numbers. An increase in osteoprogenitor numbers may also be the result of increased proliferation of osteoblasts or osteoblast progenitors as demonstrated by Miao et al.. In this study, they demonstrate that PTHrP is able to increase osteogenic cell proliferation by stimulating MAPK in osteoblastic cells through a PKC dependent pathway(258). There is also evidence that would suggest that both PTH and PTHrP may enhance differentiation of osteoblast precursors. Finally, an increase in osteoblastic numbers could also occur by increasing the commitment of MSCs towards the osteoblastic lineage.

4. BONE MORPHOGENETIC PROTEIN

4.1 Discovery and Characterization

In 1965, Dr. Marshall Urist first demonstrated that demineralized bone extracts could induce de novo bone formation when implanted in ectopic sites in rats (360, 361). Ectopic bone formation in response to these extracts was found to follow a similar sequence as endochondral bone formation **(Figure 8)**. Initially, the implant is first colonized by undifferentiated mesenchymal cells that later differentiate into chondrocytes. These differentiated chondrocytes then synthesize a chondrocytic matrix that is subsequently subjected to vascular invasion and chondroclastic resorption. Osteoblast progenitors can then be delivered to the implant site by neo-vascularization where they themselves would undergo differentiation and promote *de novo* bone formation as differentiated osteoblasts. Although Urist was unable to isolate the agent responsible for *de novo* bone formation, he named this hypothetical factor Bone Morphogenetic Protein (BMP). More then 20 years after Urist's first experiments, the first members of the BMP family were cloned. The number of peptides in the family has since grown to include over 30 BMP homologues (50, 316, 317, 377, 378, 389). Analysis of the amino acid sequence and predicted structure of these proteins determined that BMPs were similar to TGF-β proteins based on a conserved seven cysteine domain in the carboxyl region of the proteins, and are therefore, categorized as a distinct subfamily under the TGF-β superfamily of signalling molecules(201). Of the BMP family members, only BMP-2, BMP-4, BMP-6 and BMP-7 have been implicated as inducers of osteoblastic commitment for MSCs.

The demonstration of the osteoinductive properties of some members of the BMP family suggests that the expression patterns of these BMPs might be limited to sites of active skeletal formation. *In situ* analysis, however, demonstrated that this was not the case. Instead, BMPs were found to be expressed in a variety of tissues during development including monocytes, epithelial cells, mesenchymal cells and neuronal cells. In addition to bone formation, BMPs demonstrate a much more extensive range of biological activities in a variety of cell types including proliferation, differentiation, and apoptosis. BMPs may also define left-right asymmetry in a developing organism, and influence neurogenesis, mesoderm patterning and development of the kidney, gut, lung, teeth, limb, amnion and testis(93, 128, 158, 388).

Although only BMP2, BMP4, BMP6 and BMP7 have the ability to induce osteoblast commitment, genetic mouse models lacking these morphogens has revealed very little regarding their roles in osteoblast determination during development, due to the early lethality of the majority of phenotypes. Only BMP6-deficient mice are viable, however, they exhibit only a mild delay in the ossification of the sternum that can be traced to the impaired formation of mesenchymal condensations(327). Deletion of the BMP2 gene in mice results in embryonic lethality prior to the onset of skeletogenesis. The developing embryos of BMP2-deficient mice retained an open preamniotic canal resulting in the defective formation of the amnion and chorion. Defects in cardiac development were also identified in the mutant embryos of BMP2-deficient mice (411). The BMP4 null mutation like the BMP2 null mutation results in embryonic lethality prior to skeletogenesis. Embryonic lethality of BMP4-deficient mice is observed between days 6.5 and 9.5 days post-conception (dpc) and a variety of phenotypes are observed. In the

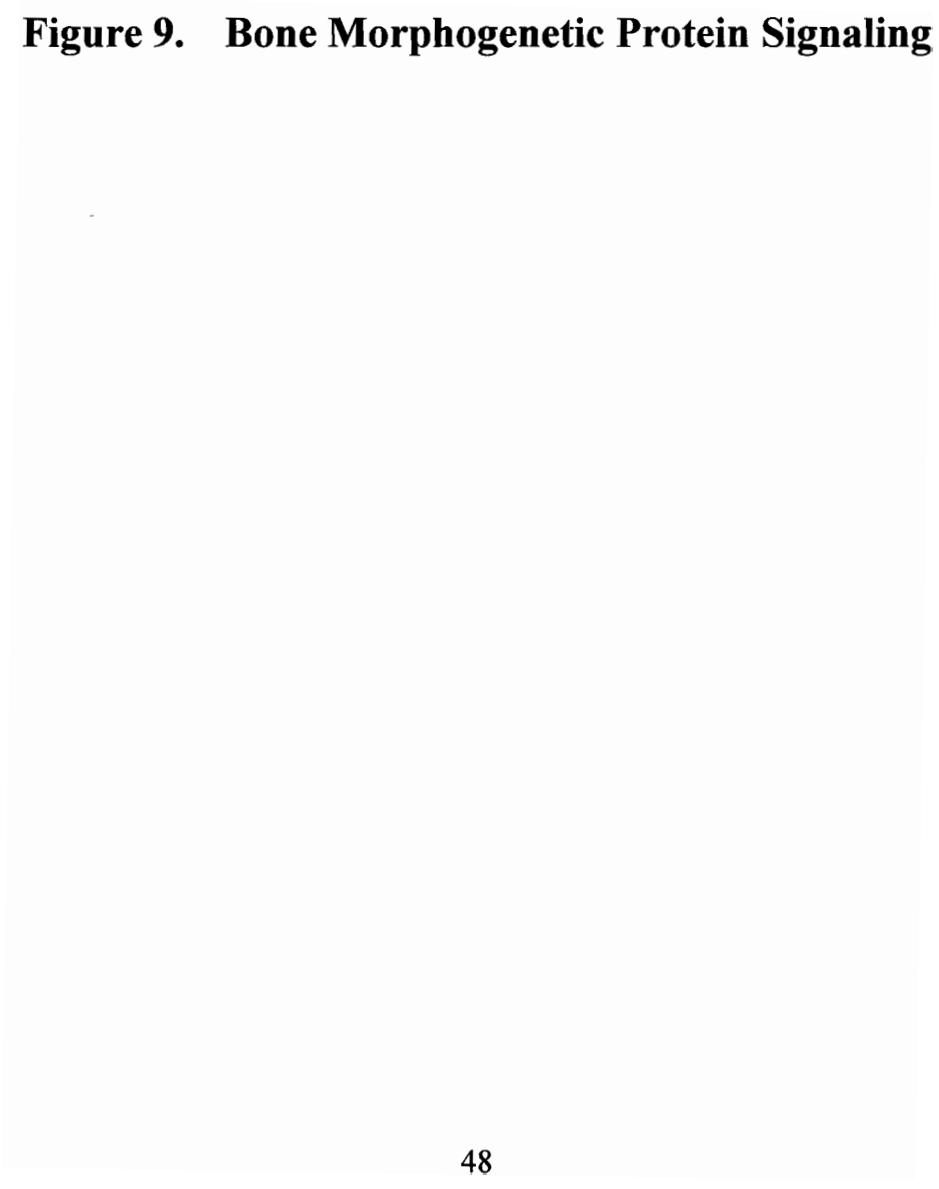
majority of cases, the embryos do not proceed beyond the egg cylinder stage and no visible signs of mesodermal differentiation are observed. The remaining homozygous mutants develop to the head fold or beating heart (early somite) stage or beyond, however, they exhibit disorganized posterior structures, reduced extraembryonic mesoderm, a complete absence of primordial germ and absent lens induction(115, 220, 387). The reason for the pleiotropic effects of BMP4 remains unclear. Deletion of the BMP7 gene also results in a lethal phenotype within 24 hours following birth. Histologic examination of the BMP7-deficient mice failed to detect any abnormalities in chondrocyte and osteoblast differentiation, however, kidney morphogenesis is stunted due to a defect in the epithelial-mesenchymal interaction that occurs between 12.0 and 12.5 dpc which results in cell death of the metanephric mesenchymal cells. BMP7 deficiency also causes patterning abnormalities with mice lacking ribs and a preaxial polydactyly in the hind limbs(91, 233), as well as an absence of lens induction and eye formation.

4.2 Bone Morphogenetic Protein Signal Transduction and Intracellular Events

BMPs are first synthesized as precursor proteins that require dimerization prior to becoming functional ligands. Dimerization is believed to occur within the cell whereupon the dimers are then proteolytically cleaved at a consensus Arg-X-X-Arg site by subtilisin-like convertases (SCPs) (63, 68) to yield carboxy-terminal mature dimers (172, 201, 389) which are then secreted from the cell. The processed secreted BMP dimer can then bind to a type II serine/threonine kinase dimer (ActR-II, ActR-IIB, or BMPR-II) whose kinase activity is constitutively active **(Figure 9)**. Upon ligand binding, the type II receptor can

recruit two type I (ALK-1, ALK-2, ALK-3, or ALK-6) receptors that allows for the formation of a BMP ligand/type II receptor/type I receptor complex(14, 230, 312). The close proximity of the type I and II receptors allows for the trans-phosphorylation of the type I receptors by the type II receptors, on serine and threonine residues within a glycine serine rich region, located on the intracellular region. The activated type I receptor then phosphorylates specific homo-oligomeric complex members of the Smad family of transcription factors known as receptor-regulated Smads (R-Smads) (143) **(Figure 9)**. Smad proteins share two highly conserved domains at their NH_2- and COOH-termini referred to as Mad-Homology domains 1 and 2 (MH1 and MH2), respectively. These two domains are separated by a divergent proline-rich region of variable length. It is through the MH2 domain that cytosolic R-Smads transiently interact with the activated type I receptor allowing for the phosphorylation of their extreme COOH-terminal serine residues at a conserved SSXS phosphorylation motif(174, 236, 341). Three R-Smads have been implicated in BMP signalling: Smad1, Smad5 and Smad8, with Smad8 being the only Smad that is exclusive to BMP activation; both Smad1 and Smad5, have also been shown to be activated by TGFβ in various cell types (41, 231, 408, 409). Following phosphorylation of the R-Smads, they are released from their receptors and through their free MH2 domains, form heteromeric complexes with a Common Mediator Smad (Co-Smad), that is, Smad 4 in mammals **(Figure 9)**. The exact stoichiometry between R-Smads and Co-Smads in the heteromeric complex are unclear(67, 194, 391). The R-Smad/Co-Smad heteromeric complex can then translocate to the nucleus where they can initiate expression of target genes in concert with other transcription factors or co-activators (146).

Figure 9. Bone Morphogenetic Protein Signaling



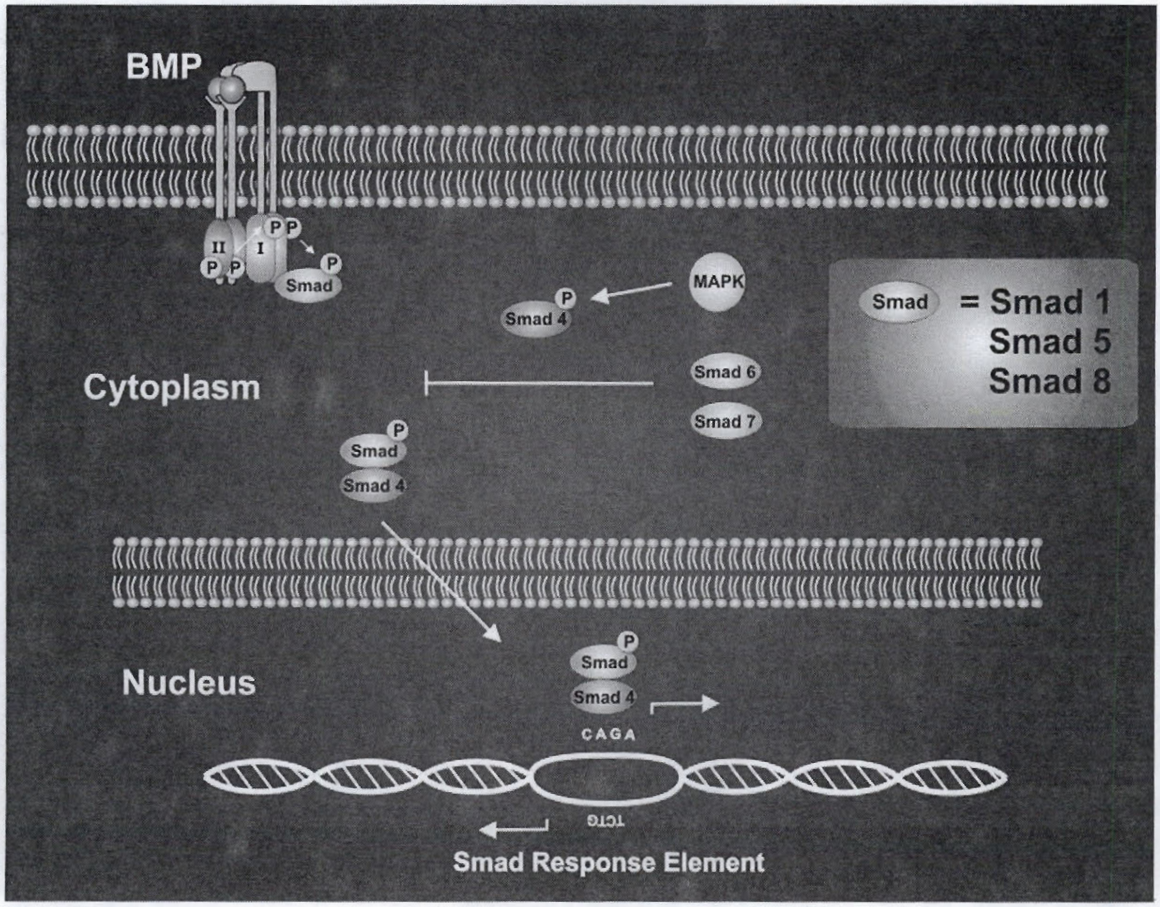


Figure 9. The processed secreted BMP dimer can bind to a type II serine/threonine kinase dimer, that can then recruit two type I receptors that allows for the formation of a BMP ligand/typeII receptor/Type I receptor complex. The type 1 receptors are activated by trans-phosphorylation via the type II receptor kinases which in turn phosphorylates specific homo-oligomeric complex members of the receptor-regulated Smads (R-Smads). Following phosphorylation, the R-Smads are released from their receptors and form heteromeric complexes with Smad 4. The R-Smad/Co-Smad heteromeric complex can then translocate to the nucleus where they can initiate expression of target genes in concert with other transcription factors or co-activators.

The binding of Co-Smads and R-Smads to specific DNA sequences occurs through their MH1 domains. This interaction occurs with a rather low affinity and therefore, other DNA-binding proteins such as Olf-1/EBF associated zinc finger (OAZ) are required to enhance DNA binding efficiency(390). OAZ has been shown to interact with Smad1 and Smad4 proteins to promote expression of the homeobox regulator of Xenopus mesoderm and neural development, Xvent-2, from BMP response elements found within the Xvent-2 promoter(143).

. Besides coordinating receptor-Smad interactions and R-Smad-Co-Smad interactions, the MH2 domain is responsible for recruiting transcriptional activators such as p300 and CBP for transcriptional activation(99, 178, 298, 323). The MH2 domains of the R-Smads (Smad 1, Smad 2, Smad3, Smad 5) and the Co-Smad, Smad4, have all been shown to interact with all three members of the core binding factor alpha (CBFA) family of transcription factor proteins(321). Each CBFA member although structurally similar, has been shown to serve unique processes initiated by TGF-β and BMP signalling. These functions include bone formation for CBFA1 (89, 205, 286), in hematopoesis for CBFA2(286, 379) and class switching by spleenic B cells to IgA for CBFA3(324).

A third group of Smads, Smad6 and Smad7, have been identified as inhibitory Smads (I-Smads) that inhibit the activation of signal-transducing R- and Co-Smads. In studies employing xenopus, overexpression of Smad6 and Smad7 presents a similar phenotype as that produced by blocking BMP signalling. Drawing from experimental data, several mechanisms have been proposed for this inhibitory effect. I-Smads have been shown to interact efficiently with activated type I receptors and can therefore compete with R-Smads for binding to the activated type I receptor (144, 168, 271). In

addition, Smad6 has been shown to compete with Smad4 in forming heterodimers with receptor activated Smad1 thereby blocking the formation of the Smad1-Smad4 transcription complex(142). In the case of Smad7, the exact mechanism of action by which it inhibits BMP signalling is less clear, however, reports have suggested that Smad7 is more directly involved in TGFβ signalling than BMP signalling. Smad7 has been shown to interact constitutively with the homologous to E6-AP C Terminus (HECT)-domain ubiquitin ligases, and Smad ubiquitination regulatory factors 1 and 2 (Smurf1 and Smurf2) (94, 193). Smurf1 was initially identified as a Smad ubiquitin ligase which targets the Smads specific for BMP signalling for ubiquitination which is followed by degradation and hence their inactivation(413). Upon recruitment of the Smad7/Smurf complex to the activated TGFβ receptors, both Smurf1 and Smurf2 can ubiquitinate the TGFβ receptor thus targeting the receptor for degradation through proteosomal and lysosomal pathways.

4.3 Modulation of BMP signalling by Growth Factors and Cytokines

The osteoinductive properties of BMPs has been well documented, such that treatment of pluripotent mesenchymal cells and committed cells of the mesenchymal lineage with BMP2, BMP4, BMP6 and BMP7 is sufficient to induce osteogenesis(5, 139, 332, 378). Certain combinations of BMPs with other growth factors have been shown to synergistically enhance osteogenesis of marrow derived MSCs. Basic FGF (bFGF) has a synergistic effect in enhancing *in vivo* bone formation of mesenchymal stem cells with BMP-2(137, 288). In direct contrast to the synergistic coupling of bFGF and BMP2, TGFβ1 and TGfβ2 have an inhibitory effect on BMP2 stimulation of osteogenesis of

human derived MSCs. Pre-treatment of primary marrow derived MSCs with TGFβ prior to BMP treatment resulted in decreased expression of indices of the osteoblast phenotype including alkaline phosphatase, osteocalcin expression and in mineralization(112, 200). It has been postulated that the inhibitory effect of TGF-β is attributed to the limited pool of intracellular Smad4, as both TGF-β and BMP signalling requires the co-Smad, Smad4 for transcriptional activation of target genes. Due to the central role that Smad4 plays in TGF-β and BMP signalling, competition occurs for free Smad4 with the outcome of the competition determining the relative strength of BMP signalling(46). The inhibitory effects of TGF-β has been assessed against the osteoinductive properties of BMP. TGF-β has been shown to hinder BMP induced osteogenesis by downregulating CBFA1 activity. TGF-β signalling has also been shown to promote the formation of a Smad3-CBFA1 complex which is able to bind to Smad responsive elements, yet, has a limited capacity to induce transcription of CBFA1 responsive genes such as osteocalcin. This reduction in the transcriptional capacity of CBFA1 has been shown to be sufficient to curtail osteoblast differentiation(283).

Mitogen Activated Protein Kinase (MAPK) may be activated by a variety of growth factors and can regulate a variety of cellular responses including that of BMP and Smad1 signaling, however, the exact influence of MAPK on BMP signalling is unclear as conflicting reports exist regarding the role of MAPK on BMP2 induced osteoblast differentiation. Epidermal growth factor (EGF) acts by binding to its cognate receptor tyrosine kinase thereby initiating a signalling cascade that leads to activation of MAPK. The osseous cell line CFK1 proved to be susceptible to EGF inhibition of BMP2 induced osteogenesis as a result of phosphorylation of Smad1(32). In contrast to this inhibitory

model, osteoblastic differentiation of MC3T3-E1 cells or the transdifferentiation of myoblastic C2C12 cells appears to require MAPK for differentiation to take place as the inhibitors of MAPK signalling PD098059 and U0126 limited the osteoinductive effects of BMP2(116, 395). In addition to the regulatory effects of MAPK activity on BMP2 signaling, BMP2 treatment of pluripotent mesenchymal C3H 10T½ cells not only initiates osteoblast commitment and differentiation, but also induces MAPK expression and activation which appears essential for osteoblast differentiation(232).

Activated MAPK may also limit the activity of BMP signalling effectors as MAPK has been shown to phosphorylate a consensus PXSP sequence in the linker region of Smad1 thereby blocking its nuclear accumulation(209). By limiting the nuclear accumulation of Smad1, the transcriptional machinery for BMP induced signalling is thereby restricted from performing its function.

4.4 Bone Morphogenetic Protein and Mesenchymal Stem Cell Commitment

The extracellular concentration of BMPs plays a role in defining cell fate in a variety of biological systems. In Xenopus, Drosophila and sea urchin, BMPs form an active gradient during embryogenesis *in vivo* thus determining cell fate that is defined by local BMP concentrations(13, 69, 385). BMP concentration also appears to be important for mesenchymal stem cell commitment. More specifically, BMP2, BMP4 and BMP7 have been shown to induce both osteogenesis and adipogenesis of pluripotent mesenchymal cells *in vitro*, however commitment to a specific lineage is dependent on BMP concentration (5, 16, 55, 184, 280, 354, 376). Higher concentrations of BMP have been shown to enhance osteogenesis, whereas lower concentrations favours adipogenesis.

One report has suggested that it is not the effective concentration of BMP that determines the lineage selection by MSCs, but rather signalling by specific type I BMP receptors that determines either adipogenesis or osteogenesis. Pluripotent mesenchymal 2T3 cells are able to undergo both adipogenesis and osteogenesis following BMP2 treatment. By generating 2T3 cells stably expressing a dominant negative form of the BMP type IB receptor (trBMPR-IB) that lacks kinase activity, osteogenesis was inhibited following treatment with BMP2. When 2T3 cells were stably transfected to express a constitutively active form of BMP receptor type IB (caBMPR-IB) induced osteogenesis was observed with no adipogenesis. Conversely, when 2T3 cells were stably transfected with a dominant negative form of the BMP receptor type IA, (trBMPR-IA), a general inhibition of adipogenesis was observed, however, stable transfections of 2T3 cells with a constitutively active form of BMP receptor type IA (caBMPR-IA) resulted in general adipogenesis and an inhibition of osteogenesis. Taken together, this data would suggest that BMPR-IB is required for mesenchymal stem cell commitment to the osteoblastic lineage and that BMPR-IA promotes adipogenic commitment(54). Arguing against such a model is the phenotype of mice with targeted disruption of the BMPR-IB gene, generated by homologous recombination in embryonic stem cells. In spite of the lack of BMPR-IB expression throughout the developing skeleton, mice homozygous for BMPR-IB gene disruption are viable with skeletons that are similar to wild-type animals save for differences largely restricted to the appendicular skeleton as a result of a defect in proliferation of prechondrogenic cells and chondrocyte differentiation in the phalangeal region(404). These findings would suggest that BMPR-IB mediated signaling is required for distal limb patterning and is not essential for osteoblastic commitment of MSCs. The

question as to whether BMPR-IA signalling is required for adipocyte formation *in vivo* is complicated by the necessity of BMPR-IA for gastrulation during mouse embryogenesis. BMPR-IA does not appear to be required for preimplantation or for initial postimplantation development however morphological and molecular examinations demonstrated that no mesoderm formed in the mutant embryos (261).

If there is a shift in MSC commitment in the bone marrow compartment favouring adipogenesis over osteogenesis such as in cases of osteopenia, this could result from cells being more or less responsive to stimuli for osteoblast commitment and differentiation. If high concentrations of BMP have been shown to enhance osteogenesis, and low concentrations enhance adipogenesis, any change in a cells ability to respond to BMP signaling would affect the differentiation process of pluripotent mesenchymal cells. In aging bone, the capacity of cells to respond to hormonal stimuli is different, as was demonstrated in the cases of Vitamin D, growth hormone, PDGF and IGF(73, 92, 310). *In vivo*, studies of BMP implantation performed in rats of advanced age have demonstrated differential effects of BMP that are age-dependent. One such BMP implantation study was performed in the calf muscle of rats and a second was performed in the bilateral palatal grooves. Aged rats when compared to their younger counterparts demonstrated reduced alkaline phosphatase activity, calcium content and bone volume surrounding the BMP implants(33, 249). It is uncertain whether the BMP implants were inducing osteogenesis from MSCs or whether BMP implants were causing transdifferentiation of surrounding cells. Whatever the precise mechanism, it appears that the osteogenic effect of BMP in these aged animals is reduced. If pluripotent MSCs

within the bone marrow were indeed less responsive to BMP signalling, based on *in vitro* data, increased adipogenesis would have been a consequence(16, 55, 376).

5. PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR GAMMA

5.1 Discovery and Characterization

Various chemicals when administered to rats and mice produce a dramatic increase in liver weight as a result of cell division and an increase in peroxisomal volume. Peroxisomes are cell organelles that serve a role in the β-oxidation of long-chain fatty acids and the catabolism of cholesterol to bile acids(152, 364). Based on their physiological actions, these compounds were designated peroxisome proliferators. When liver cells are treated with peroxisome proliferators, a coordinate and rapid change in mRNAs encoding peroxisomal β-oxidation enzymes such as acyl COA oxidase(223) and microsomal cytochrome P450 IV enzymes occurs, suggesting that peroxisome proliferators might regulate genes in a manner similar to that of steroid like transcription factors(123, 291). By using a degenerate nucleotide sequence from the estrogen, thyroid and retinoid receptor sequences a cDNA library was screened resulting in the identification of the first peroxisome proliferators activated receptor (PPAR) nuclear receptor(173). A search for other PPAR homologues led to the cloning of the three PPAR isoforms, PPARα, PPARδ and PPARγ from a xenopus cDNA library. Each isoform has since been determined to be products of a distinctive gene(82).

The PPARγ gene is well conserved between human and mice (99% similarity and 95% identity) and is comprised of nine exons spanning over 100 kilobases in humans and 105 kilobases in mouse(96, 97) **(Figure 10)**. The PPARγ_1 and PPARγ_2 mRNAs are encoded by 8 and 7 exons respectively and are products of alternative promoter usage resulting in differing 5'-ends. The 5'-untranslated sequence of PPARγ1 is comprised of exons A1 and A2, whereas the 5' end of PPARγ_2 is comprised of exon B that provides an

Figure 10. PPARγ Gene Structure

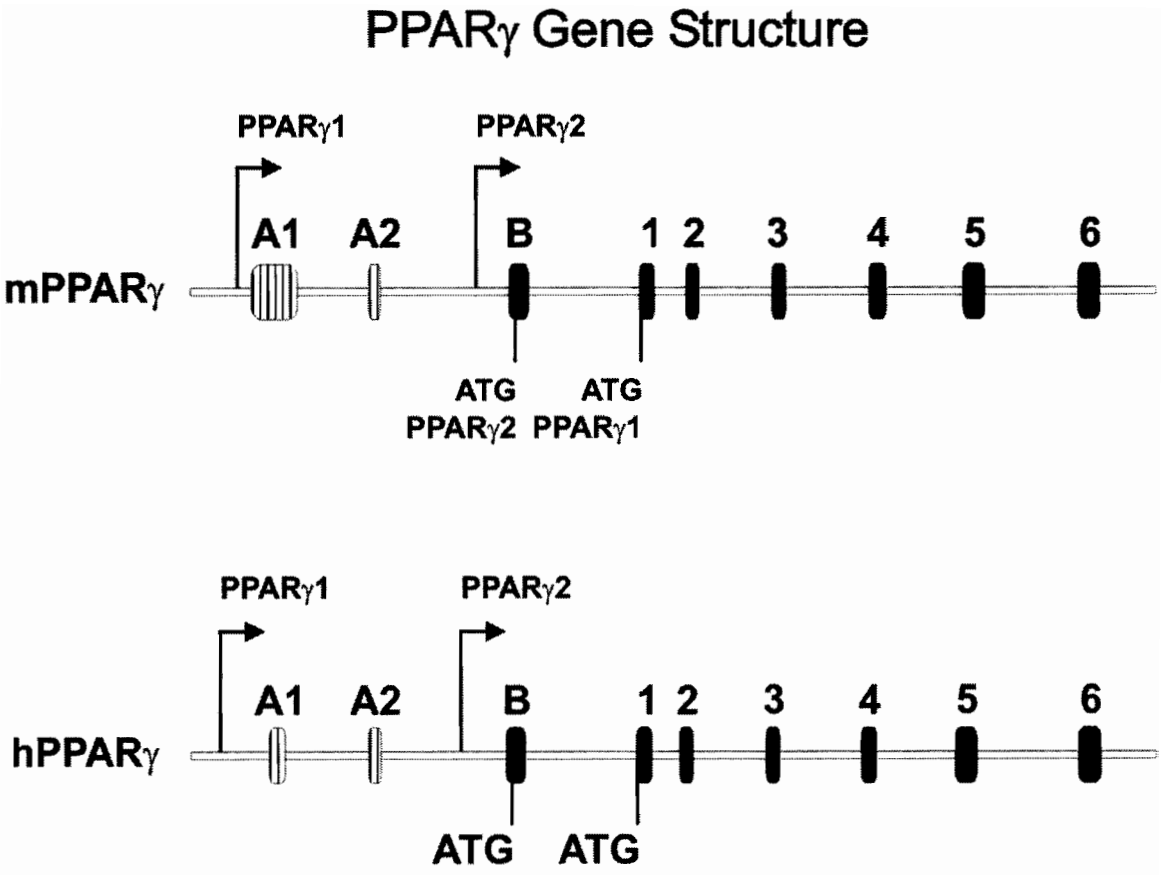


Figure 10. The PPARγ gene is well conserved between human and mice (99% similarity and 95% identity) and is comprised of nine exons spanning over 100 kilobases in humans and 105 kilobases in mouse. The PPARγ$_1$ and PPARγ$_2$ mRNAs are encoded by 8 and 7 exons respectively and are products of alternative promoter usage resulting in differing 5'-ends. The 5'-untranslated sequence of PPARγ1 is comprised of exons A1 and A2, whereas the 5' end of PPARγ$_2$ is comprised of exon B that provides an untranslated region as well as additional NH_2-terminal amino acids that are specific for PPARγ$_2$. The remaining six exons, designated 1 to 6, are common to the PPARγ$_1$ and γ$_2$ and encode PPARγ$_1$ and the majority of PPARγ$_2$. In humans, a third transcript, PPARγ$_3$, has been identified whose protein product of this mRNA is identical to that of PPARγ$_1$.

untranslated region as well as additional NH_2-terminal amino acids that are specific for PPARγ_2. The remaining six exons, designated 1 to 6, are common to the PPARγ_1 and γ_2 and encode PPARγ_1 and the majority of PPARγ_2. In humans, a third transcript, PPARγ_3, has been identified which is a product of a third promoter that is located upstream of exon A2. The protein product of this mRNA is identical to that of PPARγ_1(97).

Besides the similarities observed between the gene structure of human and mouse PPARγ, the sequence of the encoded protein is also very well conserved (99% similarity and 95% identity) with no differences in length with respect to PPARγ_2 between the human and mouse homologues(96). Human PPARγ_1 on the other hand differs from that of mouse PPARγ_1 due to a different initiation codon that results in an additional 2 amino acid residues as compared to its mouse counterpart.

The domains that are conserved amongst the various PPAR isoforms, consist of a DNA binding domain (DBD) and a ligand binding domain (LBD) **(Figure 11)**. The DBD consists of two zinc fingers that are found between amino acids 137-211 and bind specifically to PPAR response elements (PPREs) in the regulatory region of PPAR-responsive genes. The LBD is a relatively large pocket, which may allow the PPARs to interact with a broad range of structurally distinct natural and synthetic ligands, and resides in the COOH-terminal half of the receptor (277, 398). Also located in the COOH-terminus of the LBD is the ligand-dependent activation domain commonly referred to as the AF-2 domain. This region is intimately involved in the generation of the co-activator binding pocket of the receptors that allows for the recruitment of co-activators(398). A second ligand-independent activation domain has been described in the NH_2-amino terminus of both PPARγ_1 and PPARγ_2. This domain is referred to as AF-1

Figure 11. PPARγ Functional Domains and DNA Binding

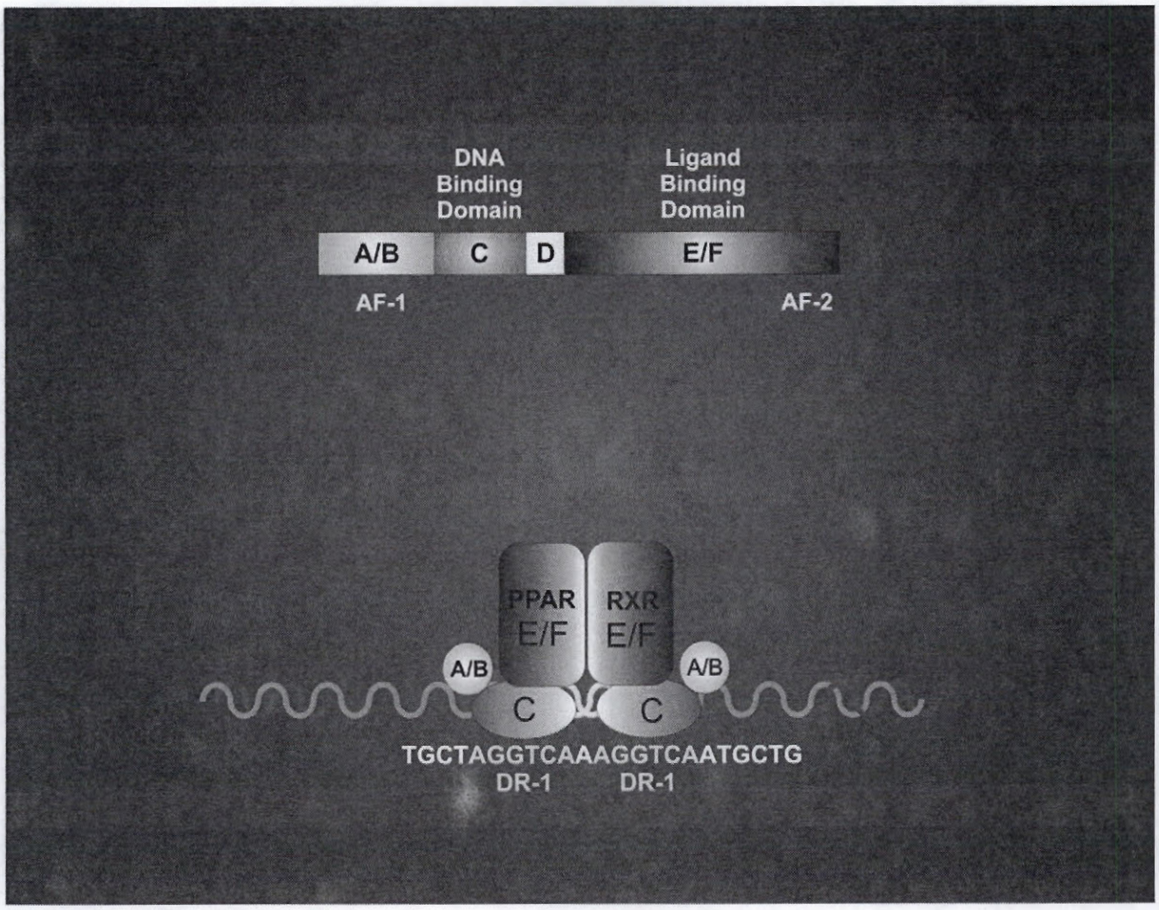


Figure 11. The various PPAR isoforms consist of a DNA binding domain (DBD) and a ligand binding domain (LBD). The DBD consists of two zinc fingers that are found between amino acids 137-211 and bind specifically to PPAR response elements (AGGTCA) in the regulatory region of PPAR-responsive genes. The LBD is a relatively large pocket which may allow the PPARs to interact with a broad range of structurally distinct natural and synthetic ligands and resides in the COOH-terminal half of the receptor. Adjacent to the LBD is the ligand-dependent activation domain referred to as the AF-2 domain. A second ligand-independent activation domain has been described in the NH_2-amino terminus of both PPARγ_1 and PPARγ_2 and is referred to as AF-1.

but differs between PPARγ_1 and PPARγ_2 with respect to their abilities to induce transcription according to reporter assays(383).

Although only small differences exist between PPARγ_1 and PPARγ_2 at the amino acid sequence level, the two isoforms differ considerably in their distribution patterns. PPARγ_1 is expressed in many tissues, whereas PPARγ_2 is expressed primarily in adipose tissue, liver and skeletal muscle. PPARγ_1 is also expressed in adipose tissue, liver and skeletal muscle and appears to be the predominant form: PPARγ_1 mRNA makes up 85% of all PPARγ mRNA, with PPARγ_2 constituting the remaining 15%(96). Cumulatively, the most prominent tissue types that express high levels of PPARγ are adipose tissue and the large intestine. Other tissues that demonstrate considerable levels of PPARγ expression include parts of the immune system, such as the spleen and Peyer's patches located in the jejunum, skeletal muscle, liver, heart and bone marrow stromal cells (18, 37, 130, 202, 245, 264, 351).

As a transcription factor, PPARγ functions as an obligate heterodimer with the retinoid X receptor (RXR) and acts by binding to PPREs that consist of direct repeat (DR)-1 elements consisting of two hexanucleotides with the consensus sequence AGGTCA separated by a single nucleotide spacer **(Figure 11)**. This heterodimer allows the formation of various protein-protein interactions with a variety of nuclear proteins known as co-activators and co-repressors, which mediate contact between the PPAR-RXR heterodimer, chromatin, and the basal transcriptional machinery, thereby promoting and repressing gene expression.

5.2 PPARγ and Adipogenesis

The currently favored hypothesis regarding adipocyte commitment involves the initial induction of PPARγ by C/EBPβ and σ (393, 394), which then initiates the adipogenic program. Adipocyte terminal differentiation then requires the concerted action of PPARγ, C/EBPα, and ADD-1/SREBP1 (199, 353). The majority of the work characterizing PPARγ as an adipogenic factor was determined using cell lines and *in vitro* differentiation assays. PPARγ$_2$ was initially identified as the trans-activating factor responsible for regulating expression of the adipocyte-specific fatty acid-binding protein, aP2. Increasing expression of PPARγ was shown to coincide with maturation of the adipocytic phenotype as PPARγ expression is upregulated during the conversion of pre-adipocytes to adipocytes. Very high expression levels of PPARγ are found in both white and brown adipose tissue(350). Overexpression of PPARγ in 3T3-L1 pre-adipocytes and NIH-3T3 fibroblasts accompanied by ligand activation is sufficient for induction of adipocyte specific gene expression as well as triacylglycerol uptake(354). Transdifferentiation of mesenchymal cell lines has also been observed as a result of ectopic overexpression of PPARγ. Murine G8 myoblasts overexpressing PPARγ$_2$ in combination with an appropriate agonist and the pro-adipogenic protein C/EBPα, induced the transdifferentiation of myoblasts to adipocytes (165).

In contrast to PPARγ overexpression studies, experiments performed with dominant negative forms of PPARγ expressed in cultured pre-adipocytic 3T3-L1 cells and cultured human pre-adipocytes demonstrate an inhibition of adipogenesis(248). PPARγ has been shown to direct the actions of many, if not most fat cell-specific genes, whereby PPARγ response elements have been identified in the regulatory sequences of

numerous adipocytic genes including lipoprotein lipase, the fatty acid transport proteins FATP and FAT/CD36, adipophilin, long chain acyl-CoA synthase, phosphoenolpyruvate carboxykinase, and liver X receptor α(214, 311).

Genetic analyses of PPARγ null mice have yielded very little information regarding PPARγ and adipogenesis due to the early lethality associated with absence of PPARγ. This lethality is attributed to defects in placental development and during the terminal differentiation stage of the trophoblast(22, 212). In an effort to bring PPARγ$^{-/-}$ mice to term, a less conventional tetraploid-rescue approach was used. This methodology produced a single PPARγ$^{-/-}$ pup that survived to term, which upon examination demonstrated a complete lack of white and brown adipose tissue(22).

Although PPARγ null mice are not viable, they have provided additional supporting evidence for a role involving PPARγ during adipogenesis. PPARγ$^{-/-}$ embryonic stem cells when cultured to undergo adipogenesis failed to differentiate into adipocytes *in vitro*(22, 310). PPARγ heterozygous null mice are viable and demonstrate a link between PPARγ gene dosage and adipogenesis. Thus, haploinsufficiency for PPARγ resulted in smaller adipocytes and resistance to a high fat diet induced adipocyte hypertrophy(212). The generation of chimeric mutant mice for wild-type and PPARγ null cells also provided evidence that PPARγ is required for adipogenesis *in vivo* as PPARγ null cells contributed little to adipose tissue despite the ability of these cells to contribute to the formation of other tissues(310).

Studies of PPARγ mouse models have been complemented by examining polymorphisms for PPARγ in humans. A rare proline→glycine^{115} mutation for PPARγ was found in 4 of 121 obese German subjects, yet was absent from 237 normal-weight

controls(307). This mutation is adjacent to a serine residue at position 114 which forms a consensus MAPK phosphorylation site and renders the consensus sequence non-functional. The consequence of phosphorylation of PPARγ by MAPK at Ser^{114} has been shown to inhibit PPARγ activity. Therefore, a mutation at Ser^{114} would lead to a PPARγ mutant that is resistant to downregulation by MAPK. These studies would support the theory that a more active PPARγ results in increased obesity(163). Further evidence exists supporting the inhibitory effect of MAPK activity on PPARγ, as another MAPK phosphorylation site at position Ser^{82} of PPARγ has been identified. Phosphorylation at this site has also been shown to limit PPARγ transcriptional activity(4, 44). In contrast to the activation mutations of PPARγ, a more common proline→$alanine^{12}$ mutation has been shown *in vitro*, to be defective with respect to DNA binding and its ability to mediate ligand-stimulated transactivation in transfected cells(75). In spite of these findings, the physiological outcome of a proline→$alanine^{12}$ is unclear as reports have shown both a predisposition to obesity(25) as well to a lower body mass index(6, 75). In other cases, no changes were detected with respect to body mass index(263, 306).

5.3 PPARγ Ligand Activation

Overexpression studies that focused on the role PPARγ plays in the terminal differentiation of pre-adipocytes as well as the transdifferentiation of fibroblasts and myoblasts, determined that ligand activation is a pre-requisite for acquiring the adipocytic phenotype. As a result, endogenous ligands for PPARγ is actively researched in order to identify markers for new adipose tissue formation and to aid in designing antagonists for PPARγ. To date, activators of PPARγ include fibrates, fatty acids and synthetic ligands,

such as the thiazolidinediones, which are all able to induce adipocyte differentiation(12, 38, 53, 106, 122, 203, 350).

The first ligands identified for PPARγ include a variety of fatty acids and their derivatives such as palmitic acid, oleic acid, linoleic acid, linolenic acid, arachidonic acid, and eicosapentaenoic acid, that have been found to bind to PPARγ, albeit at relatively low affinities (μmolar range) (398). Although serum levels for these fatty acids are within this range, it is believed that their cellular concentrations specifically in the nuclei of target cells, are likely to be too low for them to be true endogenous ligands for PPARγ. Certain eicosanoids have been shown to bind and activate PPARγ with slightly greater affinity such as 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2(304, 407). These ligands bind to PPARγ with a dissociation constant (k_D) in the low micromolar range and can activate PPARγ target genes at concentrations at or near the k_D. Unfortunately, determinations of 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 cellular concentrations cannot be made accurately as cellular concentrations are believed to be below the k_D range. Where fatty acids and eicosanoids fail to meet the criteria of a bona fide endogenous ligand, the oxidized alkyl phospholipid, hexadecyl azelaoyl phosphatidylcholine, was shown to bind to PPARγ with a K_d of ~ 40 nM and is not cross reactive with other PPAR isoforms(72). These affinities are the highest thus far reported for naturally occurring ligands and is comparable to those of synthetic ligands such as rosiglitazone which are known to be potent activators of PPARγ. Thus, oxidized low-density lipoproteins may serve as the most effective naturally occurring ligands for PPARγ activation.

Upon ligand-binding, PPARγ undergoes a conformational change, which first allows for the release of the nuclear receptor co-repressor (NCoR)(79, 281). The

conformational change also potentiates the recruitment of various co-factors that facilitate transcriptional activation. Steroid receptor co-activator 1 (SRC-1) displays histone acetyl transferase activity and serves as a prototypical co-activator by interacting in a ligand-dependent manner with multiple nuclear receptors, including PPARγ (210, 287, 328). SRC-1 also serves as a junction in the PPARγ-SRC-1 complex by interacting with another cofactor, the CREB-binding protein, CBP/p300 that also possesses histone acetyltransferase activity. CBP/p300 can interact in a ligand-dependent manner with this COOH-terminal portion of PPARγ as well as in a ligand-independent manner with amino acids 31–99 located in the A/B domain (117) **(Figure 11)**.

5.4 PPARγ and Bone

An intimate relationship between osteoblast and adipocyte biology exists within the bone marrow such that any change in total bone volume is often accompanied by a reciprocal increase in bone marrow adiposity. One hypothesis is that increased bone marrow adiposity is a result of the inappropriate differentiation or transdifferentiation of osteoprogenitors towards cells of the adipocytic lineage. As PPARγ plays a pivotal role in the commitment and differentiation of adipocytes, several studies have focused on the possibility that PPARγ may affect differentiation of osteoprogenitors and pluripotent mesenchymal cells. Osteoblastic ROS17/2.8 and SaOS-2/B10 cell lines both express PPARγ, and when maintained in culture supplemented with free fatty acids such as palmitic, oleic, and linoleic acids which are known ligands for PPARγ, they undergo conversion to adipocytes. This suggests that stimulation of PPARγ in committed pre-osteoblasts is sufficient for initiation of the adipogenic program(78). Likewise, both

primary bone marrow stromal cells and the BMS2 bone marrow stromal cell line express the PPARγ receptor. Following treatment of these cells with the synthetic ligands for PPARγ, BRL49653 and pioglitazone, increased adipocyte differentiation was observed in a dose- and time-dependent manner(126).

The influence that PPARγ exerts on adipocyte commitment and differentiation may take precedence over that of the osteoblast transcription factor CBFA1. Osteoblastic UAMS-33 cells express CBFA1 but not PPARγ$_2$. In culture, these cells express osteoblastic markers and form a mineralized matrix over time. Stable transfection of these cells with PPARγ and activation with BRL49653 not only suppressed CBFA1 expression, it rendered the cells incapable of forming a mineralized matrix. Instead, expression of markers associated with the adipocyte phenotype such as adipsin, aP2 and fat accumulation were observed. Taken together, it would appear that the effects of PPARγ supercedes those of CBFA1; that is, PPARγ exerts its influence by promoting the adipogenic phenotype and at the same time suppressing CBFA1 and the osteoblast phenotype(225).

Genetic evidence also suggests that a link exists PPARγ and osteoporosis. A silent polymorphism found at position nucleotide 161 of exon 6 of the PPARγ gene, which consists of a C→T substitution, has been shown to be associated with an increased incidence of osteoporosis(284). The mechanism by which PPARγ affects bone formation in this case, may be indirect as this silent mutation is associated with increased levels of circulating leptin, which has been shown to inhibit bone formation through a central mechanism involving the hypothalamus(86, 255).

6 CORE BINDING FACTOR ALPHA 1

6.1 Discovery and Characterization

The discovery of CBFA1 originated from a search for proteins that interact with the enhancer element of the polyoma virus promoter and its original designation was meant to reflect the functionality of the protein: polyoma virus enhancer binding protein 2αA (PEBP2αA). Since then, it has also been referred to as AML3, OSF2 and Runx2, however, CBFA1 will be used for the purposes of this introduction. CBFA1 is one of three mammalian homologues of the drosophila runt protein that are all characterized by a 128 amino acid highly conserved DNA-binding domain known as the RUNT domain(89, 188, 266, 283, 332). The three isoforms all share similar structural features which can be represented by the third isoform, CBFA1(iii) **(Figure 12)**. The RUNT domain is located at positions 107-235 of CBFA1 and serves primarily as a DNA binding motif through which CBFA1 can bind to specific stretches of DNA referred to as CBFA1 response elements. Their core recognition sequence is a (Pu-A-C-C-Pu-C-A) motif. Binding of CBFA1 to DNA allows the protein to function as either an activator or repressor of transcription based on the context of the specific promoter as well as the co-factors associated with CBFA1. The RUNT domain also serves to coordinate dimerization with CBFβ, an unrelated small protein which has no affinity for DNA itself, but has been shown to increase the DNA-binding affinity of CBFA1 (386). Specific to CBFA1 is an NH_2-terminal stretch of 29 glutamine residues in sequence followed by 18 alanine residues at positions 49-96. This is commonly referred to as the Q/A domain and has also been shown to possess transactivation found in the first 19 NH_2-terminal amino acids (AD1). These two activation domains contribute to the total transcriptional activity

Figure 12. CBFA1 Functional Domains

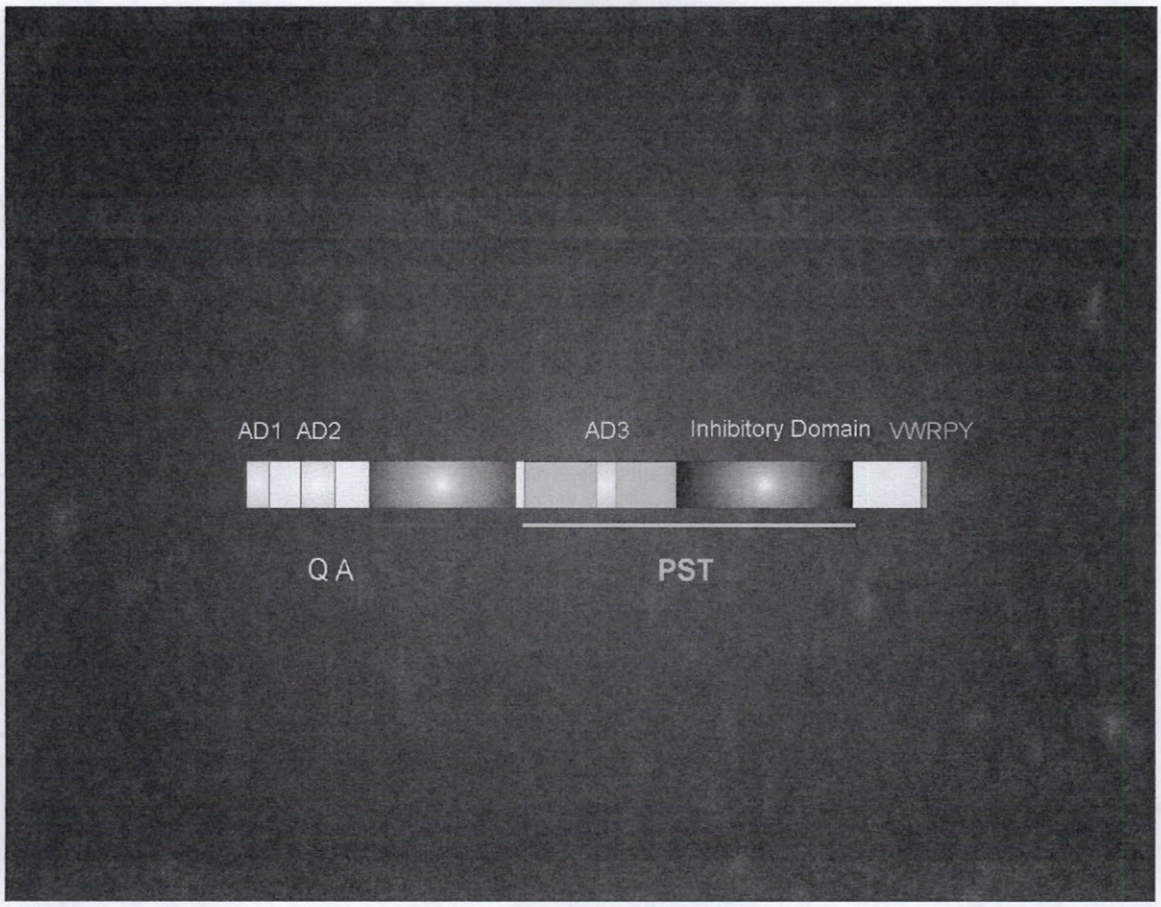


Figure 12. The three CBFA1 isoforms all share similar structural features as represented here by the third isoform. The RUNT domain is located at positions 107-235 and serves primarily as a DNA binding motif that recognizes specific stretches of DNA (Pu-A-C-C-Pu-C-A). Within the NH_2-terminal region is a stretch of 29 glutamines followed by 18 alanine residues that is referred to as the Q/A domain and has also been shown to possess transactivation potential (AD2). CBFA1 possesses a second activation domain that can be found in the first 19 NH_2-terminal amino acids (AD1). The COOH-terminal contains a proline, serine and threonine rich region known as the PST domain which also possesses an activation domain (AD3). Immediately adjacent to AD3 is a repression domain of 154 amino acids Lastly, the last five amino acids (VWRPY motif) have also been shown to act as a repressor domain by interacting with the Groucho/TLE1 class of co-repressors.

of CBFA1, however, the transactivational abilities of each domain appears to remain functional only in the context of the native protein as fusion proteins of these activation domains with different DNA binding domains demonstrate little transactivating ability(346). In examining the COOH-terminal portion of CBFA1, a proline, serine and threonine rich region extending from amino acids 258-528 is known as the PST domain. The PST domain can be further dissected into several smaller functional domains including a myc-related nuclear localization signal which borders both the RUNT domain and PST domain at positions 233-242 and a less conservative nuclear localization signal at positions 293-309. Asides from the two activation domains in the NH_2-terminus, a third activation domain has been found in the NH_2-terminal region of the PST domain from amino acid 241 to position 374 (AD3). Immediately adjacent to this activation domain is a repression domain of 154 amino acids that consist of residues 374-523(346). Finally, the last five amino acids of CBFA1 (VWRPY motif) have also been shown to act as a repressor domain by interacting with the Groucho/TLE1 class of co-repressors(15, 346).

6.2 CBFA1 and Bone Biology

The central role attributed to CBFA1 in the regulation of bone biology is a product of complementary *in vivo* and *in vitro* studies. The most recently discovered CBFA1(iii) isoform was identified as one of two key regulators for expression of the osteoblast specific gene, *osteocalcin*(88). Examination of adult mouse bone has identified expression of all three isoforms of CBFA1 in this particular tissue. Each isoform is able, although with different potencies, to increase expression levels of mRNAs related to

osteoblast differentiation in pluripotent C3H 10T½ (139). CBFA1 response elements have since been identified in the promoter region of multiple genes that contribute to the bone extracellular matrix including type I collagen, bone sialoprotein and osteopontin amongst others (89, 197). Fibroblasts and myoblasts are also susceptible to osteoblast-specific gene expression *in vitro* following overexpression of CBFA1, thereby demonstrating that CBFA1 is sufficient to initiate osteoblastic transdifferentiation of non-osteoblastic cells(89).

In addition to the evidence gathered from *in vitro* studies, *in vivo* data also supports a role for CBFA1 in bone biology. The *in vivo* expression pattern of CBFA1 coincides with and delineates the early murine skeleton . As early as E10.5, CBFA1 expression can be detected in all mesenchymal condensations before osteoblast differentiation has been initiated. It is only between E10.5 and E12.5 that CBFA1-positive cells begin to express molecular markers of chondrocyte (α1(II) collagen) and of osteoblast progenitors (α 1(I) collagen); for this reason these mesenchymal cells have been described as both osteoblast and chondrocyte progenitors. From E14.5 on, CBFA1 expression progressively increases in cells of the osteoblast lineage (both osteoprogenitors and osteoblasts) but decreases steadily in the prehypertrophic chondrocytes until it is no longer detectable in these cells at birth(169, 198, 340).

Genetic studies, however, have provided the strongest evidence that CBFA1 is a critical transcription factor for osteoblast commitment. CBFA1 maps to the same location in the mouse genome as the mutation for the skeletal dysplasia, cleidocranial dysplasia (CCD). Mice that are haploinsufficient for CBFA1 have hypoplastic clavicles and a delay in closure of the sutures of the fontanelles, two hallmarks of CCD(292). Complete

inactivation of CBFA1 in mice results in lethality at birth due to respiratory failure as a result of the absence of a rigid rib cage. Although normally patterned and of a nearly normal size, the skeleton of $CBFA1^{-/-}$ mice are entirely cartilaginous and devoid of osteoblasts and consequently bone matrix(89, 205, 292).

In humans, haploinsufficiency for CBFA1 also results in CCD. Mutational analysis of CBFA1 in CCD patients has provided insight into the mechanism of CBFA1 action. Two missense mutations have been identified, $met \rightarrow arg^{175}$ and $ser \rightarrow asn^{191}$, in the DNA-binding domain of the CBFA1, that abolishes the DNA-binding ability of CBFA1(226). Despite the phenotype of CCD, as a result of diminished levels of CBFA1, bone formation *per se* does not appear to be affected as most patients with CCD exhibit normal bone mass.

Given that CBFA1 is able to induce osteoblast-specific gene expression in non-osteoblastic cells, it is likely that CBFA1 itself may control the expression of other osteoblast-specific transcription factors such as Osx. Whether the expression of Osx is directly dependent on transcriptional regulation by CBFA1 is open to speculation, however, no functional CBFA1 response elements have been reported in the proximal promoter of the Osx gene.

CBFA1 is currently the earliest known marker of the osteoblast lineage, yet CBFA1 expression is known to precede the detection of identifiable osteoblasts by several days. These findings are in some ways a paradox, as CBFA1 is known to regulate the expression of osteocalcin, which is expressed only at the latter stages of osteoblast differentiation. This lag between CBFA1 expression and osteogenesis may be explained by several reasons. CBFA1 may regulate the expression of other transcription factors that

are required for osteoblast commitment and differentiation, or CBFA1 function is inhibited during development by posttranslational processes. Genetic analysis of patients with CCD has identified transcriptional partners which affect the ability of CBFA1 to induce osteoblast differentiation. A novel CBFA1 nonsense mutation referred to as CCDalphaA376, results in a truncated CBFA1 protein that functions as a dominant negative(228). Although CBFA1 is able to efficiently bind DNA, this premature termination affects 2 aspects of CBFA1 function. The first involves the subcellular localization of CBFA1, where the deletion results in the inappropriate nuclear localization of CBFA1. The second involves the loss of the SMAD interacting domain from CBFA1 thereby limiting its transactivation potential and its ability to transdifferentiate myoblasts(228, 265, 412). It would therefore appear that the COOH-terminus of CBFA1 serves multiple roles in the osteogenic determining potential of CBFA1.

Core-binding factor β (CBFβ), (also referred to as polyomavirus enhancer binding protein 2β (PEBP2β)) is a non-DNA-binding protein which dimerizes with CBFA1 thereby allowing for efficient DNA binding of CBFA1 and for CBFA1-dependent transcriptional activation. Although mice deficient in CBFβ die at midgestation owing to an absence of fetal liver hematopoesis and hemorrhaging in the central nervous system (264, 287, 371), transgenic rescue of CBFβ deficient animals was accomplished by introducing a 7.7-kb Gata1 promoter that directs CBFβ expression to primitive and definitive erythroid cells allowing the mice to survive until birth. However, these mice demonstrated dwarfism with shortened limbs and died soon after birth from respiratory failure(405). Although mesenchymal cells were shown to differentiate into immature

osteoblasts, intramembranous bones were poorly formed. The maturation of chondrocytes into hypertrophic cells was also markedly delayed, and no endochondral bones were formed. Primary calvarial cells from $CBF\beta^{-/-}$ mice demonstrated little alkaline phosphatase activity and neither osteocalcin expression nor mineralization was observed. When CBFβ is re-introduced into $CBF\beta^{-/-}$ calvarial cells by retroviral infection alkaline phosphatase activity, osteocalcin expression and mineralization is restored thereby demonstrating that CBFβ is required for the proper functioning of CBFA1 in skeletal development as well as osteoblast differentiation. A similar study examining the function of CBFβ involved a gene replacement-knock-in strategy that substituted a CBFβ-GFP fusion protein in place of wild-type CBFβ. $CBF\beta^{GFP/GFP}$ pups died within the first day after birth with a phenotype similar to but less severe than that of CBFA1with a delay in endochondral and intramembranous ossification as well as in chondrocyte differentiation(213).

Several consensus phosphorylation motifs have been identified in CBFA1 which have been shown to play a role in regulating CBFA1 activity. MAPK stimulation is a product of numerous stimulatory events and its activity has been shown to facilitate osteoblast commitment(176). CBFA1 has also been shown to be phosphorylated by MAPK *in vitro*, at a consensus phosphorylation site, resulting in an increase its transcriptional activity(396). Protein kinase A (PKA) signalling has also been implicated in CBFA1 biology. PTH stimulation of the osteosarcoma cell line, UMR 106-01 resulted in stimulation of adenylyl cyclase and cAMP accumulation. cAMP acts as a secondary messenger that activates PKA, which may phosphorylate CBFA1 at ser^{346} in the AD3 domain resulting in increased activity(321). In contrast to these findings, cAMP and

cAMP analogues were shown to inhibit differentiation of osteoblastic MC-3T3-E1 cells. cAMP accumulation in MC-3T3-E1 osteoblastic cells was associated with a decrease in CBFA1 DNA binding as well as well as a reduction in the expression of CBFA1-regulated osteoblast-specific genes including osteopontin, type I collagen, bone sialoprotein, and osteocalcin(347). The reduction in CBFA1 protein levels was found to be mediated in part by proteolytic degradation involving a ubiquitin/proteosome-dependent mechanism which had been previously described for PTH stimulation of adenylyl cyclase and cAMP accumulation(268). Although these findings would suggest divergent roles for cAMP and PKA activation for CBFA1 activity, it is conceivable that under specific circumstances, PKA signalling could modulate both transcriptional activity as well as CBFA1 degradation.

Protein-protein interactions have also been shown to contribute to the overall transcriptional activity of CBFA1. To date, only inhibitory co-factors have been identified. TLE2 is the mammalian homologue of the Drosophila melanogaster protein Groucho and is expressed in osteoblasts. TLE2 exerts a repressive effect on the transactivation function of CBFA1 by interacting with the terminal 5 amino acids of CBFA1 (VWRPY)(346). The VWRPY domain does not only interact with TLE2 but appears to be a preferred motif for co-repressors as demonstrated by the affinity of HES1 for the same amino acids as well as for the PST domain. HES1 is a basic helix-loop-helix protein that can bind to CBFA1 through the PST repressor and VWRPY domain, thereby inhibiting CBFA1 function(253). Because both the TLE and HES bind to the same region of CBFA1, competition can occur between both proteins for binding to CBFA1 as these factors are usually co-expressed with CBFA1.

Regulation of CBFA1 may also occur by blocking access to its respective DNA response elements. The zinc-finger protein AJ18 has been recently shown to bind to the same consensus sequence as CBFA1(182). As a result, AJ18 can modulate the activity of CBFA1 by acting as a transcription factor agonist via competition for binding to CBFA1 response elements.

6.3 CBFA1 and Chondrocyte Hypertrophy

CBFA1 is expressed early in development (E12.5) in cells that demonstrate both osteoblastic and chondrocytic traits. In mice, the pattern of CBFA1 expression from E12 to birth in mice in cartilage is restricted to prehypertrophic and hypertrophic chondrocytes(340). This restricted expression pattern of CBFA1 during development would suggest that CBFA1 serves a role in the transition of pre-hypertrophic chondrocytes to a more differentiated hypertrophic state. Studies of mice deficient for CBFA1 partially support this hypothesis as hypertrophic chondrocytes are absent in some skeletal elements(169, 198). Because CBFA1 expression decreases in chondrocytes during gestation, a strategy involving the targeted overexpression of CBFA1 in nonhypertrophic chondrocytes was employed. CBFA1 under the transcriptional control of the α1 type II collagen (coll II) promoter maintains CBFA1 expression in nonhypertrophic chondrocytes throughout development(340). In these animals, premature chondrocyte hypertrophy and ectopic hypertrophic chondrocyte differentiation was detected in skeletal elements where it normally never occurs(340).

Crossing of the CBFA1 transgenic (coll II) animals with CBFA1-deficient mice results in a partial rescue of the CBFA1 deficient mice. On this background, the transgene

restored chondrocyte hypertrophy and vascular invasion in the bones of the mutant mice but did not induce osteoblast differentiation (340). Taken together, these studies indicate that in addition to functioning as an osteoblast differentiation factor, CBFA1 also serves a function in regulating chondrocyte hypertrophy.

6.4 Regulation of CBFA1 Expression

In view of the fact that CBFA1 plays a central role in osteoblast differentiation, the identification of transcription factors that regulate CBFA1 expression is paramount to understanding the mechanism of osteoblast commitment and differentiation. Studies of the CBFA1 promoter have, however, yielded very few candidate transcription factors. To date, only CBFA1, the vitamin D receptor and both estrogen receptor α and β have been shown to interact with the CBFA1 promoter and to directly control its expression(83, 84, 356). Within the promoter region of the rat CBFA1 gene there are at least three CBFA1 recognition motifs. Forced expression of CBFA1 protein results in downregulation of CBFA1 promoter activity demonstrating autoregulation by negative feedback upon its own promoter. The proximal promoter sequences (-92 to -16) of the CBFA1 gene contains a functional vitamin D-responsive element (VDRE) that binds a vitamin D receptor/retinoid X receptor heterodimer. Reporter assays in MC 3T3-E1 and ROS 17/2.8 cells demonstrate decreased promoter activity from cells following treatment with $1,25(OH)_2D_3$. Mutation of the VDRE completely abolished responsiveness of the CBFA1 promoter to $1,25(OH)_2D_3$ treatment suggesting that $1,25(OH)_2D_3$ mediates suppression of CBFA1 expression(84).

Although no classical estrogen-response element (ERE) binding sites were identified in the human CBFA1 proximal promoter region, ER alpha and ER beta have been shown to regulate gene activity from AP1 response elements in the CBFA1 promoter. In examining the effects of selective estrogen receptor modulators (SERMs) on human CBFA1 gene promoter activity, three characterized SERMs, tamoxifen, raloxifene, and ICI 178,180, all upregulated CBFA1-luciferase gene activity in a dose-dependent manner(356).

Animal genetic models have also provided evidence of several candidate genes that act upstream of CBFA1. Msx2 is a homeobox containing transcription factor that is expressed in osteoblasts during development. Msx2 when inactivated in mice results in severe skeletal abnormalities associated with a decrease in CBFA1 expression suggesting that it could act upstream, directly or indirectly of CBFA1(319).

Bapx1 is another homeobox containing protein that is required for axial skeleton formation. Bapx1-deficient mice demonstrate decreased expression of CBFA1 in the axial skeleton, suggesting that Bapx1 is another activator of CBFA1 expression, however, this time in the prospective vertebral column(358).

To date, only one gene has been identified *in vivo* to inhibit CBFA1 expression during development. Inactivation of *Hoxa-2*, a homeobox-containing protein, results in ectopic CBFA1 expression and ectopic bone formation in the second branchial arch(190).

Some secreted molecules have also been proposed to regulate CBFA1 expression. Indian Hedgehog (Ihh) was shown to be required for osteoblast differentiation and to be involved in the control of CBFA1 expression. In addition to defects in chondrocyte differentiation, Ihh-deficient mice have intramembranous but no endochondral

ossification, which was shown to be associated with the absence of CBFA1 expression(333). Because Ihh is not expressed in skeletal elements that form through intramembranous bone formation, osteoblast differentiation occurs normally in these structures. This would suggest that CBFA1 expression is differentially regulated in intramembranous and endochondral bone. Furthermore, the position of CBFA1 relative to Ihh in the signalling scheme varies during endochondral bone formation. CBFA1 can act upstream of Ihh in the control of chondrocyte hypertrophy but downstream in the control of osteogenesis.

Consequently, the regulation of CBFA1 expression does not appear to be regulated in a linear fashion by an orderly defined set of genes. Instead, CBFA1 expression appears to be regulated by different transcription factors and secreted molecules that are context dependent and specific to the different skeletal structures.

Chapter 2

PTHrP inhibits adipocyte differentiation by down-regulating PPAR gamma activity via a MAPK-dependent pathway

ABSTRACT

We examined the capacity of PTHrP to modulate the terminal differentiation of the pre-adipocytic cell line, 3T3-L1. These cells express endogenous PTHrP and its receptor, but expression levels were undetectable following differentiation into mature adipocytes. Cells stably overexpressing PTHrP failed to differentiate when induced to undergo adipogenesis and proliferated at a faster rate. MAPK activity was elevated in PTHrP transfected 3T3-L1 cells and treatment with the PKA inhibitor H-8 decreased this activity. Inhibition of MEK with PD098059 permitted the terminal differentiation of the PTHrP transfected 3T3-L1 cells to proceed. Although PPARγ gene expression levels remained relatively constant in the PTHrP transfected cells, PPARγ phosphorylation was enhanced. Furthermore, the capacity of PPARγ to stimulate transcription in the presence of troglitazone was diminished by PTHrP. Expression of the PPARγ regulated adipocyte specific gene aP2 transiently rose and then fell in the PTHrP transfected cells. These results indicate that PTHrP can increase MAPK activity in 3T3-L1 cells via the PKA pathway thereby enhancing PPARγ phosphorylation. This modification can inactivate the transcriptional enhancing activity of PPARγ and diminish expression of adipocyte specific genes. These studies therefore demonstrate that PTHrP may inhibit the terminal differentiation of pre-adipocytes and describes a molecular pathway by which this action can be achieved.

INTRODUCTION

Osteoblasts, the principle bone forming cells, and adipocytes are believed to originate from the same pluripotent bone marrow stromal stem cells, and their relative numbers in bone are ultimately determined by the number of stem cells that commit to each of these lineages. (30, 256) Signals for cell fate determination therefore regulate whether pluripotent stem cells pursue one specific differentiation program relative to another. One hypothesis is that the increase in bone marrow adiposity observed in many forms of osteoporosis arises as a consequence of a shift in the differentiation program of the common precursor cell. Such a preferential differentiation process is detrimental, for it leads to a reduction in functional osteoblasts, decreased bone formation and ultimately the osteopenic state.

Evidence also exists that the relationship between adipocytes and osteoblasts may extend beyond that of simply sharing the same precursor. Committed adipocytes and osteogenic cells, for example, exhibit a form of plasticity that allows for transdifferentiation between the two cell types. Thus, after cells have assumed an adipogenic phenotype, they are capable of reverting to a more immature state and pursue an osteogenic fate(27). Furthermore, primary osteogenic cultures can undergo adipogenic differentiation when treated with glucocorticoids or thiazolidinediones, which activate the glucocorticoid receptor and the receptor for the adipocyte master differentiation factor, PPARγ respectively(280). Alternatively, when the same osteogenic cells are treated with 1,25-dihydroxyvitaminD_3 (1,25$(OH)_2D_3$), the cells resist adipogenesis, and an increase in the expression of phenotypic markers of bone, i.e. osteocalcin, type I collagen and

alkaline phosphatase is observed. It is therefore critical to understand the molecular switches regulating cell fate determination within the bone marrow microenvironment.

Parathyroid hormone (PTH) and parathyroid hormone related protein (PTHrP) have empirically been shown to have potent anabolic effects on bone. Although presently, there is little understanding of the factors that tend to favor these anabolic effects, by careful selection of the dose and pattern of administration, these agents stimulate bone formation in adult and aged animals of either sex, and in animals with osteopenia induced by disuse, denervation, and immobilization (for review see (303)and references therein). On the other hand, we have observed that young heterozygous mice carrying a targeted PTHrP null allele display reduced PTHrP expression in bone and a premature form of osteoporosis characterized by decreased trabecular bone volume and increased bone marrow adiposity(9). Given that osteoblasts and adipocytes originate from the same pluripotent stem cells, (131, 295) the increased number of adipocytes observed in the bone marrow of these mice could be the result of pluripotent mesenchymal cells committing to the adipocytic lineage with a concomitant decrease in osteoblastogenesis, as a consequence of PTHrP haploinsufficiency within the skeletal microenvironment.

We show here that PTHrP and the PTH/PTHrP receptor are expressed in cells of the adipocytic lineage and that PTHrP signaling by the cAMP-dependent protein kinase A (PKA) enhances mitogen activated protein kinase (MAPK) activity leading to phosphorylation of PPARγ, the master regulator of adipocyte differentiation and thereby repression of the adipogenic differentiation program.

These studies, therefore, identify inhibition of adipogenesis within the bone marrow as a novel mechanism for at least part of the anabolic action of PTHrP, PTH and their analogues in bone and in the treatment of osteoporosis.

MATERIAL AND METHODS

Cell Culture-The 3T3-L1 cell line was obtained from ATCC and cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), with fresh medium being applied every second day. To induce adipocytic differentiation of 3T3-L1 cells, cells were allowed to grow to confluence followed by treatment with DMEM-10% FCS supplemented with 0.5 M isobutylmethylxanthine (IBMX), 1 μM dexamethasone, and 5 μg/ml insulin. In preliminary experiments, periods of induction from 18 to 48 hours were assessed. Inasmuch as no increase in differentiation was observed in our system after 18 hours of induction, this time was employed in subsequent studies. Following 18 hours of exposure to differentiation medium, cells were subsequently cultured in DMEM-10% FCS supplemented with 5 μg/ml insulin which was changed every second day. In some experiments, forskolin was applied at a concentration of 100 nmoles per ml of differentiation medium and was reapplied at the same concentration of culture medium.

The C3H10T½ cell line was obtained from ATCC and cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), with fresh medium being applied every second day. To induce adipocytic differentiation of C3H10T½ cells, cells were allowed to grow to confluence followed by treatment with alpha minimum essential medium (αMEM)-5% FCS supplemented with 100 μg/ml ascorbic acid, 5mM β-glycerophosphate and 100 ng/ml of bone morphogenetic protein 2

(Genetics Institute, Cambridge, Massachusetts) with fresh medium being applied every three days.

For the proliferation assay, 3T3-L1 cells were plated in triplicates at an initial density of 10,000 cells per well in 6-well plates and then trypsinized and counted every second day for 6 days, once again at day 10 and then again on day 14. For treatment with PD098059 (Sigma), differentiation and post differentiation medium was supplemented with 20 nmoles of PD098059 per ml of medium. The PKA inhibitor H8 (10 nmole per ml of culture medium) and the protein kinase C (PKC) inhibitor chelerythrin chloride (5 nmole per ml of culture medium) were added for a period of 24 hours to sub-confluent cells prior to lysis.

Oil Red O Staining-Cells were washed twice with PBS then fixed for 30 minutes with 10% formalin. Oil red O stain (5% oil red 0 in 70% pyridine) was applied for 30 minutes, and cells were then washed three times with PBS.

Vectors and transfections-The PTHrP/pCDNA3 plasmid was constructed, as previously described. (149) 3T3-L1 cells were stably transfected with 5 μg of either PTHrP/pCDNA3 or pCDNA3 (Invitrogen) plasmid DNA, using the Bio-Rad Gene Pulser (0.2 kV and 960 μF). Cells were allowed to recover for 36 hours and selection for stable transformants was accomplished using 400 μg/ml of Geneticin (Gibco BRL). For subsequent experiments, populations of stably transfected cells were used. C3H10T½ cells were stably transfected with 1 μg of either PTHrP/pCDNA3 or pCDNA3 plasmid DNA using FUGENE6 reagent (Boehringer Mannheim). Stable transfected cells were selected using 400 μg/ml of Geneticin. COS7 cells were stably transfected with the cDNA of the PTH/PTHrP receptor cloned into the expression plasmid pCDNA3.1

(Invitrogen) kindly provided by Dr. Geoff Hendy. Transfection was performed using FUGENE6 reagent (Boehringer Mannheim). Cells were then selected for stable transformants with 400 μg/ml of Geneticin.

RT-PCR and Northern Blot Analysis-Total RNA was isolated by a variation of the CsCl method, as previously described. (241)

For reverse transcription of RNA, 100ng of total RNA in 10 μl of DEPC H_2O was used as template. RNA was denatured in the presence of 1mM oligo(dT) for 5 minutes at 80°C and then allowed to cool to room temperature. To the reaction, 6 μl 2.5 mM dNTPs, 6 μl of first strand buffer, 4 μl DTT (100mM), 0.5 μl BSA (5 μg/ml), 1.0 μl RNasin inhibitor (Promega) and 1.0 μl Superscript Polymerase (Boerhinger Manneheim) were added. The mixture was incubated at 40°C for 1 hour and then amplified in *Ready to Go,* PCR tubes (Pharmacia) for 45 cycles (melting 94°C, annealing 60°C, extension 72°C, each for 30 seconds followed by an extension cycle at 72°C for 7 minutes). Primers for PTHrP (5'-TAC AAA GAG CAG CCA CTC-3' and 5'- GAT CCC AAT GCA TTT ACA GT-3', forward and reverse, respectively) and PTH/PTHrP receptor (5'-TGG TGA GGT GCA GGC AGA GAT TAG-3'and 5'-AAA CAC TGG CTT CTT GGT CCA TC-3', forward and reverse, respectively) were designed to span splice sites in their respective cDNAs. The amplification products for PTHrP were fractionated on agarose gel and subjected to Southern blot analysis using as probe an internal oligonucleotide (5'-GGA CTC GGT CTG CCT GGC CAG G-3') end labeled with ^{32}P, which complements the expected RT-PCR product.

For Northern blot analysis, 6 μg of total RNA per sample was electrophoresed through a formaldehyde/agarose gel. (241) The RNA was transferred to Bio-Trans nitrocellulose

membrane and probed with an internal EcoRI fragment of PPARγ cDNA and the full-length cDNA of aP2, both labeled by the random priming method. (98)

Immunoprecipitation and Immunoblotting-Cells were lysed in 50mM Tris/HCI (pH7.5), 1mM EDTA, 1mM EGTA, l0mM sodium glycerophosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1% Triton X-100, 0.1% β-mercaptoethanol and 1mM sodium vanadate with the protease inhibitors leupeptin (2 μg/ml) and PMSF (100 μg/ml). 20 μg of total cell lysate was incubated with 2.5 μg of monoclonal anti-PPARγ antibody (Research Diagnostics Inc.) in 100 μl of PBS at 4°C overnight. The sample was then incubated in 50 μl of protein A sepharose for 4 hours, washed with immunoprecipitation buffer (50mM Tris-HCI pH 7.5, 0.1% Triton X-100, l50mM NaCl), boiled in 60 μl loading buffer and 5 μl aliquots were used for loading. Proteins were electrophoresed through a 10% SDS/polyacrylamide gel and transferred at 45V for 16 hours to Bio Trans nitrocellulose membranes. After blotting with either the anti-PPARγ antibody or a monoclonal anti-phosphoserine antibody (Gibco BRL), detection was performed using the ECL chemiluminescent kit (Boerhinger Manneheim).

For phosphorylated MAPK and actin detection, 20 μg of cell lysate was similarly analyzed using anti-phosphorylated p42/44 (NEB) and anti-actin (Amersham) antibodies.

PKA Assay- Cells were plated at an initial density of 50000 cells per 35 mm plate and allowed to grow to 80% confluence. Cells were then cultured in serum free medium overnight and the next day, were incubated with fresh serum free DMEM with or without PTHrP 1-34 ($1x10^{-7}$M) for 5 minutes. The cells were then lysed and assayed for PKA activity using a PKA Assay Kit (Upstate Biotechnology) in which ^{32}P is

incorporated into Kemptide (Upstate Biotechnology) by PKA. Results were obtained from six independent samples and their means and standard deviations were determined. Fisher's test was performed to determine P value.

MAPK Assay-MAPK activity assays were conducted using cell lysates from pCDNA3 and PTHrP transfected 3T3-L1 cells by measuring ^{32}P incorporation into a MAPK specific substrate from the Biotrak p42/p44 MAP kinase enzyme assay system (Amersham). Three readings were taken per determination and their means and standard deviations were determined. Fisher's test was performed to determine the p value.

PPARγ Luciferase Assay-The luciferase assay was performed by cloning the PPARγ response element from the aP2 promoter (349)in the luciferase vector pXP2. COS7 cells stably transfected with the PTH/PTHrP receptor were plated at a density of 75,000 cells per 35mm plate and transiently transfected with PPARγ2 cDNA in the expression plasmid pSVSPort, (353) with the aP2 luciferase reporter, and a β-galactosidase reporter plasmid to determine transfection efficiency. All transfections were performed using FUGENE6 reagent. Two days following transfection, the cells were serum deprived for 4 hours then treated with or without l00 μM troglitazone and with or without PTHrP (1-34) ($1x10^{-6}M$). Two hours after the first treatment, the cells were treated once again with PTHrP (1-34) ($1x10^{-6}M$) due to the high degrading activity of COS7 cells. Fifteen minutes after the second treatment, the cells were lysed. Luciferase activity was assessed using the Promega luciferase detection assay. All luciferase assay readings were performed in triplicate and were corrected for β-galactosidase expression levels in each cell population.

RESULTS

Expression of PTHrP and its receptor in 3T3-L1 preadipocytes-First, we examined whether PTHrP and the PTH/PTHrP receptor are expressed in 3T3-L1 preadipocytes. We selected these cells because although they are committed to the adipocytic lineage, they require further stimulation to terminally differentiate into adipocytes. We induced the differentiation of these cells and isolated total RNA at three different time points, day 0 (uninduced cells), day 3 (induced cells), and day 14 (post differentiated cells). RNA from 3T3-L1 cells transfected with PTHrP cDNA was used as a positive control. Total RNA was subjected to RT-PCR and examined for the presence of PTHrP and PTH/PTHrP receptor transcripts. The RT-PCR products of PTHrP and its receptor were verified by probing with a radiolabeled oligonucleotide specific for the PTHrP product and by sequencing the amplified receptor product, respectively.

PTHrP mRNA was detected prior to differentiation (day 0) by RT-PCR. Two weeks following induction (day 14), PTHrP transcript levels were undetectable by Southern blot analysis of the RT-PCR products (Fig. la). PTH/PTHrP receptor mRNA was shown to follow a similar pattern, with expression observed prior to induction (day 0), but not after differentiation (day 14) of 3T3-L1 adipocytes (Fig lb). Therefore, expression of both PTHrP and its receptor were coordinately decreased during progression of the adipocyte differentiation program.

Determination of Immunoreactive PTHrP-PTHrP was determined in cell conditioned medium using a two site immunoradiometric assay (Nichols Institute Diagnostics). The detection limit of the assay is 4.51 pg/ml.

Characteristics of PTHrP transfected 3T3-L1 cells-Stable PTHrP transfectants were generated in order to maintain delivery of intact PTHrP to 3T3-L1 cells. In contrast to untransfected cells, 14 days after induction PTHrP protein was still detected in conditioned medium by radioimmunoassay at levels of 16.7±.8 pg/ml (mean±SE of triplicate determinations). PTH/PTHrP receptor mRNA levels were also present in transfected cells (Fig 1c) in contrast to untransfected cells, and were sustained throughout the culture period. Furthermore, PTHrP stimulated substantial PKA activity (Fig. 1d) demonstrating the functional integrity of the receptor. As shown in Fig. 2, PTHrP transfected cells proliferated at a higher rate than control 3T3-L1 cells.

PTHrP inhibits differentiation towards the adipocytic lineage-To determine whether PTHrP might also alter the differentiation program of preadipocytes, PTHrP transfected 3T3-L1 cells and control cells were induced to differentiate, as described in *Materials and Methods*. After two weeks of treatment, differentiation was assessed by staining of lipid droplets with oil red. As shown in Fig. 3a, the control pCDNA3/3T3-L1 cells differentiated readily and intense staining of fat cells that had accumulated large amounts of lipid was observed. In contrast, differentiation in PTHrP/3T3-L1 cells was barely detectable, as demonstrated by the low intensity of staining for lipid accumulation (Fig. 3b).

To provide further evidence for the influence of PTHrP, the effects of PTHrP were also examined in the pluripotent mesenchymal cell line C3H10T½. PTHrP and pCDNA3 stable transfected cell lines were generated and induced to differentiate with 100 ng/ml of BMP2. Members of the BMP family including BMP2 have been shown to induce adipogenesis in a variety of pluripotent mesenchymal cell lines including the C3H10T½

(5, 16, 376). Stable Transfection of these cells with PTHrP also inhibited the capacity of these cells to differentiate into adipocytes when compared to control cells (Fig. 4).

MAPK activity in PTHrP/3T3-L1 cells-The increased proliferative capacity of the PTHrP transfected 3T3-L1 cells suggested that PTHrP expression might lead to activation of MAPK in these cells. To assess the degree of MAPK activation, a monoclonal antibody recognizing dually phosphorylated MAPK (Thr 202/Tyr 204) was employed on whole cell lysates from both control and PTHrP transfected cells. A 5-fold increase of activated MAPK (determined by densitometric scanning) was observed in PTHrP/3T3-L1 cell lysates as compared to pCDNA3/3T3-L1 control cell lysates (Fig. 5).

PTHrP activates MAPK through a PKA dependent pathway-PTHrP binds to its G-protein coupled PTH/PTHrP receptor thereby activating both protein kinase A (PKA) and protein kinase C (PKC) intracellular signal transduction pathways. (3, 39) To determine if there is a preferred pathway for signaling to MAPK via the PTHrP G-coupled receptor in 3T3-L1 cells, we treated PTHrP transfected cells with either H8 or chelerythrin chloride to inhibit PKA or PKC signaling, respectively. (150, 153) Following treatment for 24 hours with the respective inhibitors, cells were lysed and assayed for MAPK activity by measuring ^{32}P incorporation into a MAPK-specific substrate. Inhibition of PKC by chelerythrin resulted in a very slight increase in MAPK activity. In contrast, inhibition of PKA by H8, caused a significant (39%) decrease in MAPK activity (Fig. 6a). These results suggest that PTHrP is able to stimulate PKA activity in 3T3-L1 cells and the increase in MAPK activity by PTHrP is through a PKA dependent pathway.

Different iation *of 3T3-L1 cells with Forskolin*-To further determine whether the ability of PTHrP to inhibit adipocyte differentiation can be attributed to PKA signaling, 3T3-L1 cells were induced to differentiate with the adipocyte differentiation medium with and without the addition of the PKA activator. Two weeks following induction, the cells were fixed and stained with Oil Red O. 3T3-L1 cells treated with forskolin resisted terminal differentiation as assessed by the lack of oil red O staining (Figure 7), providing further evidence that activation of PKA by forskolin is able to inhibit the terminal differentiation of 3T3-L1 pre-adipocytes.

Differentiation of PTHrP/3T3-L1 cells with PDO98059-If excessive MAPK signaling is responsible for the inhibition of differentiation by PTHrP, then inhibition of MAPK signaling should enhance the differentiation potential of PTHrP/3T3-L1 cells. We therefore induced adipocyte differentiation in the presence of the MAPK kinase (MEK) inhibitor PD098059 that blocks the phosphorylation and activity of p42 and p44 MAPK isoforms. When PTHrP transfected 3T3-L1 cells were induced to differentiate in the presence of PD098059, they were able to terminally differentiate into adipocytes (Fig. 8a), whereas DMSO treated control cells remained resistant to differentiation (Fig. 8b). This data would therefore suggest that activation of the MAPK cascade is involved in the pathway leading to inhibition of adipogenesis by PTHrP.

PTHrP alters the phosphorylation status of PPARγ-PPARγ has a consensus MAPK site (PASP) and its transcriptional activity is downregulated upon phosphorylation by MAPK. (163) Therefore, we next examined the phosphorylation status of PPARγ in PTHrP transfected and control cells, to determine whether PPARγ phosphorylation was altered by PTHrP expression. Whole cell lysates were isolated from both PTHrP

transfected and control 3T3-L1 cells, and PPARγ was immunoprecipitated using a monoclonal anti-PPARγ antibody. Following SDS-PAGE and probing of PPARγ immunoprecipitates with an anti-PPARγ antibody, densitometric scanning showed that there was 43% more PPARγ protein in control pCDNA3/3T3-L1 cells compared to PTHrP transfected 3T3-L1 cells (Fig. 9a). However, when the same immunoprecipitates were probed with an anti-phosphoserine antibody, there was a 78% increase in phosphorylated PPARγ in PTHrP transfected cells (Fig. 9b). The ratio of phosphorylated to total (phosphorylated and dephosphorylated) PPARγ in PTHrP transfected cells was greater than 2.5 times that of control pCDNA3 cells. This would suggest that a substantially greater ratio of inactive to active PPARγ was present in PTHrP transfected cells, as compared to control 3T3-L1 cells

PTHrP inhibits the capacity of PPARγ to enhance transcription-We next determined whether exogenous PTHrP is able to reduce the capacity of PPARγ to regulate transcription. Due to the low transfection efficiency of 3T3-L1 cells, and the low levels of PPARγ expression prior to induction, we tested this hypothesis in the COS7 cell line stably transfected with the cDNA encoding PTH/PTHrP receptor. These cells were transiently transfected with PPARγ cDNA and a luciferase reporter plasmid with the PPARγ response element from the aP2 gene. A β-galactosidase reporter plasmid was used as a transfection control. Following PPARγ activation by the addition of the PPARγ ligand troglitazone, the cells were incubated with or without PTHrP ($1x10^{-6}$M). We found that cells treated with PTHrP displayed a marked reduction in luciferase activity (Fig 10). This would suggest that acting via its receptor, exogenous PTHrP is able to directly inhibit the transcriptional activity of PPARγ.

Effects of PTHrP on PPARγ and aP2 expression-We next examined mRNA levels of fat specific genes in these cells. Total RNA was isolated from pCDNA3/3T3-L1 and PTHrP/3T3-L1 cells at various time points following incubation with the differentiation medium, and assessed by Northern blot analysis using labeled probes for PPARγ and for the adipocyte specific gene aP2. (353) Tissue specific expression of the fatty acid binding protein aP2 is directly regulated by the transcriptional activity of PPARγ; therefore any changes in aP2 expression levels will be a result of changes in PPARγ activity. (349) We observed that upon induction of differentiation, PPARγ transcript levels increased and remained relatively constant in both 3T3-L1 and PTHrP/3T3-L1 cells (Fig. 11a). In contrast, aP2 levels initially increased faster in PTHrP expressing cells. However, the levels plateaued earlier and thereafter fell and remained at a lower level than in the control pCDNA3/3T3-L1 cells (Fig. 11a-b).

DISCUSSION

An inverse relationship generally exists between the total number of osteoblasts and adipocytes within bone marrow. (30) This inverse relationship is reflected in virtually every form of osteopenia, such that a decrease in bone mass is always accompanied by an increase in adiposity within the bone marrow. It has been postulated that this accumulation may be a consequence of the inappropriate differentiation of pluripotent stem cells that are diverted from other lineages such as osteoblasts. (125) While the molecular signals that are ultimately involved in this process are poorly understood, our studies on the bone alterations arising in mice heterozygous for targeted disruption of the PTHrP gene have suggested the possibility that PTHrP expressed within the skeletal microenvironment functions as one such signal in regulating cell fate determination. (9)

We show here that cells of the adipocytic lineage, the 3T3-L1 preadipocytic cell line, express PTHrP and the PTH/PTHrP receptor. Under conditions that promote terminal differentiation of 3T3-L1 cells, expression levels for both PTHrP and its receptor decrease until they are not detectable in the terminally differentiated cells. This sequence of PTHrP expression in adipocytes is reversed in osteoblasts where PTHrP transcripts are detected after commitment to the osteoblast lineage, and their levels increase as the cells proceed through the differentiation program. (192)

When PTHrP levels are induced by stable transfection to remain constant throughout differentiation of the pre-adipocytic 3T3-L1 cells, this appears sufficient to perturb the progress of the cells in acquiring an adipocytic phenotype. If PTHrP limits adipocyte differentiation by signaling via the PTH/PTHrP receptor, then it is likely that inhibition of adipogenesis within the bone marrow could result not only from PTHrP derived from pre-adipocytes, but also from neighboring developing osteoblasts secreting PTHrP (10, 192, 335)thereby diminishing their adipogenic potential. Systemic PTH that also acts on the PTH/PTHrP receptor might also contribute to the inhibition of bone marrow adiposity.

PTH and PTHrP bind to the common PTH/PTHrP receptor leading to activation of two signal transduction systems, a Gαs-mediated increase in cAMP and activation of PKA, and a Gαq-mediated increase in intracellular calcium and inositol triphosphate levels and activation of PKC. Although the MAPK cascade represents the basic mechanism used by many growth factors to transduce mitogenic and differentiation signals by receptors with intrinsic tyrosine kinase activity, this pathway is also subject to regulation or "cross talk" by G protein-coupled receptor signaling. PKA has been reported for example to directly

activate the small G protein Rap1 that in turn activates B-Raf leading to the sequential activations of MEK and MAPK in a Ras-independent pathway. (374, 375) While activation of PKC also stimulates increased MAPK activity, the signaling pathways involved are less well defined. All three groups of PKCs (conventional, novel, and atypical) are able to activate MAPK and MAPK kinase (MEK) but only conventional and novel PKCs are potent activators of c-Raf1. (320, 374) Here we show that MAPK activity in 3T3-L1 cells is under the regulation of PTH/PTHrP receptor signaling as a consequence of increasing PKA activity. PTH/PTHrP signaling has previously been reported to either trigger or inhibit MAPK activity, depending on the cell type examined. Thus, PTH inhibits growth factor-induced MAPK activation in UMR 106 and ROS 17/2.8 osteosarcoma cells (369)and MAPK in F9 embryonal carcinoma cells (370)through activation of PKA, while it enhances activation of MAPK in Chinese hamster ovary R15 and parietal yolk sac carcinoma cells. (368) Activation of MAPK by PTH in these cell lines was also mediated by cAMP and was independent of Ras. This argues either for a cAMP site of action downstream of Ras in the Ras-Raf-MEK-MAPK cascade or a parallel Ras-independent pathway such as Rap1 and B-Raf. In 3T3-L1 preadipocytes, PTHrP signaling via cAMP is also a positive regulator of the MAPK pathway at a level upstream of MEK, as indicated by our findings. Whether Raf1 or B-Raf kinase are involved in the activation of MAPK by the cAMP-dependent PKA in these cells remains to be determined. Nevertheless, activation of MAPK, irrespective of the pathway utilized, had profound effects on the differentiation program of 3T3-L1 pre-adipocytes by increasing phosphorylation of PPARγ, the master controller of the adipogenic program. Conversely, inhibition of MEK activity by PD098059 was sufficient

to restore the adipogenic differentiation program. This observation is consistent with a recent report showing that MAPK inhibition in pluripotent cells enhanced adipogenesis. (176)

It has been reported that activation of PPARγ, is able to induce adipocyte differentiation in fibroblasts and myoblasts as well as in bone marrow stromal cells. (125, 163, 353) This appears to occur by enhancing transcription of a variety of adipocyte specific genes such as aP2 and phosphoenolpyruvate carboxykinase (PEPCK). (352) PTHrP was shown in our studies to diminish the capacity of ligand bound PPARγ to act as a transcriptional enhancer at an aP2 promoter site. Consequently, it also decreased gene expression of aP2. Another mechanism by which PPARγ functions, although less well understood, is by acting synergistically with other fat regulatory factors, such as CAAT/enhancer binding protein alpha (C/EBPα) to drive forward the adipogenic program. (353) Therefore, inactivation of PPARγ could also reduce the response of these cells to other fat determining factors, such as C/EBPα. PPARγ therefore is a potent target by which PTHrP signaling can promote its inhibitory effects on the development of the adipocyte phenotype.

Our findings illustrate that while PTHrP does not affect the transcription of PPARγ, it does however downregulate its activity. A striking feature of our study was the rapid initial increase in aP2 expression observed in PTHrP transfected cells as compared to control cells, following induction of differentiation. This rapid early increase in aP2 expression could be the result of activated MAPK in PTHrP transfected cells, as these cells are more likely to proceed at an accelerated rate through the mitotic divisions required to take place prior to adipocyte differentiation, thereby allowing the

differentiation program to begin earlier. (64) This conclusion is further substantiated by the increased proliferative capacity of PTHrP transfected 3T3-L1 cells, as illustrated by the proliferation assay. Despite this early increase in aP2, however, PTHrP overall downregulates transcript levels of this marker. These observations are consistent with previous reports that cAMP has dual effects on the differentiation of preadipocytes in that it potentiates early events but exerts potent inhibitory effects on terminal differentiation. (400) Therefore, early aP2 expression followed by its downregulation in PTHrP/3T3-L1 cells would be consistent with cells being initially directed towards an adipogenic fate but are subsequently prevented from further differentiation. This ability to withdraw from the adipogenic differentiation program has been observed even with mature adipocytes derived from primary bone marrow cultures as they are capable of reverting to a more proliferative state and undertake an osteogenic fate, (28) indicative of the plasticity that allows for transdifferentiation between adipocytes and osteoblasts.

PTH and PTHrP analogues are considered one of the most effective forms of anabolic treatment of osteoporosis. When PTH and PTHrP are administered continuously, their effect on bone is catabolic. However, when PTH or PTHrP are administered intermittently, an increase in bone formation follows. (45, 111, 303, 331) Current theories regarding the anabolic action of PTH and PTHrP action in bone suggest that these agents act primarily on cells of the osteoblast lineage. PTHrP and the PTH/PTHrP receptor are expressed in osteoblast precursors and differentiated osteoblasts. (10, 227, 313, 359) Consequently, PTH and PTHrP are also likely to influence the biology of cells of the osteoblast lineage. Prevention of osteoblast apoptosis was reported recently to be a mechanism of increased bone formation observed following PTH administration *in*

vivo(185). Here, we present evidence for another possible mode of action for PTH and PTHrP analogues, specifically altering the commitment and differentiation of pluripotent bone marrow stromal cells. While PTHrP was shown here to inhibit the adipogenic differentiation program, the question remains whether PTHrP reciprocally influences the commitment of stem cells to the osteoblast lineage. Recent findings demonstrate that PTHrP is able to stimulate the osteoblast differentiation factor CBFA1 by PKA signaling. (321) These findings taken together with our findings, would suggest that PTHrP is able to inhibit adipogenesis and at the same time enhance osteogenesis by PKA signaling.

In summary, our findings here provide a molecular mechanism to explain the increased bone marrow adiposity observed with PTHrP haploinsufficiency and perhaps with the osteopenic state in general. The capacity of PTHrP to inhibit adipocyte differentiation and ultimately enhance osteogenesis may provide novel targets and strategies for the treatment of osteoporosis.

FIGURES

Figure 1

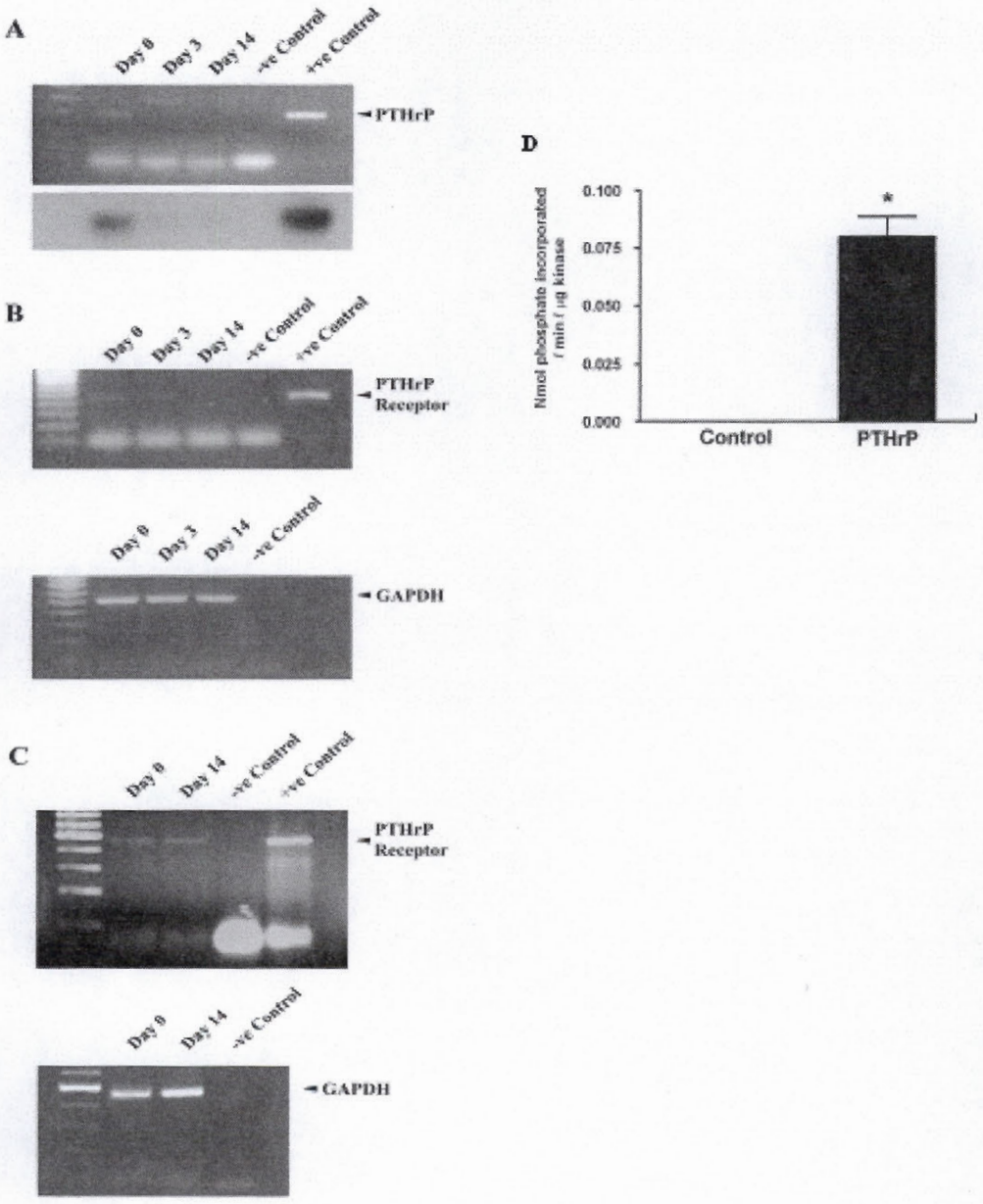


Figure 1. **RT-PCR analysis of PTHrP and PTH/PTHrP receptor expression in 3T3-L1 pre-adipocytes.** Numbers above each lane represent days following induction of differentiation. RNA was isolated from three different time points during adipocyte differentiation, reverse transcribed, and amplified by PCR for (A) PTHrP. Negative control (-ve) represents the PCR product of oligos alone and positive control represents amplification of the RT-PCR product of 3T3-L1 cells transfected with PTHrP cDNA. for PTHrP and the PCR product of the cDNA for the PTHr. (B) PTH/PTHrP receptor. RT-PCR was performed on wild-type 3T3-L1 pre-adipocytes for the PTH/PTHrP receptor on the indicated days. Positive control (+ve) represents the analogous PCR amplification of the cDNA for the PTH/PTHrP receptor. (C) RT-PCR for PTHr expression in PTHrP transfected cells show persistent expression of the receptor 14 days following induction of differentiation. 10 µl aliquots of the RT-PCR samples were analyzed by gel electrophoresis. The identity of the products was confirmed either by sequencing for the receptor or by probing with an internal radiolabeled oligonucleotide specific for the PTHrP product (A, lower panel). The RT products of 3T3-L1 and PTHrP transfected 3T3-L1 cells were evaluated based on concurrent amplification of GAPDH transcripts (B and C lower panels respectively). Each experiment is representative of three triplicate experiments which provided the same result. (D) PTHrP stimulates PKA activity in 3T3-L1 cells. 3T3-L1 cells were plated at an initial density of 50 000 cells per 35 mm plate and allowed to grow to 80% confluence. The cells were then serum starved overnight with fresh DMEM applied the followed by treatment with PTHrP 1-34 ($1x10^{-7}$M) for 5 minutes and then assayed for PKA activity by monitoring incorporation of ^{32}P into PKA specific substrate. Readings are representative of six independent samples. * indicates

that the differences with respect to PTHrP treatment and control cultures are statistically significant ($p < 0.05$)

Figure 2

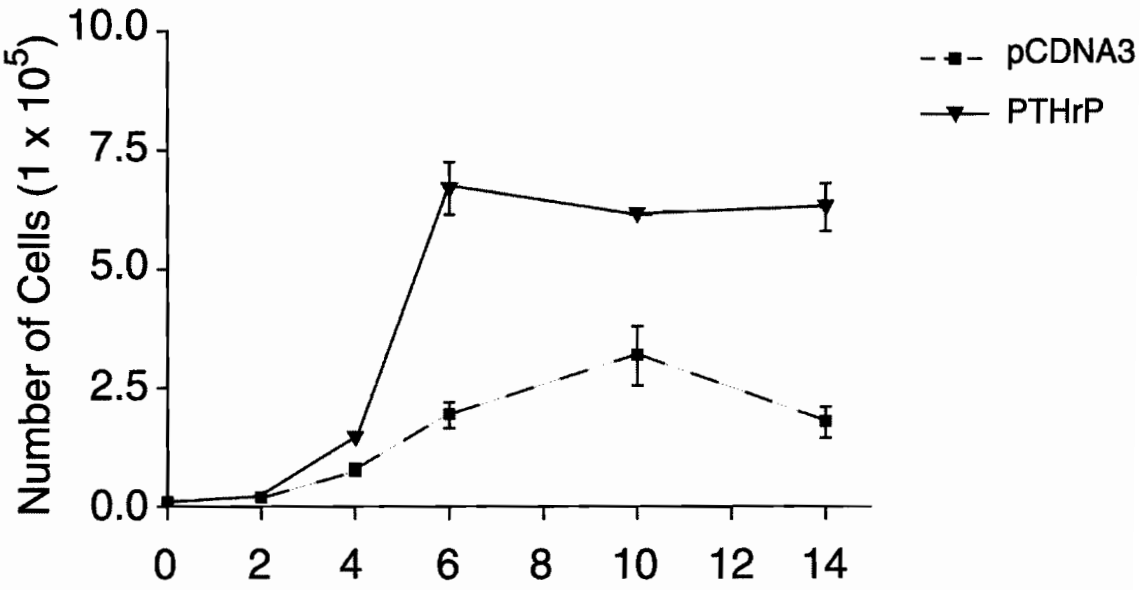

Number of Cells (1 x 10^5)
10.0
7.5
5.0
2.5
0.0
0
2
4
6
8
10
12
14
pCDNA3
PTHrP

Figure 2. **Proliferation assay of pCDNA3 and PTHrP transfected 3T3-L1 cells.** Both cell populations were plated at an initial density of 10 000 cells per well in 6-well plates. Cells were then trypsinized and counted every two days for 6 days then once at day 10 and once again on day 14. Cell numbers were then plotted as (■) for pCDNA3 transfected 3T3-L1 cells and (▼) for PTHrP transfected 3T3-L1 cells. Each data point is the mean ± standard error of triplicate determinations.

Figure **3**

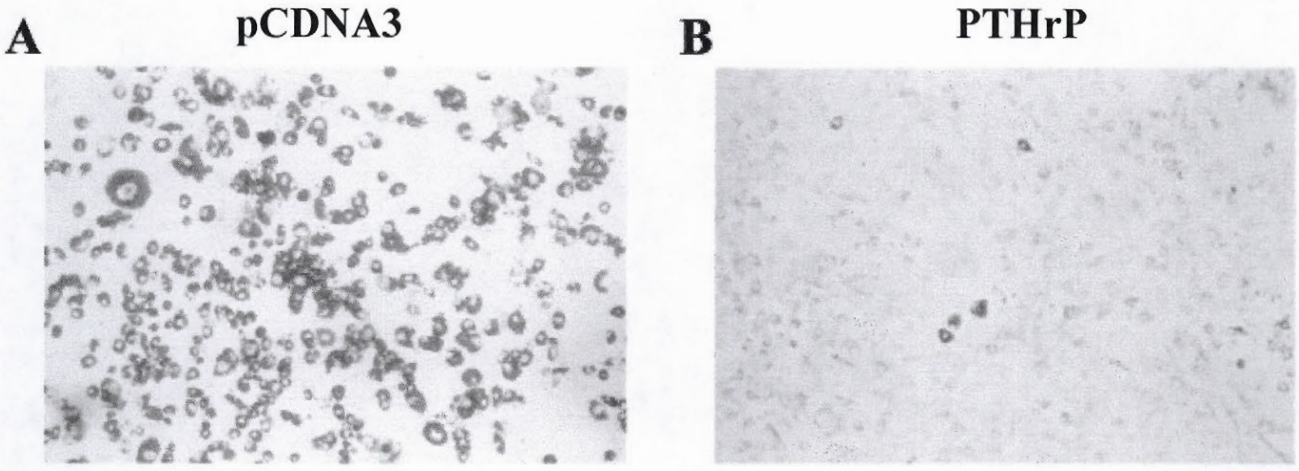


Figure 3. **Differentiation of pCDNA3 and PTHrP transfected 3T3-L1 cells.** pCDNA3 and PTHrP transfected cells were plated at an initial density of 250 000 cells per 100 mm plate and allowed to grow to confluence. Cells were then induced to differentiate. Following differentiation, cells were fixed and stained with oil red O. (A) pCDNA3 transfected cells and (B) PTHrP transfected cells following differentiation. The data shown is representative of triplicate experiments.

Figure 4

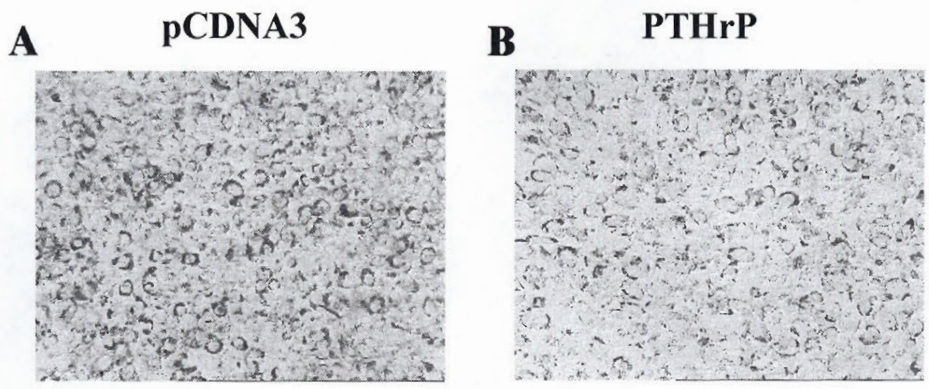


Figure 4. Differentiation of pCDNA3 and PTHrP transfected C3H10T½ cells. pCDNA3 and PTHrP transfected cells were plated at an initial density of 50 000 cells per 35 mm plate, allowed to grow to confluence and then induced to differentiate. 14 days after the initial induction, the cells were fixed and stained with oil red O. (A) pCDNA3 transfected cells and (B) PTHrP transfected cells following differentiation. Each result is representative of those obtained from triplicate determinations.

Figure 5

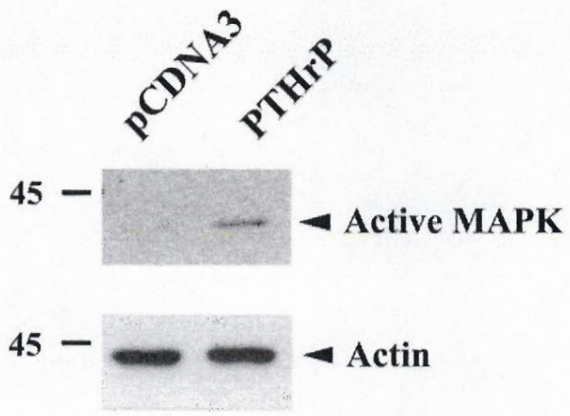


Figure 5. **Activation of MAPK in PTHrP transfected 3T3-L1 cells.** pCDNA3 and PTHrP transfected cells were plated at an initial density of 250,000 cells and allowed to grow to confluence. Cells were then lysed and 20 μg of whole cell lysate was assessed by western blot analysis for activated forms of MAPK. Loading was verified by probing the same membrane for actin. Each blot is representative of data obtained from triplicate experiments.

Figure 6

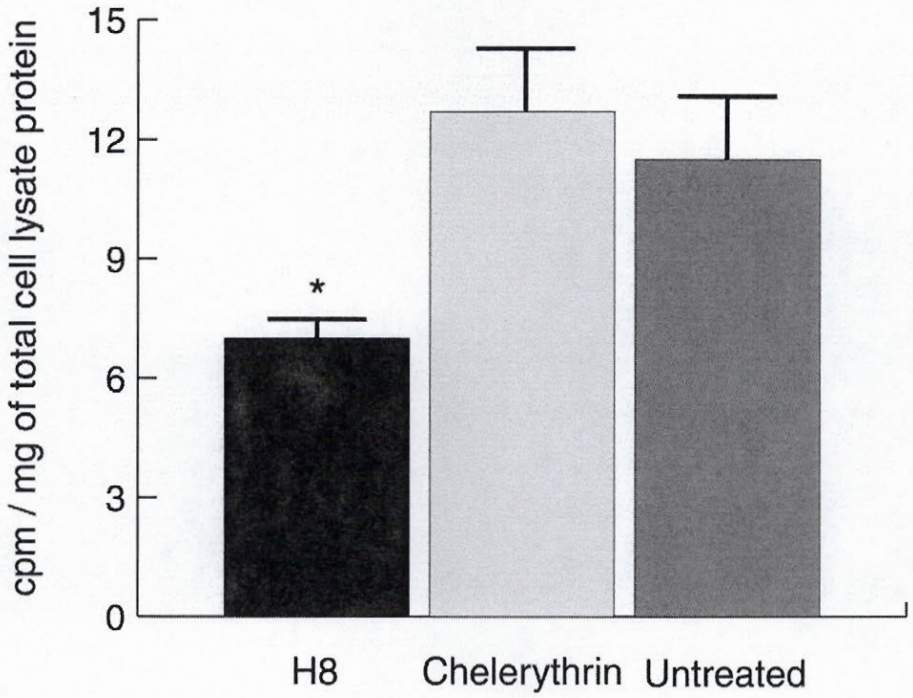

cpm / mg of total cell lysate protein
15
12
9
6
3
0
*
H8
Chelerythrin
Untreated

Figure 6. **MAPK activity in PTHrP transfected cells treated with PKA and PKC inhibitors, H8 or chelerythrin chloride, respectively.** PTHrP transfected 3T3-L1 cells were plated at a density of 250 000 cells and treated with either the PKA inhibitor H8, the PKC inhibitor chelerythrin chloride, or DMSO as vehicle for a period of 24 hours. MAPK activity was then assessed by assaying incorporation of ^{32}P into MAPK specific substrate. Total cpms were standardized against the amount of cell lysate protein per sample. Each bar represents the mean ± SE of three determinations. * indicates that the differences with respect to H8 treatment and control cultures are statistically significant ($p< 0.05$).

Figure 7

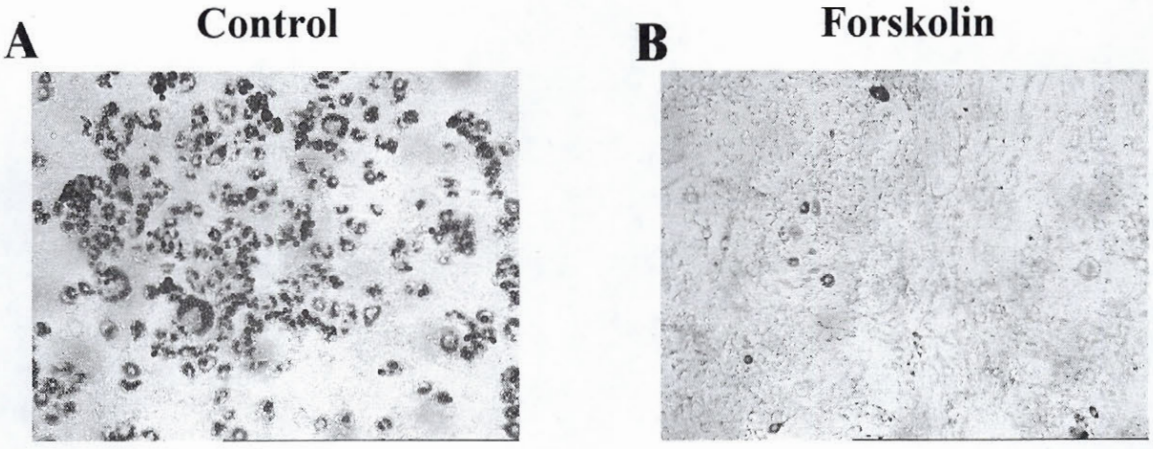


Figure 7. **Forskolin inhibits the terminal differentiation of 3T3-L1 cells.** 3T3-L1 cells were induced to differentiate in the presence of the PKA activator forskolin at a concentration of 100 μg per ml. Following differentiation, cells were fixed and stained with oil red O. (A) Forskolin treated cells and (B) control untreated cells following differentiation. Each result is representative of those obtained from triplicate experiments.

Figure 8

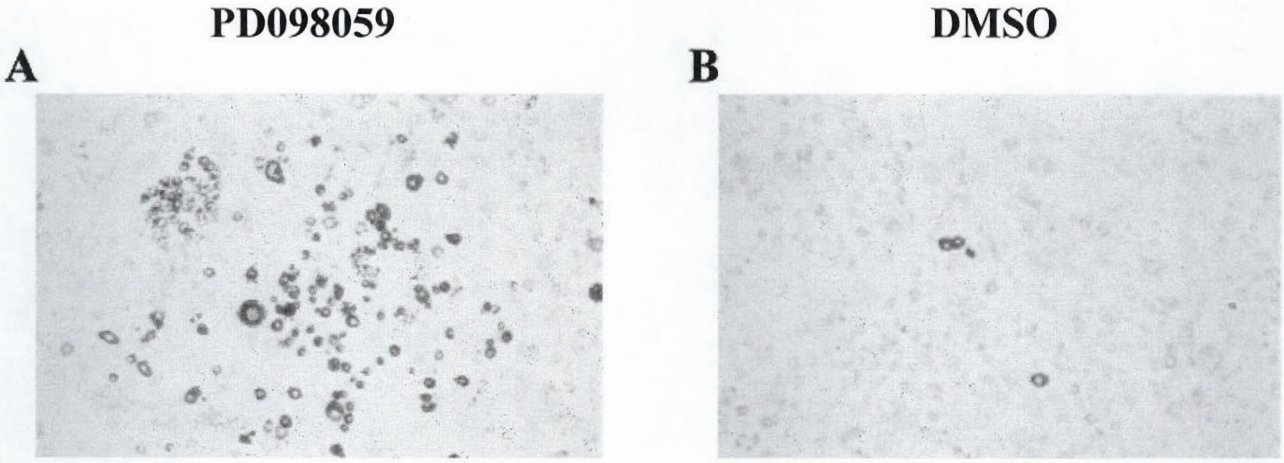


Figure 8. **Differentiation of PTHrP transfected 3T3-L1 cells occurs by inhibiting MAPK signaling.** PTHrP expressing 3T3-L1 cells were induced to differentiate in the presence of (A) PD098059 (20 μM) or (B) DMSO as control. Fourteen days later, the cells were fixed then stained with oil red O. Each result is representative of those obtained from triplicate experiments.

Figure 9

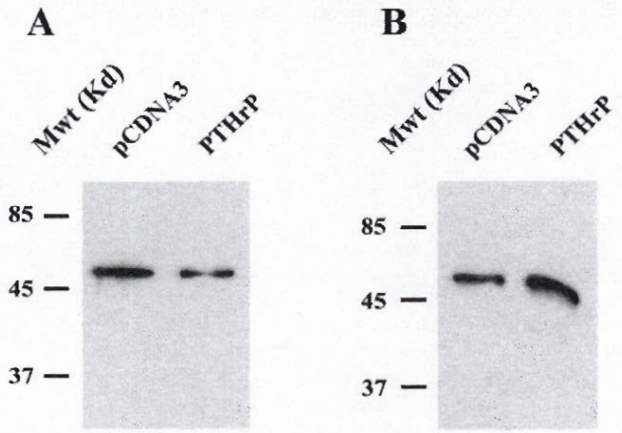


Figure 9. **Effects of PTHrP on the phosphorylation status of PPARγ.** 5 μl of immunoprecipitates from lysates of pCDNA3 and PTHrP transfected 3T3-L1 cells, which were obtained 14 days after induction with IBMX, dexamethasone and insulin, were examined by western blot analysis. Following SDS-PAGE, immunoprecipitates were transferred onto membranes, and probed with the anti-PPARγ (A) to quantify the total PPARγ loaded onto each lane and an anti-phosphoserine antibody (B) to quantify the serine phosphorylation status of PPARγ. Each blot is representative of triplicate determinations.

Figure 10

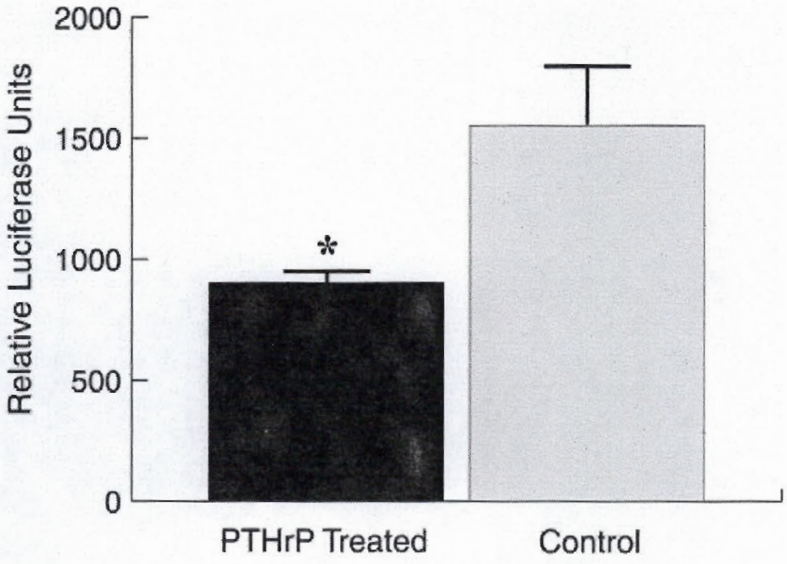

2000
1500
1000
500
0
Relative Luciferase Units
*
PTHrP Treated
Control

Figure 10. **PTHrP inhibits PPARγ transcriptional activity.** COS7 cells expressing the PTH/PTHrP receptor were transfected with PPARγ, and then stimulated with the PPARγ ligand, troglitazone (l00 μM) in the presence of PTHrP 1-34 ($1x10^{-6}$M). Untreated cells were maintained as a control. Luciferase activity was assessed and standardized against β-galactosidase expression. Each bar represents the mean ± SE of three determinations. * indicates that the difference between with PTHrP treated and control cultures are statistically significant ($p<0.05$).

Figure 11

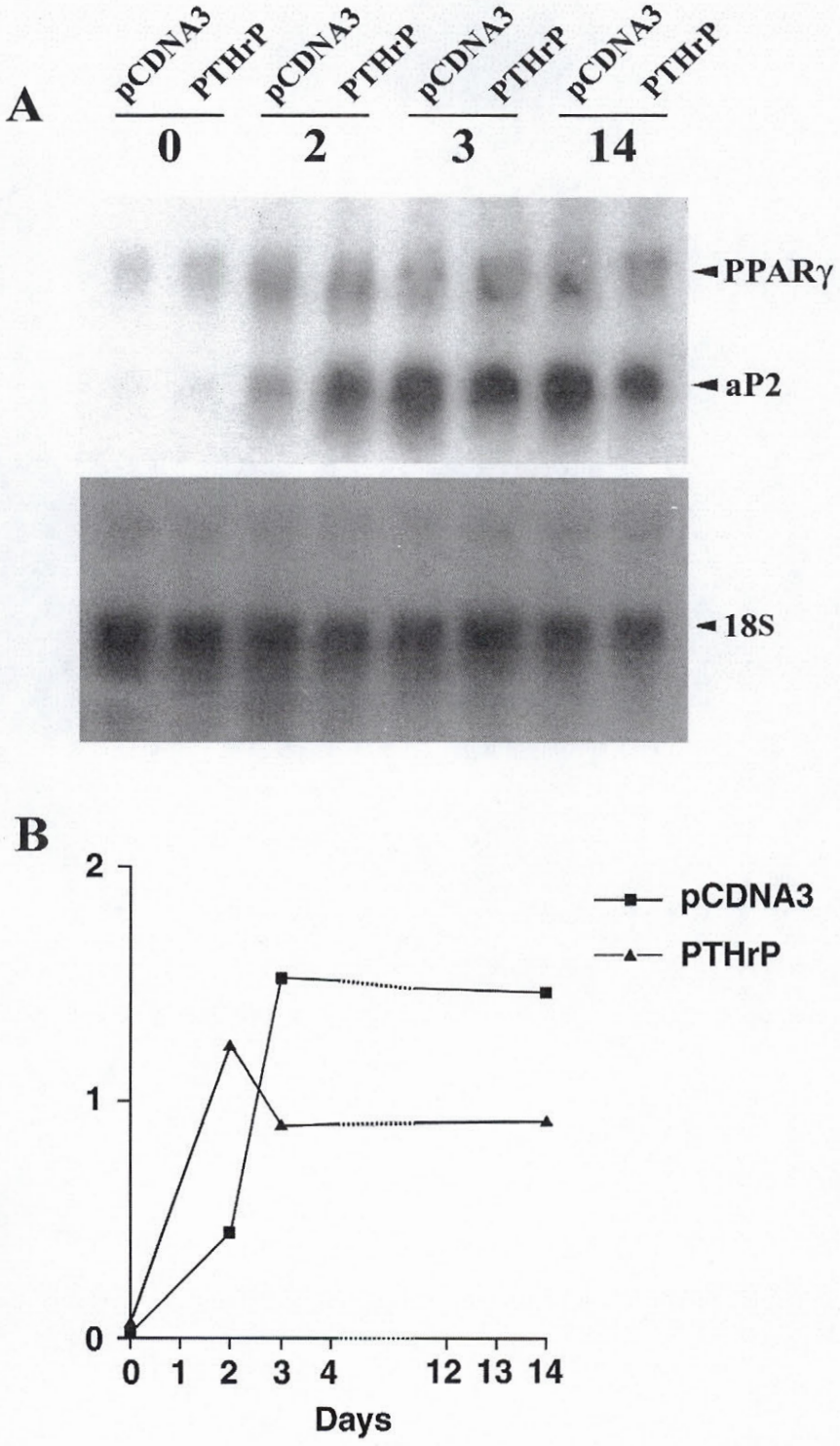

A
pCDNA3
PTHrP
pCDNA3
PTHrP
pCDNA3
PTHrP
pCDNA3
PTHrP
0
2
3
14
PPARγ
aP2
18S
B
2
1
0
pCDNA3
PTHrP
0 1 2 3 4 12 13 14
Days

Figure 11. **PPARγ and aP2 expression levels in pCDNA3 and PTHrP transfected 3T3-L1 cells.** Total RNA was isolated at four different time points during the differentiation program of pCDNA3 and PTHrP transfected 3T3-L1 cells. 6 μg of RNA was loaded per sample, and (A) probed with a radiolabeled probe for PPARγ and aP2, concurrently. Numbers above lanes represent days following induction of differentiation. The membrane was probed for ribosomal 18s expression as a loading control (lower panel). (B) Graph depicting relative aP2 levels vs 18s expression as determined by densitometric scanning. Results shown are representative of triplicate experiments.

PREFACE TO CHAPTER 3

PTHrPs ability to inhibit adipogenesis of pre-adipocytic 3T3-L1 and pluripotent mesenchymal C3H 10T½ cells are consistent with previous *in vivo* findings that demonstrate that haploinsufficiency for PTHrP results in enhanced bone marrow adipogenesis. In light of the fact that PTHrP heterozygous null animals also demonstrate reduced bone volume, we proceeded to investigate whether PTHrP might also serve a role in the commitment and differentiation of osteoblasts.

Chapter 3

Parathyroid Hormone Related Peptide Interacts with Bone Morphogenetic Protein 2 to increase Osteoblastogenesis and decrease Adipogenesis in Pluripotent C3H10T½ Mesenchymal Cells

ABSTRACT

We examined the effect of PTHrP on modulating adipogenesis and osteoblastogenesis in the pluripotent mesenchymal stem cell line C3H10T½. These cells express the type 1 PTH/PTHrP receptor thereby allowing PTHrP to inhibit Bone Morphogenetic Protein 2 (BMP2) from enhancing gene expression of peroxisome proliferator-activated receptor γ (PPARγ) and the adipocyte specific protein aP2, and from augmenting the accumulation of lipid. In the presence of BMP2, PTHrP or a protein kinase C (PKC) stimulator (phorbol ester), increased expression of indices of the osteoblast phenotype including alkaline phosphatase, type I collagen and osteocalcin, whereas a PKC inhibitor (chelerythrin chloride) inhibited PTHrP action. PTHrP and a phorbol ester increased gene expression of the BMP-IA receptor and both enhanced BMP2 dependent increases in SMAD6 promoter activity. Overexpression of the BMP-IA receptor facilitated the capacity of BMP2 to increase osteoblastogenesis in the absence of PTHrP and a dominant negative BMP-IA receptor variant inhibited this effect of BMP2. These results demonstrate that PTHrP can direct osteoblastic rather then adipogenic commitment of mesenchymal stem cells, implicates PKC signaling in this activity and shows that PTHrP action involves enhanced gene expression of the BMP-IA receptor which facilitates BMP2 action in specifying an osteoblastic fate for a pluripotent mesenchymal stem cell.

INTRODUCTION

Osteoblasts the principal bone forming cells and adipocytes the main fat storing cells are believed to originate from the same pluripotent mesenchymal stem cells(131). Both cell types also contribute to the architectural structure of bone and a decrease in bone volume is generally accompanied by an increase in adipocyte numbers within the bone marrow(256). This reciprocal relationship is exemplified in the development of age related osteopenia. Thus, bone marrow at a very early age is virtually devoid of adipocytes, however as aging progresses, a decrease in bone volume occurs with a reciprocal increase in fat deposits within the marrow(125, 256). One hypothesis to explain these phenomena is that the reduced number of osteoblasts and increased number of adipocytes is a result of increasing commitment of pluripotent mesenchymal cells along the adipocytic as opposed to the osteoblastic lineage(280). Regulation of this process may involve bone morphogenetic proteins (BMPs). BMPs are expressed within the bone marrow stroma and are the only known morphogens that are able to induce both adipogenesis and osteogenesis of pluripotent mesenchymal cells(5). BMP2, BMP4 and BMP7 have all been shown to be effective inducers of both osteoblast and adipocyte commitment *in vitro* however the cell lineage that is specified is often determined by the concentration of applied BMP. BMP family members may therefore serve to activate transcription of distinct target genes in a dose-dependent manner leading to distinct cellular phenotypes. BMPs act by binding to a type I and type II receptor. BMP binding to the type II receptor enhances the affinity for the type I receptor and heterodimerization of the two receptors follows. Once heterodimerization takes place, the type II receptor trans-phosphorylates and activates the type I receptor. It is the type I receptor that

contains the functional kinase domain that is essential for serine phosphorylation and activation of downstream SMAD transcription factors(262).

Parathyroid hormone (PTH) and PTH related peptide (PTHrP) analogues have been shown to effectively induce bone formation both *in vivo* and *in vitro*(303). Both peptides interact at a common G-protein coupled receptor termed the type I PTH/PTHrP receptor (PTHR) which is linked to the adenylyl cyclase/protein kinase A (PKA) signaling system and the phospholipase C/protein kinase C systems. (189) Several mechanisms have been put forward regarding the action of PTH and PTHrP on increasing bone formation. These include proliferation of osteogenic progenitor cells(258), enhancing osteoblast differentiation(48) and inhibiting apoptosis of osteoblastic cells(185). PTH has also been shown to enhance the transcriptional activity of the osteoblast differentiation factor CBFA1 via phosphorylation through an adenylyl cyclase-protein kinase A(PKA) dependent mechanism(321).

We recently demonstrated that PTHrP is able *in vitro* to inhibit the terminal differentiation of committed pre-adipocytes by stimulating MAPK, which in turn can phosphorylate and downregulate the adipogenic determining factor peroxisome proliferator-activated receptor γ (PPARγ) (51). We also reported that, *in vivo*, PTHrP heterozygous null mice that are haploinsufficient for PTHrP develop a premature form of osteopenia characterized by reduced trabecular bone volume and increased bone marrow adiposity(9). In the current studies, we investigated whether PTHrP might play a role in the commitment of mesenchymal cells towards the adipocytic or osteoblastic lineages. For these studies we employed the pluripotent mesenchymal cell line C3H10T½. These cells are derived from mouse embryo connective tissue and can be induced to

differentiate along several mesenchymal cell lineages(5, 342). When treated with low concentrations of BMP2, C3H10T½ cells commit to the adipocytic lineage, however, with higher concentrations of BMP2, osteogenesis is enhanced(376).

We therefore employed the C3H 10T½ cells to study the early commitment process of these cells. Our studies show that in this system, PTHrP can inhibit the capacity of BMP to increase adipogenesis and can facilitate the action of BMP to direct commitment towards the osteoblastic lineage. The PKC pathway is implicated in the early commitment process and the mechanism of the PTHrP effect involves upregulation of the BMP-IA receptor which enhances sensitivity to BMP and favors specification of the osteoblastic rather than the adipocytic lineage.

MATERIALS AND METHODS

Cell Culture and Cell Transfections - C3H 10T½ cells were obtained from ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). Fresh medium was applied every second day. To induce commitment and differentiation, cells were grown to confluence then maintained in alpha minimum essential medium (αMEM) supplemented with ascorbic acid (100 μg/ml), β-glycerophosphate (5mM) and BMP2 (Research Diagnostics Inc, Flanders, NJ) ($6x10^{-9}$M) unless stated otherwise. Fresh medium was applied every two days. When required, PTHrP 1-34 was added at a concentration of $1x10^{-7}$ M unless otherwise stated. The PKC inhibitor chelerythrin chloride was added at a concentration of $1x10^{-6}$ M.

C3H 10T½ cells expressing the BMP-IA receptor were generated after cloning cDNA encoding the rat BMP-IA receptor(339) (provided by Dr. Gideon Rodan, Merck

Laboratories, West Point, PA) into the pcDNA3 expression plasmid. The expression plasmid was then used for stable transfection as previously described(51). C3H 10T½ cells expressing a BMP-IA dominant negative receptor (BMP-IA-DN) lacking kinase activity were generated after cloning the cDNA (provided by Dr. Gideon Rodan) into the pcDNA3 expression plasmid. Stably transfected cells were generated in a similar manner and control cells were generated by stable transfection with empty pCDNA3 plasmid. Selection was performed using 400μg Geneticin per ml (Gibco BRL). All transfections were carried out using FUGENE6 reagent (Boerhinger Mannheim). SMAD6 luciferase reporter transformants were generated by cotransfection of C3H10T½ cells with 5 μg of a SMAD6 promoter fused to a luciferase reporter as previously described(170) and 0.5μg of pCDNA3 due to the lack of a selection marker for the SMAD6 luciferase reporter construct. Serial dilutions were performed and selection of individual clones was performed using 400μg Geneticin per ml of medium.

Northern Blot Analysis - Total RNA was isolated using TRIZOL reagent (Gibco BRL). For Northern blot analysis, 10 μg of total RNA per sample was electrophoresed through a formaldehyde/agarose gel. The RNA was transferred to Bio-Trans nitrocellulose membrane(Amersham Biosciences). For detection of adipocytic genes, the membrane was probed with an internal EcoRI fragment of PPARγ mouse cDNA (51)and the full-length cDNA of mouse aP2(51). For detection of osteoblastic genes, the membrane was probed with a 1135bp fragment encoding the amino terminus of the mouse alkaline phosphatase cDNA, a 1600 bp fragment of the rat α1R1 collagen 1 cDNA(118) and the entire 467 bp coding region of the rat osteocalcin cDNA. For detection of the BMP-IA mRNA, a 563 bp fragment of the mouse BMP-IA receptor cDNA was generated by PCR

(5'-ttg ctg tat tgc tga cct gg-3' and 5'-CAT CCT GGG ATT CAA CCA TT-3'). Northern analysis detection was performed using a Packard Cyclone Storage Phosphor System (Packard Instrument Company, Inc., Meriden, Connecticut). Levels of mRNA were quantified using Scion Image (Scion Corp, Frederick, Maryland) and standardized by comparison with 18S RNA levels that were detected simultaneously as previously described(51).

To determine the effects on BMP-IA receptor expression, cells were incubated for 4 days with BMP2 ($6x10^{-9}$M) alone or with BMP-2 plus PTHrP (10^{-7}M). Alternatively, cells were incubated with BMP-2 alone and on day 4, the cells were treated with either forskolin (100 μM) or TPA (1 μM) for 24 hours. RNA was then harvested from all cells.

BMP2 Responsive Luciferase Assays – SMAD6 promoter-luciferase reporter assays were performed by a modification of a previously described method. (170)Briefly, C3H 10T½ cells stably transfected with the SMAD6 promoter-luciferase reporter, were pretreated with BMP2 ($6x10^{-9}$M) alone for 5 days. To examine PTHrP effects, in some culture plates PTHrP (10^{-7}M) was added with BMP-2. To examine TPA effects, in some plates, TPA (1μM) was added with BMP2 on the 4th day for 24 hours. After a further 24 hours of serum deprivation, all cells were then stimulated with PBS vehicle or with BMP2 ($6x10^{-9}$M) for 24 hours and luciferase activities were measured.

PKA and PKC Activity Assay - C3H 10T½ cells were incubated with BMP2 ($6x10^{-9}$M) for 4 days. The day before assays were to be performed, the cells were serum deprived overnight. The following day, PKA and PKC response to PTHrP were measured intermittently after treatment of cells for 15 minutes with PTHrP 1-34 followed by lysis.

No readings were taken for day 1 as cells were treated for a minimum of 24 hours with BMP2 prior to serum deprivation. PKA and PKC activities were assessed with a PKA Assay Kit (Upstate Biotechnology, Lake Placid, NY) and a PKC Assay Kit (Upstate Biotechnology, Lake Placid, NY) respectively. For the PKA assay, Kemptide (S6 kinase substrate) (Upstate Biotechnology, Lake Placid, NY) was used in place of the provided substrate. Six samples were prepared for each time point

Oil Red O Staining - After 14 days of incubation with BMP-2 ($6x10^{-9}$M), C3H 10T½ cells were washed twice with PBS then fixed for 30 minutes with 10% formalin. Oil red O stain (5% oil red O in 70% pyridine) was applied to the cells for 30 minutes, and then washed three times with PBS.

Alkaline Phosphatase Staining - Cytochemical staining for alkaline phosphatase (ALP) was performed by incubating the cells for 15 minutes at room temperature in 100 mM Tris-maleate buffer containing 0.2 mg/ml naphthol AS-MX phosphate (Sigma, St. Louis, MO) dissolved in ethylene glycol monomethyl ether (Sigma, St. Louis, MO) as a substrate, and fast red TR (0.4 mg/ml) (Sigma, St. Louis, MO) as a stain for the reaction product.

Immunocytochemistry - Cultured cells were stained for type I collagen, osteopontin and osteocalcin using the avidin-biotin-peroxidase complex (ABC) technique as described previously(258). The cells were first treated with 0.5% bovine testicular hyaluronidase (Sigma, St. Louis, MO) for 30 minutes at 37°C, followed by application of primary antibodies affinity-purified goat anti-human type I collagen antibody (Southern Biotechnology Associates, Inc., Birmingham, AL); goat anti-mouse osteocalcin (Biomedical Technologies, Inc., Stoughton, MA); and PPARγ (Research Diagnostics Inc.,

Flanders, NJ) overnight at room temperature. As a negative control, the preimmune serum was substituted for the primary antibody. After washing with high salt buffer (50mM Tris-HCl, 2.5% NaCl, 0.05% Tween 20, pH 7.6) for 10 min at room temperature followed by 2x10 min washes with TBS (50mM Tris-HCl, 150 mM NaCl, 0.01% Tween 20, pH 7.6), the cells were incubated with a secondary antibody (biotinylated rabbit anti-goat IgG (Sigma, St. Louis, MO)). Cells were then washed as before and incubated with the Vectastain ABC-AP kit (Vector Laboratories, Ontario, Canada) for 45 min. After washing as before, red pigmentation to identify regions of immunostaining was produced by a 10-15 min treatment with Fast Red TR/Naphthol AS-MX phosphate (Sigma, St. Louis, MO) containing 1 mM levamisole as endogenous ALP inhibitor.

Quantification for both cytochemistry and immunocytochemistry by image analysis was performed as previously described(258).

Computer-assisted image analysis - Computer-assisted image analysis was performed as described previously(258). Briefly, images of stained culture dishes were photographed with transmitted light over a light box. All images were processed using Northern Eclipse image analysis software, version 5.0 (Empix Imaging Inc., Mississauga, ON). For determining the area of positive colonies in cultured cells, thresholds were set using green and red channels. The thresholds were determined interactively and empirically on the basis of three different images. Subsequently, this set threshold was used to automatically analyze all recorded images of all sections that were stained in the same staining session under identical conditions.

Statistical Analysis – Statistical analysis of cell cultures are based on 3 random fields.

Statistical analysis was performed using a Student T test, Fisher's test or ANOVA followed by Bonferroni adjustment as appropriate. A P value of ≤ 0.05 was taken as significant.

RESULTS

Evidence for functional Type I PTH/PTHrP receptors (PTHR) in C3H10T½ cells – Northern analysis revealed PTHR mRNA expression in C3H 10T½ cells which remained constant throughout a 5 day incubation with BMP2(Fig 1a and 1b). PTHrP is known to stimulate both PKA and PKC after binding to its G-protein coupled receptor (PTHR). To determine the functionality of the PTHR expressed in C3H 10T½ cells, we determined basal and PTHrP stimulated PKA and PKC activity over a 5 day incubation with BMP2. In the absence of PTHrP, PKA and PKC levels increased and then stabilized by day 4 (Fig 1c and 1e). In the presence of PTHrP, significant increases in both PKA and PKC activity were observed at each time point (Fig 1c and 1e). Furthermore, when assessed at day 4, PTHrP elicited a concentration-dependent increase in both PKA and PKC activity (Fig 1d and 1f).

PTHrP inhibits adipogenesis of pluripotent C3H10T½ cells - We incubated C3H 10T½ cells over a 2-week period with either a concentration of BMP2 known to induce adipocytes differentiation (control) (376)or with the same concentration of BMP2 in combination with PTHrP. Control cultures demonstrated extensive adipogenesis as assessed by oil red O staining of the cells (Fig 2a and 2c). In contrast, markedly reduced adipogenesis was observed in the presence of PTHrP (Fig 2b and c).

To examine the mechanism of this effect, mRNA was isolated prior to cell treatment and 7 days after treatment of the cells with either BMP2 alone (control) or BMP2 and PTHrP. Northern blots were performed to examine expression of the adipogenic transcription factor PPARγ and the fat specific gene aP2. BMP2 alone increased both PPARγ and aP2 expression by day 7, whereas with PTHrP treatment, a reduction in both PPARγ and aP2 mRNA levels was observed (Fig 2d and 2e).

Expression of PPARγ was further confirmed by immunocytochemistry of cells that were incubated with either BMP2 or BMP2 in combination with PTHrP (Fig 2f, 2g and 2h). Control cells demonstrated abundant expression of PPARγ protein (Fig 2f) whereas cells treated with both BMP2 and PTHrP (Fig 2g) showed limited expression of PPARγ. Consequently PTHrP appeared capable of curtailing the adipogenic program induced by BMP2 in these cells.

PTHrP enhances osteoblastogenesis in pluripotent C3H10T½ - C3H10T½ cells were incubated over a 2-week period, with either an adipogenic dose of BMP2 alone or with an adipogenic dose of BMP2 in combination with PTHrP. Indices of osteoblastic differentiation were then examined. Evidence for expression of alkaline phosphatase, type I collagen and osteocalcin protein and mRNA were observed with PTHrP treatment

(Fig 3a, b, c and d) whereas only minimal expression of these markers was observed in cultures treated with BMP2 alone (Fig 3a, b, c and d). Consequently PTHrP appeared to reciprocally stimulate osteoblastogenesis while inhibiting adipogenesis.

To determine whether PTHrP might act by preferentially stimulating the proliferation of pre-existing committed osteoblast progenitors within the C3H10T½ clonal population(305), we examined cell numbers after addition of either BMP2 alone, or BMP2 plus PTHrP. PTHrP did not stimulate cell proliferation over that observed with BMP2 alone (data not shown). Consequently, the effect on osteoblastogenesis appeared to be due to induction of commitment of pluripotent cells to the osteoblast lineage.

PTHrP acts by a PKC mechanism to increase osteoblastogenesis and BMP-IA receptor expression and signaling – To assess whether the PKA pathway or the PKC pathway or both mediate the effects of PTHrP, C3H10T½ cells were treated with low levels of BMP2. On day 4, vehicle alone (control), the PKA activator forskolin, the PKC activator TPA, or both forskolin and TPA, were added for 24 hours. After an additional 10 days in culture, cells were examined for expression of the osteoblast markers alkaline phosphatase, type I collagen and osteocalcin. Treatment with forskolin had only a slight effect on expression of these markers whereas treatment with TPA produced dramatic increases (Fig 4a and b). Cells treated with both forskolin and TPA did not enhance treatment as with TPA alone (Fig 4a and b). Consequently the PTHrP effect on osteoblastogenesis appeared to be predominantly due to a PKC mediated response. Furthermore there seemed to be no synergy between the PKC and PKA pathways in mediating this action.

To further determine whether PTHrP enhances osteoblast commitment as a result of enhanced PKC signaling, C3H10T½ cells were induced to differentiate with BMP2 alone, or BMP2 and either PTHrP alone or PTHrP and the PKC inhibitor chelerythrin chloride. After culturing, cells were stained for the osteoblast specific protein osteocalcin. Cells treated with PTHrP and chelerythrin demonstrated significantly lower levels of osteocalcin expression compared to cells treated with PTHrP alone (Fig 4c and d). Consequently, the PTHrP effect on osteoblastogenesis appeared to be due at least in part to a PKC mediated response.

In view of the fact that even in the absence of PTHrP, higher concentrations of BMP2 have been reported to enhance commitment to the osteoblast lineage(16), we next assessed whether PTHrP might augment the sensitivity of the cells to BMP2 by amplifying its signaling. We therefore first examined the effect of PTHrP on expression of the BMP I receptors which transduce the BMP signal via its serine/threonine kinase activity. Cells were incubated with low (adipogenic) concentrations of BMP2 alone, or with BMP2 plus PTHrP, or with BMP2 plus forskolin or TPA (added on day 4). On day 5, RNA was isolated and examined for expression of the BMP I receptors. The BMP IB receptor was undetectable by Northern blot or by RT-PCR (data not shown). The BMP IA receptor was detected in all four samples and expression was standardized against the 18S levels of each respective lane. Equivalent expression was observed in control cells and in forskolin-treated cells. BMP IA receptor expression was markedly increased, however, in both PTHrP-treated and in TPA-treated cells (Fig 5a and b).

To determine whether the increased BMP IA receptor expression was associated with increased post-receptor signaling, we stably transfected C3H10T½ cells with a SMAD 6

promoter-luciferase reporter construct which has been demonstrated to be responsive to BMP2 induced signaling(170). Cells were pre-treated with an adipogenic dose of BMP-2 alone (control) or with BMP-2 plus PTHrP for five days. A third set was pretreated with an adipogenic dose of BMP-2 alone for five days with TPA added to the pretreatment mixture for 24 hours on day 4 to mimic PTHrP stimulation of PKC. Pre-treatment with PTHrP or TPA in combination with BMP-2 significantly increased BMP-2-stimulated SMAD6 promoter-reporter activity (Fig 5c) as compared to control. Consequently, BMP-IA receptor signaling appeared to be enhanced by PTHrP and PKC activation.

The BMP IA receptor mediates BMP2-induced osteoblastogenesis - To determine whether increased BMP IA receptor expression can induce commitment to the osteoblast lineage, we stably transfected pluripotent C3H10T½ cells with the cDNA encoding the BMP IA receptor or with the empty vector (pcDNA3) as a control (Fig. 6a and b). When cells overexpressing the BMP IA receptor were treated with BMP2 ($6x10^{-9}$M) in the absence of PTHrP, these cells expressed considerably higher levels of alkaline phosphatase, collagen type I and osteocalcin than the pcDNA3 transfected cells (Fig 6a and b). To further confirm the role of the BMP IA receptor in this process we also stably transfected wild-type C3H10T½ cells with a dominant negative form of the BMP IA receptor (DNBMP-IA). These cells, BMP-IA overexpressing cells and empty-vector transfected cells (control) were then treated with a higher dose ($1x10^{-8}$M) of BMP2 (Fig. 6c and d). Control cells readily expressed alkaline phosphatase when treated with the higher dose of BMP2 even in the absence of PTHrP (Fig 6c and d). Cells overexpressing the functional BMP-IA receptor when treated with the higher concentration of BMP2 demonstrated even greater expression of the osteoblastic marker. In contrast, cells

transfected with DNBMP-IA failed to express the osteoblast marker alkaline phosphatase even in the presence of the higher dose of BMP2 (Fig 6c and d), confirming the critical role of the BMP IA receptor in modulating commitment of C3H10T½ cells to the osteoblastic lineage.

DISCUSSION

Previous studies have shown that BMP2 can induce in mesenchymal stem cell lines the adipocytic phenotype(54, 127, 184, 260, 285, 293, 330, 376). We showed increased activity of PKA and PKC activity as a result of PTHrP stimulation of the PTHR in the mesenchymal cell line C3H 10T½ cells and that this responsiveness to PTHrP appeared to manifest itself in part by inhibiting BMP2-induced commitment of these cells to the adipocytic lineage. The inhibition by PTHrP involved a reduction in BMP2 induced increases in mRNA encoding PPARγ and aP2 as well as a decrease in cytological staining of lipid. We also found that in the presence of PTHrP, concentrations of BMP2 that normally induce an adipocytic phenotype now induced markers of the osteoblast lineage. Consequently, the effect appeared to involve the specification of multipotent precursor cells to the osteoblast lineage.

To explore the signaling pathway involved in the PTHrP-induced effect, we employed activators of both the PKA and PKC pathways and found that the PKC pathway predominantly mediated commitment to the osteoblast phenotype. To further support the role of PTHrP stimulation of PKC as being the mechanism for enhanced osteogenesis, the PKC inhibitor chelerythrin chloride was found to limit the osteogenic potential of PTHrP. Our findings that PTHrP enhances the commitment of C3H10T½ along the osteoblastic lineage by a PKC dependent mechanism differs from recent results

in which the effect of PTH (1-34) to increase alkaline phosphatase-positivity in C3H10T½ cells transfected with both BMP2 and PTHR appeared to be mediated by forskolin rather than TPA(159). However, those studies employed C3H10T½ cells constitutively expressing BMP2 making comparison of the two systems difficult.

Other mechanisms for enhanced osteogenesis have also been ascribed to the capacity of PTHrP to stimulate PKA. The osteoblast differentiation transcription factor CBFA1 is essential for osteogenesis *in vivo* (89, 266, 292)and has been shown to be a target of PKA i.e. post-translational phosphorylation of CBFA1 via PTHrP signaling enhances the transcriptional activity of CBFA1(321). It is possible that in our system enhanced osteoblastic commitment by PTHrP is independent of CBFA1 activation by PKA or that PKC may also activate CBFA1. Alternatively, CBFA1 function may occur downstream of PTHrP action. Thus, BMP2 induced osteoblastic differentiation of C3H10T½ cells appears to involve CBFA1 mRNA and protein expression(127, 139, 397). CBFA1 can enhance the transcriptional activation of Smad proteins(138, 412) and our studies do show that PTHrP-stimulated osteoblastic commitment requires a BMP2-dependent response. Consequently, CBFA1 stimulation may still converge on the pathway of PTHrP-induced osteogenesis although further downstream in the signaling pathway.

The mechanism of PTHrP action in the C3H10T½ cells appears to involve PKC-induced gene expression of the BMP-IA receptor. This differs with results obtained in the T-antigen immortalized clonal cell line, 2T3 in which the expression of a constitutively active BMP-IA receptor induced adipocyte differentiation whereas expression of a constitutively active BMPIB receptor induced formation of mineralized bone matrix. However, both receptor sub-types are expressed in developing bone and targeted deletion

of the BMPIB receptor gene has revealed no significant change in osteoblast differentiation(404). Additionally, gene array analysis of differentiating osteoprogenitor cells demonstrates that increased BMP-IA expression correlates with the terminal differentiation of an osteoprogenitor cell(26). Consequently, an increase in BMP-IA receptor expression may well regulate specification of osteoblast development.

The receptor-regulated SMADs, SMAD1, SMAD5 and SMAD8 are directly activated by the BMP type I receptor and both SMAD1(187) and SMAD5(275) have been implicated in BMP2 induced osteoblastic differentiation. SMAD4 may then associate with the activated SMADs forming an activated SMAD complex that can then translocate to the nucleus and participate in the regulation of target genes. SMAD complexes can also increase transcriptional regulation of inhibitory SMADs that include SMAD6(170). To examine the functional integrity of the BMP-IA receptor that was increased by PTHrP, we assessed the capacity of BMP2 to increase SMAD6 promoter activity after pre-treatment of target cells with either PTHrP or a PKC agonist. The results demonstrate the enhanced promoter activity induced by BMP2 after pre-treatment with PTHrP or a PKC activator and illustrate the functional capacity of the receptor.

We demonstrated that ectopic overexpression of BMP-IA receptors in C3H10T½ cells enhances osteoblastic differentiation in the presence of concentrations of BMP2 which are generally ineffective in the absence of PTHrP. Furthermore, the higher concentrations of BMP2 that are effective in inducing osteoblast commitment even in the absence of PTHrP became ineffective in the presence of a dominant negative form of the BMP-IA receptor. These findings support the view that PTHrP acts to increase sensitivity

to BMP2 via enhancing expression of functional BMP-IA receptors that then signals the initiation of a genetic osteogenic program in an adipo/osteo progenitor.

Sonic hedgehog (Shh) is a member of the hedgehog family of morphogens that includes Indian hedgehog (Ihh), an important regulator of skeletal development. Both Shh and Ihh share substantial amino acid sequence homology and Shh has been employed *in vitro* to mimic the effects of Ihh. During endochondral bone formation, Ihh can stimulate PTHrP production that then mediates the inhibitory effect of Ihh on chondrocytic differentiation. Shh has recently been shown to inhibit BMP2 induced adipogenesis in C3H10T½ cells(330, 410). Furthermore, recombinant N-terminal Shh (N-Shh) has also been reported to enhance osteoblastic commitment in the presence of BMP2. This synergistic effect was mediated at least in part by BMP-stimulated SMAD signaling to increase gene transcription. Although it would be tempting to hypothesize that the effects of hh analogs on enhancing BMP2 induced osteoblastic commitment are in fact mediated by PTHrP, it appears that N-Shh has no effect on PTHrP or PTHR expression in C3H 10T½ cells(330), nor does exogenous PTHrP appear to affect N-Shh induced endochondral bone formation(211). Consequently, these effects may independently converge on the BMP pathway.

Our studies therefore demonstrate that PTHrP plays a critical role in regulating an inverse relationship between adipocytes and osteoblasts by inhibiting commitment of a multipotential mesenchymal cell to the adipocytic lineage and synergizing with BMP2 to restrict differentiation towards the osteogenic lineage. This supports a role for PTHrP in cell fate determination that may prove to be an important component of its anabolic effect on the skeleton.

Figures

Figure 1

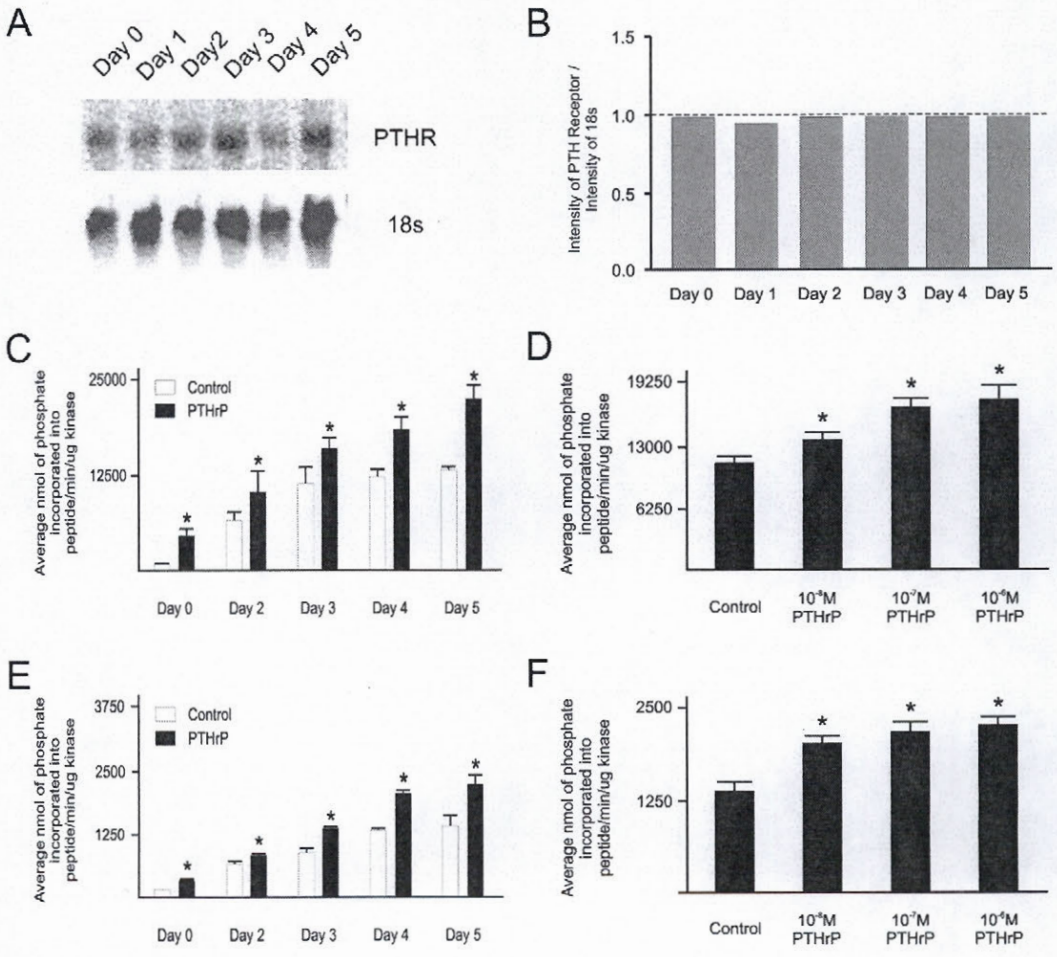
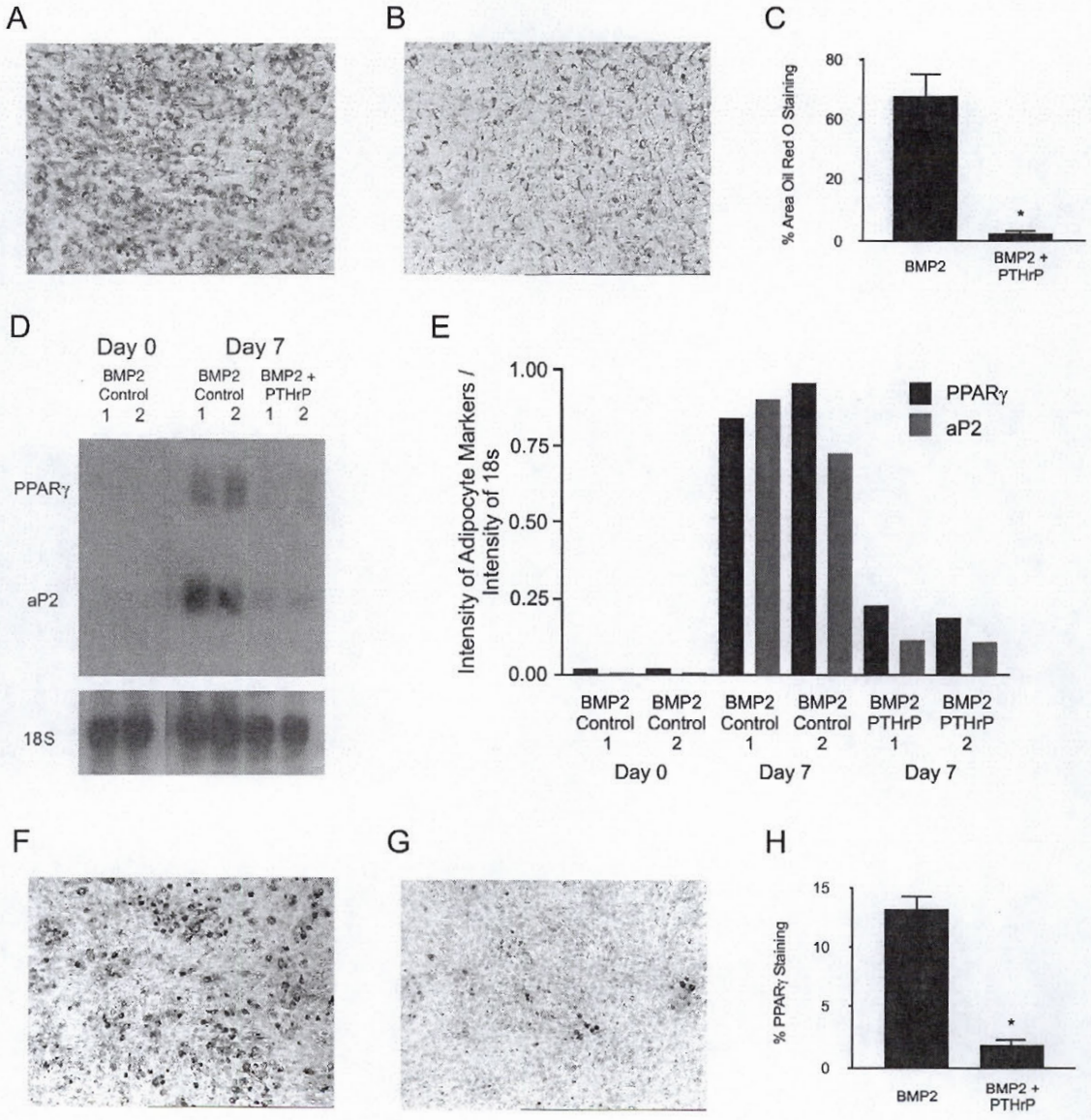


Fig 1. Influence of BMP2 on PTHR expression and function in C3H 10T½ cells. A. Northern analysis was performed to determine expression of PTHR over a 5 day incubation with BMP2 ($6x10^{-9}$M). 18s RNA levels were assessed as a control. B. Computer assisted quantification of Northern analysis was performed as described in MATERIALS AND METHODS. Relative levels of PTHR expression was standardized versus relative levels of 18s. C. PKA activity was measured as described in MATERIALS AND METHODS. Cells were incubated over 5 days with BMP-2 ($6x10^{-9}$M) and enzyme activity was determined after treatment with BMP2 plus vehicle (control) or with BMP2 plus PTHrP (10^{-7}M). D. Following 4 days of incubation with BMP-2, increasing concentrations of PTHrP were added and PKA activity was assessed. E. PKC activity was measured as described in MATERIALS AND METHODS and was determined under the same conditions as described for PKA. F. The effect of increasing concentrations of PTHrP on PKC activity was determined under the same conditions as described for PKA. For C, D, E and F, each bar represents the means ± SE of six replicates and asterisks represent significant differences from control at $p<0.05$.

Figure 2


A
B
C
% Area Oil Red O Staining
80
60
20
0
*
BMP2
BMP2 + PTHrP
D
Day 0
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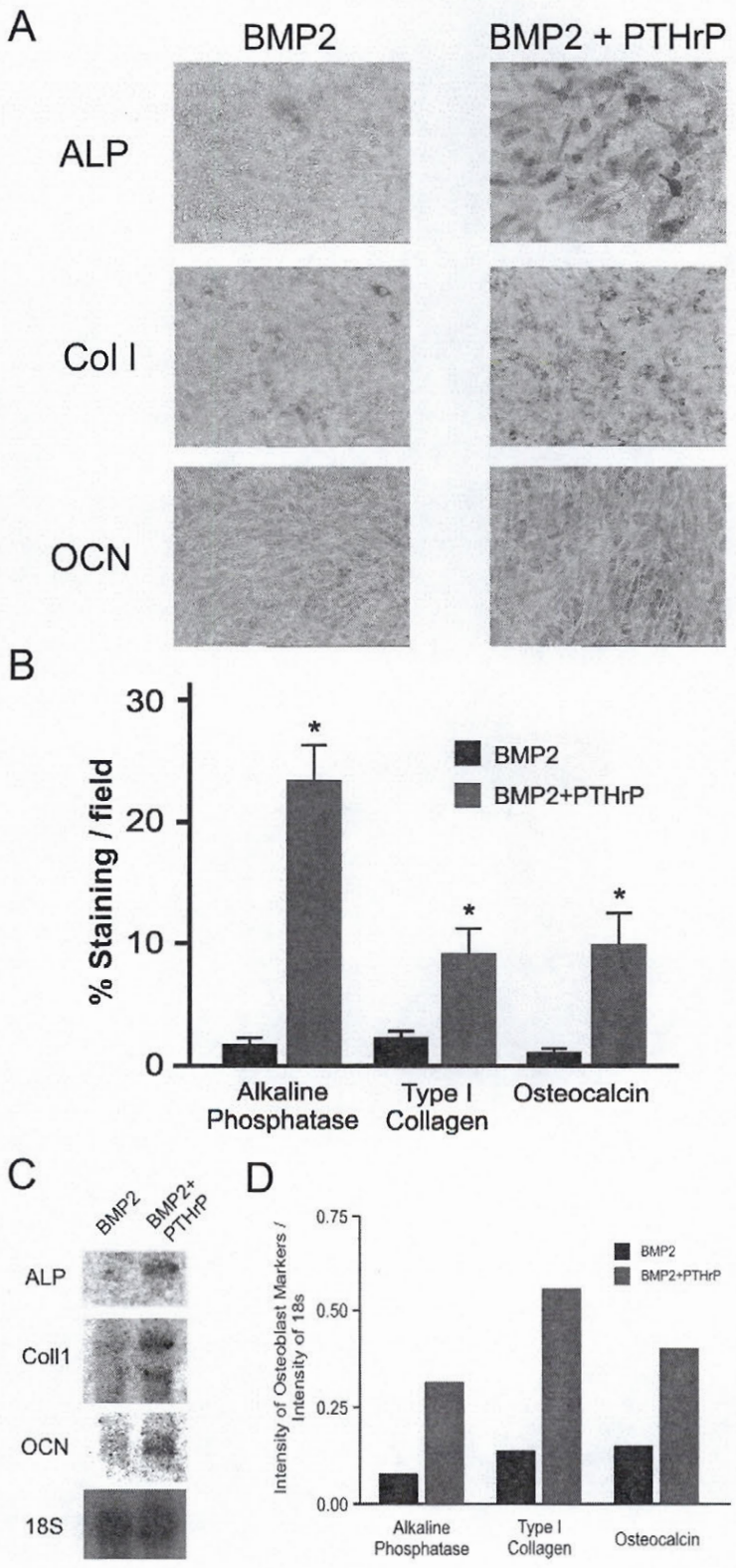
BMP2 Control 1 2
BMP2 Control 1 2
BMP2 + PTHrP 1 2
PPARγ
aP2
18S
E
Intensity of Adipocyte Markers / Intensity of 18s
1.00
0.75
0.50
0.25
0.00
PPARγ
aP2
BMP2 Control 1
BMP2 Control 2
BMP2 Control 1
BMP2 Control 2
BMP2 PTHrP 1
BMP2 PTHrP 2
Day 0
Day 7
Day 7
F
G
H
% PPARγ Staining
15
10
5
0
*
BMP2
BMP2 + PTHrP

Fig 2. Influence of BMP2 and PTHrP on adipogenesis in C3H 10T½ cells. A. Cells were incubated with BMP2 ($6x10^{-9}M$) alone for 14 days and then stained with oil red O as described in MATERIALS AND METHODS. B. Cells were incubated with BMP2 ($6x10^{-9}M$) and PTHrP ($10^{-7}M$) for 14 days and then processed as described in A. Each photomicrograph in A and B is representative of 3 random fields from 3 different cultures. C. Quantification of oil red O staining per field was performed with Northern Eclipse software as described in MATERIALS AND METHODS. Cells were treated with BMP2 ($6x10^{-9}M$) alone (left bar) or with BMP2 and PTHrP ($10^{-7}M$) (right bar). Each bar represents the mean ± SE of triplicate determinations using 3 random fields from 3 different cultures and the asterisks represent a significant difference at $P < 0.05$. D. Northern blots of PPARγ and aP2 were performed as described in MATERIALS AND METHODS and are shown in the upper panel. RNA was extracted from cells prior to treatment (Day 0) and on Day 7 after treatment. Cells were treated with BMP2 ($6x10^{-9}M$) alone (control) or with BMP2 plus PTHrP ($10^{-7}M$) (PTHrP). Numbers represent two different experiments. Levels of mRNA encoding 18S were determined as a loading control (lower panel). E. Computer assisted quantification of Northern analysis was performed, whereby relative levels of the adipocytic markers PPARγ and aP2 were standardized versus relative levels of 18S. F. Cells were treated with BMP2 ($6x10^{-9}M$) alone for 14 days and then analyzed for PPARγ protein expression as described in MATERIALS AND METHODS. G. Cells were incubated with BMP2 ($6x10^{-9}M$) and PTHrP ($10^{-7}M$) for 14 days followed by analysis for PPARγ protein expression. Photomicrographs in F and G are each representative of 3 random fields from 3 different cultures. H.

Quantitation of PPARγ protein expression was performed using Northern Eclipse software as described in MATERIALS AND METHODS. C3H 10T½ cells were treated with BMP2 ($6x10^{-9}$M) alone (left bar) or with BMP2 and PTHrP (10^{-7}M) (right bar). Each bar represents the mean ± SE of triplicate determinations using 3 random fields from 3 different cultures and the asterisks represent a significant difference at $P < 0.05$.

Figure 3


A
BMP2
BMP2 + PTHrP
ALP
Col I
OCN
B
% Staining / field
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BMP2
BMP2+PTHrP
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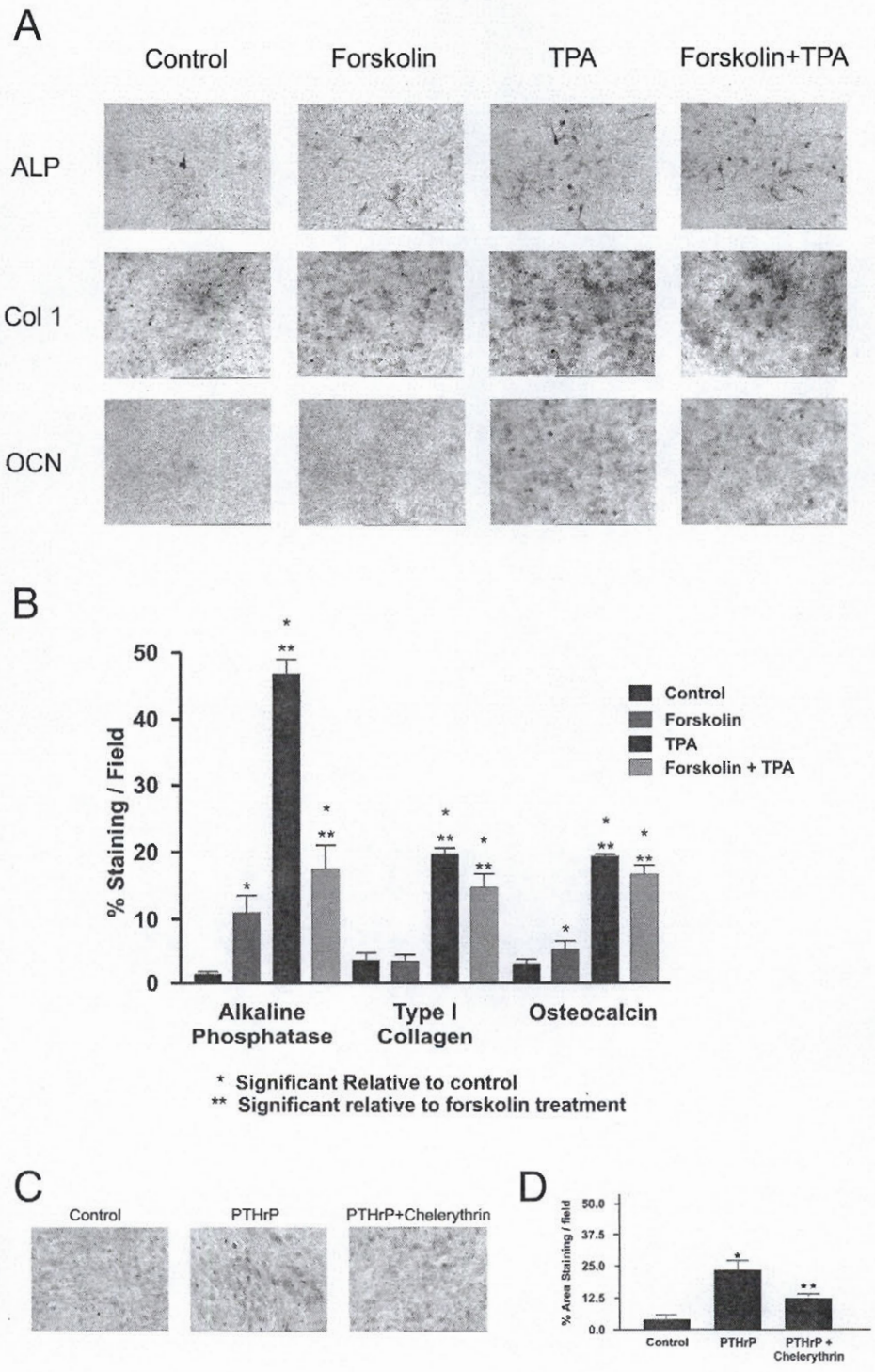
Alkaline Phosphatase
Type I Collagen
Osteocalcin
C
BMP2
BMP2+ PTHrP
ALP
Coll1
OCN
18S
D
Intensity of Osteoblast Markers / Intensity of 18s
0.75
0.50
0.25
0.00
BMP2
BMP2+PTHrP
Alkaline Phosphatase
Type I Collagen
Osteocalcin

Fig 3. Effects of BMP2 and of PTHrP on osteoblast commitment/differentiation. A. C3H 10T½ cells were incubated with BMP2 ($6x10^{-9}$M) alone or with BMP2 plus PTHrP (10^{-7}M) and after 14 days, were examined for expression of alkaline phosphatase (ALP), type I collagen (Col I) and osteocalcin (OCN) protein as described in MATERIALS AND METHODS. Photomicrographs are representative of 3 different fields from 4 different cultures. B. Expression of alkaline phosphatase, type I collagen and osteocalcin after treatment with BMP2 ($6x10^{-9}$M) alone or with BMP2 and PTHrP was quantified using Northern Eclipse imaging software as described in MATERIALS AND METHODS and is represented as a per cent of the field which stained positively for each osteoblastic marker (% staining / field). Each bar represents the mean ± SE of 3 random fields per culture from 4 different cultures. Asterisks represent significant differences from control (BMP2 alone) at $p < 0.05$. C. Expression of mRNA encoding alkaline phosphatase, type I collagen and osteocalcin was determined in control and PTHrP treated cells as described in MATERIALS AND METHODS. mRNA loading was assessed by probing with 18S. D. Quantification of Northern analysis was performed to determine expression of osteoblastic markers in cells treated with BMP2 ($6x10^{-9}$M) and cells treated with BMP2 and PTHrP. Computer assisted quantification of Northern analysis was performed as described in MATERIALS AND METHODS. Relative levels of alkaline phosphatase, type I collagen and osteocalcin expression was standardized versus relative levels of 18S.

Figure 4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Control
Forskolin
TPA
Forskolin+TPA
ALP
Col 1
OCN
B
% Staining / Field
50
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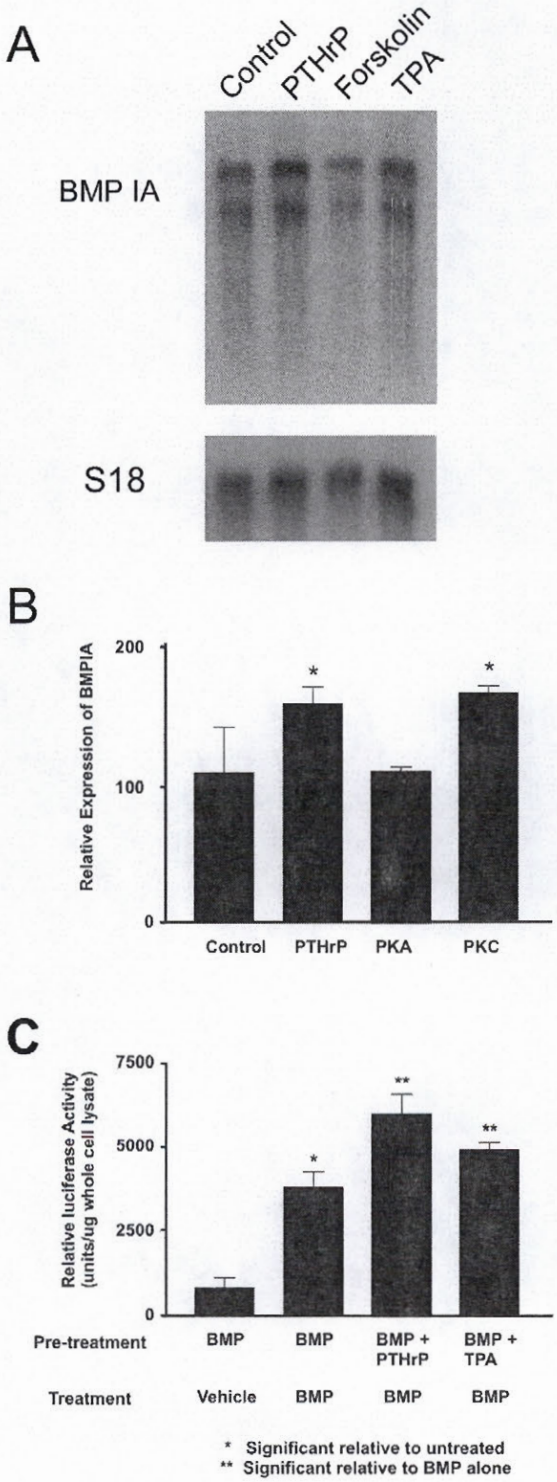
30
20
10
0
Control
Forskolin
TPA
Forskolin + TPA
Alkaline Phosphatase
Type I Collagen
Osteocalcin
* Significant Relative to control
** Significant relative to forskolin treatment
C
Control
PTHrP
PTHrP+Chelerythrin
D
% Area Staining / field
50.0
37.5
25.0
12.5
0.0
Control
PTHrP
PTHrP + Chelerythrin

Fig. 4. Effect of PKA and PKC pathways on osteoblast commitment/differentiation. A. C3H 10T½ cells were incubated for 14 days with BMP2 ($6x10^{-9}$M). On day 4 of this incubation, vehicle (control), forskolin (100 μM), TPA (1 μM) or both forskolin (100 μM) and TPA (1 μM) were added for 24 hours. After the 14-day incubation period, cells were examined for expression of alkaline phosphatase (ALP), type I collagen (Col 1) and osteocalcin (OCN) as described in MATERIALS AND METHODS. Photomicrographs are representative of 3 random fields from 3 different experiments. B. Expression of the markers shown in A was quantitated using Northern Eclipse imaging software as described in MATERIALS AND METHODS. The results are represented as a percent of the field that stained positively for each osteoblastic marker (% staining / field). Each bar represents the mean ± SE of triplicate fields from 3 different experiments. Asterisks represent significant differences from control (BMP2 alone) at $p < 0.05$. Double asterisks represent significant differences relative to BMP2 and forskolin treatment at $p < 0.05$. C. C3H 10T½ cells were incubated for 14 days with BMP2 ($6x10^{-9}$M) alone or in combination with PTHrP (10^{-7}M) or PTHrP and Chelerythrin Chloride (10^{-6}M). Following the 14 day incubation period, expression of osteocalcin was determined by immunocytochemistry. Photomicrographs are representative of 3 random fields from 3 different experiments. D. Quantitation of osteocalcin staining was performed as described in MATERIALS AND METHODS. Each bar represents the mean ± SE of 3 fields per culture from 3 separate cultures. The asterisk and double asterisk represent significant differences ($p < 0.05$) from BMP alone (control) and from BMP plus PTHrP respectively.

Figure 5


A
Control
PTHrP
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Control
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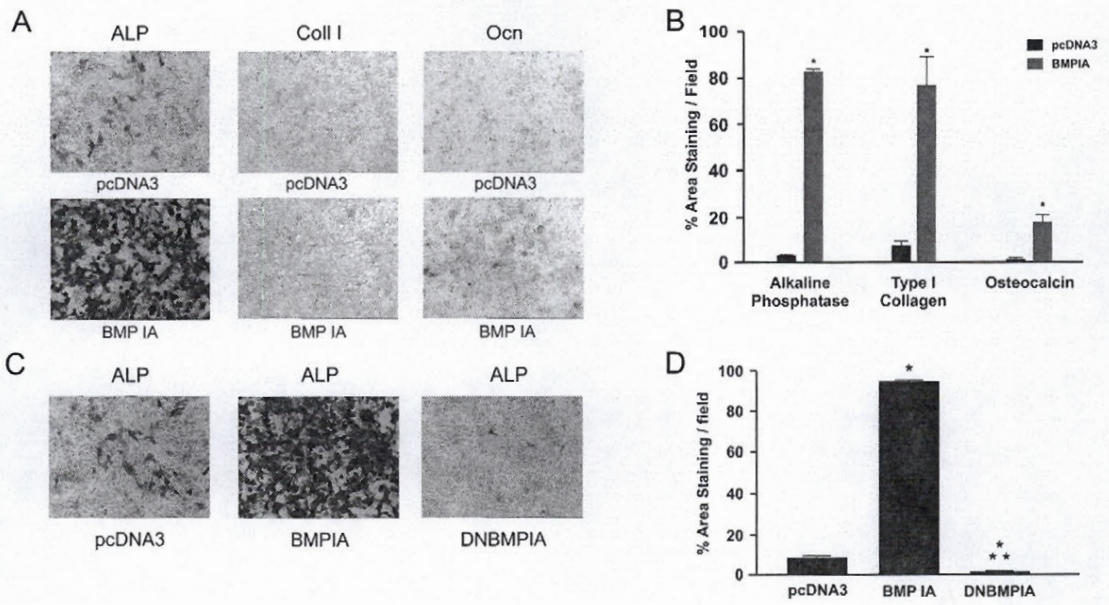
PKA
PKC
C
Relative luciferase Activity (units/ug whole cell lysate)
7500
5000
2500
0
*
**
**
Pre-treatment
BMP
BMP
BMP + PTHrP
BMP + TPA
Treatment
Vehicle
BMP
BMP
BMP
* Significant relative to untreated
** Significant relative to BMP alone

Fig. 5. Effect of PTHrP and of PKA and PKC agonists on the BMP-IA receptor. A. Northern blot of the BMP-IA receptor (upper panel). RNA was extracted from cells treated with BMP2 ($6x10^{-9}$M) alone (control), BMP2 plus PTHrP (10^{-7}M) (PTHrP); BMP2 plus forskolin (100 μM) (Forskolin); or BMP2 plus TPA (1 μM) (TPA) under conditions preparing them for a luciferase reporter assay as described in MATERIALS AND METHODS. Levels of 18S mRNA were concomitantly determined as a loading control (lower panel). Incubations and blots were performed as described in MATERIALS AND METHODS. B. Quantification of BMP-IA expression from 3 independent Northern blots. Analysis was performed using Scion image with values obtained by determination of relative BMP-IA expression divided by their respective 18S mRNA levels. Forskolin treatment is denoted as Fsk. C. Luciferase reporter activity in C3H 10T½ cells stably transfected with a SMAD 6 promoter-luciferase reporter construct as described in MATERIALS AND METHODS. Cells were pre-treated with BMP2 ($6x10^{-9}$M) alone; or with BMP2 plus PTHrP (10^{-7}M); or with BMP2 plus TPA (1 μM) as described in MATERIALS AND METHODS. Cells were then treated with vehicle (PBS) or with BMP2 ($6x10^{-9}$M) alone. Each bar represents the mean ± SE of quadruplicate determinations. Single asterisks represent significant differences relative to treatment with vehicle at $p< 0.05$. Double asterisks represent significant differences relative to pre-treatment with BMP2 alone followed by treatment with BMP2 alone at $p < 0.05$.

Figure 6


A
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BMP IA
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% Area Staining / Field
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pcDNA3
BMPIA
Alkaline Phosphatase
Type I Collagen
Osteocalcin
C
ALP
ALP
ALP
pcDNA3
BMPIA
DNBMPIA
D
% Area Staining / field
100
80
60
40
20
0
pcDNA3
BMP IA
DNBMPIA

Fig. 6. Effect of overexpression and of inhibition of the BMP-IA receptor on the osteoblastic phenotype. A. C3H 10T½ cells were transfected with the empty pcDNA3 vector (top three panels) or with the vector expressing the BMP-IA receptor (bottom three panels), then incubated with BMP2 ($6x10^{-9}$M) and then stained for alkaline phosphatase, collagen type I and osteocalcin as described in MATERIALS AND METHODS. Photomicrographs are representative of 4 random fields from 4 different experiments. B. Quantitation of alkaline phosphatase(ALP), collagen type I(CollI) and osteocalcin(OCN) staining as described in MATERIALS AND METHODS after treatment with BMP2 ($6x10^{-9}$M), of cells transfected with the empty pcDNA3 vector or with the vector expressing the BMP-IA receptor. Each bar represents the mean ± SE of 4 random fields per culture from 4 separate cultures. The asterisk represents a significant difference of $p < 0.05$. C. C3H 10T½ cells were transfected as described in MATERIALS AND METHODS with the empty pcDNA3 vector (left panel) or with the vector expressing the BMP-IA receptor, BMP-IA (middle panel) or with the vector expressing a dominant negative BMP-IA receptor, DNBMP-IA (right panel). Cells were then incubated with BMP2 ($1x10^{-8}$M) and stained for alkaline phosphatase. Photomicrographs are representative of 4 random fields from 4 different experiments. D. Quantitation of alkaline phosphatase staining, as described in MATERIALS AND METHODS, after treatment with BMP2 ($1x10^{-8}$M) of cells transfected with the empty pcDNA3 vector, the vector expressing the BMP-IA receptor or the vector expressing the DNBMP-IA receptor. Each bar represents the mean ± SE of measurements of 4 random fields per culture from 4

separate cultures. The single asterisk and the double asterisk represent a significant difference ($p < 0.05$) from pcDNA3 and BMP-IA respectively.

PREFACE TO CHAPTER 4

Previous studies have described PTHrPs ability to localize to the nucleus of various cell types where it has demonstrated pleiotropic effects. To date, no nuclear partners have been identified for PTHrP. We therefore performed a yeast two-hybrid search using PTHrP (1-141) as the bait protein, to screen an osteosarcoma yeast two-hybrid library for nuclear partners that may serve a role in the commitment and differentiation of mesenchymal cell lineages.

Chapter 4

Nuclear PTHrP and the Nuclear Orphan Receptor Rev ErbA Coordinate Adipocytic and Osteoblastic Differentiation of Progenitor Cells *In Vitro*

ABSTRACT

Parathyroid Hormone Related Peptide (PTHrP) is a secreted factor that has demonstrated multiple functions in the differentiation processes of adipocytes, osteoblasts and chondrocytes(48, 51, 166). Asides from functioning in an autocrine/paracrine manner by stimulation of the PTH/PTHrP G-coupled receptor (PTH-1R), an intracrine nuclear function for PTHrP has been identified in chondrocytic CFK-2 cells, the breast cancer cell line MCF-7, the prostate cancer cell line PC-3 and primary cultures of vascular smooth muscle cells (VSMCs) (74, 133, 148, 247, 357). Although many functions have been attributed to nuclear PTHrP, such as enhancing proliferation and survival of cells under conditions that favor apoptosis(74, 133, 148, 247, 357), its mechanistic action is uncertain. We therefore employed the yeast two-hybrid system to identify potential nuclear partners for PTHrP. By screening an osteosarcoma yeast two-hybrid library, we identified the nuclear orphan receptor, Rev ErbA, as being a potential nuclear partner for PTHrP. We determined that the interaction of Rev ErbA with PTHrP is dependent on amino acid residues (1-36) of PTHrP. Overexpression analysis of a nuclear form of PTHrP with Rev ErbA during the differentiation process in 3T3-L1 pre-adipocytes and C3H 10T½ pluripotent mesenchymal cells demonstrated that overexpression of nuclear PTHrP alone is able to inhibit development of the adipocytic phenotype of 3T3-L1 pre-adipocytes and pluripotent mesenchymal C3H 10T½ cells as assessed by Oil O Red staining for triacylglycerol uptake. Furthermore, overexpression of Rev ErbA in combination with a nuclear form of PTHrP significantly enhanced osteogenesis of C3H 10T½ cells when treated with low levels of BMP2. We therefore describe a novel nuclear

function for nuclear PTHrP involving the inhibition of adipogenesis and for both nuclear PTHrP and Rev ErbA in coordinating osteoblast differentiation.

INTRODUCTION

The commitment and differentiation of mesenchymal stem cells is a regulated process that is guided by factors that either promote or inhibit development of terminally differentiated cell types such as adipocytes, fibroblasts, osteocytes and chondrocytes(47). Parathyroid Hormone Related Peptide (PTHrP) is a developmental factor that serves an essential role in endochondral bone formation(191) and has been shown to influence the differentiation process of chondrocytes, osteoblasts and adipocytes in an autocrine/paracrine manner. Stimulation of the G-protein coupled PTH/PTHrP receptor (PTH1-R) by PTHrP results in the inhibition of chondrogenesis and adipogenesis(51, 166)and enhanced osteogenesis(48). In addition to functioning in an autocrine/paracrine manner, *in vivo* and *in vitro* data suggests that PTHrP serves an intracrine function as well, by gaining access to the nucleus through a non-classical nucleolar localization sequence(NLS) (149) or by an NLS independent mechanism(133). The intracrine function of PTHrP appears to serve multiple functions in various cell types. In the chondrocytic cell line CFK-2 and the breast cancer cell line MCF-7, overexpression of a non-secreted nuclear form of PTHrP results in reduced apoptosis and enhanced cell survival under conditions of serum deprivation(149, 357). The intracrine effect of PTHrP has also been shown to be mitogenic in vascular smooth muscle cells (VSMCs), in as much as VSMCs of PTHrP deficient animals demonstrate reduced proliferation even when cultured in the presence of PTHrP(74, 247). This mitogenic effect of nuclear

PTHrP has also been demonstrated in the prostate cell line PC-3, whereby overexpression of both PTHrP (1-87) and (1-173) resulted in increased cell proliferation apparently via a nuclear mechanism(133).

In this study, we employed a yeast two hybrid screen using PTHrP as bait to identify functional nuclear partners for the intracrine function of PTHrP. A search of an osteosarcoma library led to the identification of Rev ErbA as being an intracellular partner for PTHrP. The interaction of PTHrP and Rev ErbA appears to be dependent upon the amino-terminal region of PTHrP (1-36) and co-expression of both a nuclear form of PTHrP and Rev ErbA was found to synergistically promote osteogenesis in the clonal C3H 10T½ cell line suggesting a role for Rev ErbA and intracrine PTHrP in osteoblast differentiation. Furthermore, we found that nuclear PTHrP by itself is able to inhibit adipocyte differentiation in 3T3-L1 pre-adipocytes and pluripotent mesenchymal C3H 10T½ cells.

MATERIALS AND METHODS

Plasmids and Constructs – The full length cDNA for Rev ErbA (provided by Vincent Giguere) was cloned into the pBudCE4 (Invitrogen) and the pCDNA3 (Invitrogen) plasmid in the unique HindIII site. A *myc* tag was introduced at the carboxy-terminus of Rev ErbA by excising the carboxy terminus of Rev ErbA with ScaI and SapI from the pBudCE4 plasmid which was subsequently replaced with a *myc* PCR product generated with oligos 5'CCGGGAGTACTCTGGGCGTCCACCCGGAA3' and 5'CATGGAGAATTCCGCTTCGGTGGA3' digested with ScaI and SapI. The full length cDNA for rat PTHrP 1-141 lacking the leader sequence (PTHrP(-SS))was excised from

the previously described PTHrP(-SS)-pCDNA1(149) construct using restriction enzymes KpnI and XhoI and cloned into the respective restriction sites in the pBudCE4 plasmid. pBudCE4-Rev ErbA+PTHrP(-SS) was generated by sequentially cloning PTHrP(-SS) into the KpnI-XhoI sites of pBudCE4 followed by cloning of the cDNA for Rev ErbA into the unique HindIII site.

Bait constructs were generated by cloning in frame, various cDNAs representing different functional domains of the rat PTHrP. The different cDNAs were generated by PCR as follows. PTHrP (1-36), PTHrP (1-87) were generated using the 3' oligos, 5'GGCCCCGGATCCGATTTCAGCTGTGTGGATCTCC3' and 5'GGCCCCCGGATCCCCCGGGCGTTCTTGAGTGGCTGC' respectively. Both PTHrP (1-36) and PTHrP (1-87) employed a similar 5' oligo composed of 5'CGGGGGAATTCGCGGTGTCTGAGCACCAGCTAC3'. The PCR products were digested with BamHI and EcoRI and cloned into their respective sites in the pAS2-1 plasmid (Clontech, BD Biosciences), placing the coding sequence in frame with the Gal4 DNA binding domain. The PTHrP (87-107) cDNA was excised from the p87-107bgal construct(148) using PstI and ligated into the complementary restriction site in the pAS2-1 plasmid, placing the sequence in frame with the DNA binding domain of Gal4.

Yeast Two Hybrid – The yeast two hybrid screening was performed using the plasmids provided by the MATCHMAKER Two Hybrid System 2 (Clontech, BD Biosciences) with the yeast strain PJ69-4a that bears the two reporter genes GAL2:ADE2 and GAL1:HIS3(177) (provided by Philip James). Media for maintaining the yeast strain and for detecting PTHrP interacting proteins was prepared as previously described(309). Transformation of yeast was performed using the high efficiency LiAC method, as

previously described(124). Identification of interacting partners for PTHrP was determined using a yeast two hybrid osteosarcoma library (Clontech, BD Biosciences). The cDNA of positive clones was amplified using oligos and PCR conditions as described by the manufacturer (Clontech, BD Biosciences). β-galactosidase activity of yeast strains was determined, as previously described(177).

Co-Immunoprecipitation of PTHrP and Rev ErbA – Radiolabelled *in vitro* translation products of PTHrP(-SS), Rev ErbA and Rev ErbA(myc) were performed using TNT Quick Coupled Transcription/Translation Systems as described by the manufacturer (Promega) with the PTHrP(-SS)-pCDNA1 construct, Rev ErbA-pCDNA3 construct and the Rev ErbA(myc)-pBudCE4 construct serving as template. Co-immunoprecipitation of proteins was performed using the MatchMaker Co-IP Kit (Clontech) and a monoclonal myc antibody (Santa Cruz Biotechnology).

Cell Culture – The C3H 10T½ Clone 8 cell line was obtained from ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat inactivated fetal calf serum (FCS). Fresh medium was applied every second day. To induce adipocyte commitment and differentiation, these cells were grown to confluence then maintained in alpha minimum essential medium (αMEM) supplemented with ascorbic acid (100 μg/ml), β-glycerophosphate (5mM) and BMP2 (Research Diagnostics Inc, Flanders, NJ) ($2x10^{-9}$M), unless stated otherwise. Fresh medium was applied every two days. The 3T3-L1 pre-adipocytic cell line was obtained from ATCC and maintained in DMEM containing 10% heat inactivated FCS, with fresh media being applied every second day. To induce adipocytic differentiation of 3T3-L1 cells, cells were allowed to grow to confluence followed by treatment with DMEM-10% FCS supplemented with 0.5

M isobutylmethylxanthine (IBMX), 1 μM dexamethasone, and 5 μg/ml insulin. Following 18 hours of exposure to differentiation medium, cells were subsequently cultured in DMEM-10% FCS supplemented with 5 μg/ml insulin which was refreshed every second day for 14 days.

Transfection of cells was performed using FUGENE6 reagent (Boerhinger Mannheim) and selection was performed using 300 μg of Zeocin (Invitrogen) per ml of medium.

Cell Staining and Immunocytochemistry - Cultured cells were stained for type I collagen and osteopontin using the avidin-biotin-peroxidase complex (ABC) technique, as described previously. Cells were first treated with 0.5% bovine testicular hyaluronidase (Sigma, St. Louis, MO) for 30 minutes at 37°C which was followed by presentation of primary antibodies affinity-purified goat anti-human type I collagen antibody (Southern Biotechnology Associates, Inc., Birmingham, AL) overnight at room temperature. As a negative control, the pre-immune serum was substituted for the primary antibody. Washing was performed with high salt buffer (50mM Tris-HCl, 2.5% NaCl, 0.05% Tween 20, pH 7.6) for 10 min at room temperature followed by 2x10 min washes with TBS (50mM Tris-HCl, 150 mM NaCl, 0.01% Tween 20, pH 7.6) followed by incubation of the cells with a secondary antibody (biotinylated rabbit anti-goat IgG (Sigma, St. Louis, MO)). Cells were then washed as before and incubated with the Vectastain ABC-AP kit (Vector Laboratories, Ontario, Canada) for 45 min. The washing was repeated as before, and red pigmentation was used to identify regions of immunostaining following 10-15 min treatment with Fast Red TR/Naphthol AS-MX phosphate (Sigma, St. Louis,

MO) containing 1 mM levamisole as endogenous ALP inhibitor. Oil Red O and alkaline phosphatase staining was performed, as previously described(51, 258).

RESULTS

PTHrP and Rev ErbA Direct Expression of the Adenine and Histidine and β-Galactosidase Reporter Genes in the PJ69-4a yeast Strain – The PJ69-4a yeast strain was first transformed with the PTHrP bait plasmid (pAS2-1-PTHrP 1-141) with positive clones employed for screening of the osteosarcoma library. In total, $1x10^6$ independent clones were screened, of which, 15 were viable on media lacking histidine and adenine and tested positive for B-galactosidase activity. Of the 15 clones, 1 represented the partial sequence of the cDNA for Rev ErbA (Fig 1a) spanning 1775 nucleotides (Rev1775) of the coding sequence and representing 591 amino acids. To determine the regions of PTHrP that are important for the observed interaction we generated bait fusion proteins employing truncated forms of PTHrP (1-36), (1-87) as well as the NLS (87-107) (Figure 1b). We found that Rev1296 was able to direct expression of the reporter genes adenine, histidine and β-galactosidase in combination with all the PTHrP bait constructs except for the NLS (87-107) fusion protein suggesting that the amino-terminus of PTHrP is required for coordinating the PTHrP-Rev ErbA transcriptional activating complex (Table 1).

PTHrP and Rev ErbA do not Co-Immunoprecipitate - To further characterize the interaction of PTHrP and Rev ErbA, we attempted to co-immunoprecipitate the two proteins. Full length Rev ErbA was *myc*-tagged in its carboxy terminus to facilitate the antibody recognition of the protein. The radio-labeled *in vitro* translated products in combinations of PTHrP and Rev ErbA(*myc*), and PTHrP and Rev ErbA were co-

immunoprecipitated using a *myc* antibody. Although Rev ErbA(*myc*) was effectively recognized by the *myc* antibody, PTHrP did not co-precipitate suggesting that either the interaction is very weak or that a coordinating protein may be involved in the trans-activating potential of the two proteins in the yeast system.

Nuclear PTHrP Inhibits the Terminal Differentiation of 3T3-L1 Pre-Adipocytes – Previous studies examining the expression profile of Rev ErbA demonstrated the upregulation of its mRNA during the terminal differentiation of pre-adipocytic 3T3-L1 cells(52). As PTHrP has also been shown to affect the differentiation of 3T3-L1 cells, we examined the effects of overexpressing Rev ErbA and of a non-secreted leaderless form of PTHrP (PTHrP(-SS)) on the terminal differentiation of these cells. PTHrP(-SS)) had been previously shown to localize exclusively to the nucleus in COS-7 and CFK2 cells(148). We stably transfected 3T3-L1 cells with the pBudCE4-PTHrP(-SS), pBudCE4-Rev ErbA, pBudCE4-PTHRP(-SS)-Rev ErbA and empty pBudCE4 plasmid as control. Following differentiation, the cells were stained with Oil O Red to assess lipid accumulation which is consistent with the mature adipocyte phenotype. 3T3-L1 cells transfected with pBudCE4-PTHrP(-SS) and pBudCE4- Rev ErbA+PTHRP(-SS) were resistant to triacylglycerol uptake, whereas pBudCE4-Rev ErbA and the control pBudCE4 transfected 3T3-L1 were able to accumulate lipid droplets (Figure 2). The effect of pBudCE4-Rev ErbA+PTHrP(-SS) was similar to that of pBudCE4-PTHrP(-SS) alone (Figure 2).

PTHrP and Rev ErbA Enhance Osteogenesis of C3H 10T½ Cells – Due to the inhibitory effect of PTHrP(-SS) and PTHrP(-SS) in combination with Rev ErbA on lipid accumulation in 3T3-L1 cells, we further examined whether PTHrP(-SS) and Rev ErbA

might influence the effects of adipogenic doses of BMP2 on the differentiation of C3H 10T½ cells. C3H 10T ½ cells were stably transfected with the empty pBudCE4 plasmid, pBudCE4-PTHrP(-SS), pBudCE4-Rev ErbA, pBudCE4-PTHRP(-SS)-Rev ErbA. Following 14 days of induction with BMP2, the cells were stained with Oil O Red to assess triacylglycerol accumulation. Cells transfected with PTHrP(-SS) alone and Rev ErbA+PTHrP(-SS) were resistant to BMP2 induced adipogenesis of C3H 10T½ cells whereas Rev ErbA alone and control pBudCE4 transfected cells had no effect on adipogenesis (Figure 3). The effect of the combination of Rev ErbA+PTHrP(-SS) was again similar to that of PTHrP(-SS) alone (Figure 2)

Asides from the inhibitory effect that PTHrP has demonstrated on adipogenesis, PTHrP has also demonstrated a positive effect on osteogenesis(48). We therefore investigated whether Rev ErbA and PTHrP(-SS) can affect osteoblastic differentiation of C3H 10T½ cells. The same transfected C3H 10T½ cells were induced to undergo adipogenesis with low levels of BMP2. Following 14 days of induction, immunocytochemistry was performed on these cells to determine expression of the osteoblastic markers Collagen Type I (Coll I) and alkaline phosphatase (ALP). Neither PTHrP(-SS) nor the control pBudCE4 transfected cells demonstrated expression of the Coll I or OPN following induction with low levels of BMP2. On the other hand, Rev ErbA alone was able to significantly induce these indices of osteoblast differentiation. The combination of PTHrP(-SS)+Rev ErbA however demonstrated significant osteoblast differentiation as assessed by Coll I and ALP staining (Figure 4).

Discussion

Osteoblasts, chondrocytes, adipocytes and myoblasts all represent specific cell lineages that are derived from mesenchymal stem cells(299). The differentiation of each of these cell types follows an orderly progression from an immature committed progenitor cell which culminates in a functional cell with a phenotype that is able to serve a specific biological function such as lipid storage for adipocytes, and bone matrix synthesis by osteoblasts(47). The terminal differentiation of their respective progenitor cell can be influenced by secreted factors that function in an autocrine/paracrine manner to either facilitate or disrupt their terminal differentiation(62, 76). PTHrP is a secreted factor that has demonstrated the ability to influence the differentiation process of adipocytes, osteoblasts and chondrocytes by stimulation of the PTH-1R G-coupled receptor(48, 51, 166). In addition to functioning in an autocrine and paracrine manner by stimulating its cognate cell membrane receptor, PTHrP has also been shown to localize to the nucleus *in vivo* in murine bone cells and can affect cell biology in a variety of ways.

In vitro, PTHrP has been shown to localize to the nucleolus in chondrocytic CFK2 cells, thereby protecting them from undergoing serum deprivation induced apoptosis(148). This ability of PTHrP does not appear to be limited to chondrocytic cells as the breast cancer cell line MCF-7 also demonstrates enhanced survival under similar conditions(357). In other cell types, nuclear PTHrP appears mitogenic as VSMCs from PTHrP deficient mice demonstrate reduced proliferation in spite of the addition of exogenous PTHrP; whereas in the prostate cancer cell line, PC-3, nuclear accumulation of PTHrP was shown to upregulate interleukin-8 (IL-8) production, resulting in increased cell proliferation(133).

Intracellular partners have been identified for PTHrP that are associated with trafficking of PTHrP to the nucleus, however, it remains unclear what intranuclear factors may mediate the nuclear function of PTHrP. In order to address this question, we employed the yeast two-hybrid methodology to identify nuclear partners for PTHrP. We found that PTHrP is able to interact with the nuclear orphan receptor Rev ErbA, via its NH_2-terminal domain. Rev ErbA is a nuclear orphan receptor that belongs to the steroid receptor superfamily of transcription factors(221, 222). It functions as a dominant transcriptional repressor by interacting with the nuclear co-repressors (N-Cors) RIP13a and RIP13delta1(80) and by binding as a monomer to an asymmetric 11 base pair (bp), WAWNTAGGTCA (W = A or T) motif(141) or as a homodimer to two tandemly arranged AGGTCA motifs separated by 2 bp with unique 5' flanking and spacer nucleotides (RevDR-2) (140). Although Rev ErbA is expressed in a variety of cell tissue types, mRNA for this protein is expressed at its highest levels in skeletal muscle, brain and adipose(105, 221). Although high levels of Rev ErbA expression in these particular tissues would suggest a role for Rev ErbA in myoblast and adipocyte biology, ablation of the Rev ErbA gene in mice demonstrate no defects of fat tissue or skeletal muscle(56). Instead, these animals demonstrate alterations in the development of Purkinje cells, delay in the proliferation and migration of granule cells from the external granule cell layer and increased apoptosis of neurons in the internal granule cell layer(56). *In vitro* studies on the other hand, have found that Rev ErbA expression decreases during the differentiation of C2C12 myoblasts and that overexpression of Rev ErbA in these cells abolishes their terminal differentiation and suppresses expression of myoD(81). Although no functional consequences have been observed for overexpressing Rev ErbA in pre-adipocytic cells,

expression profiling of Rev ErbA during adipocyte differentiation demonstrates that Rev ErbA mRNA levels increase significantly during the differentiation of 3T3-L1 and 3T3-F442A cells into mature adipocytes with induction resembling the expression pattern of C/EBP alpha, an important transcriptional regulator in adipocytes(52).

Previously, a role for PTHrP had been described in the differentiation process of adipocytes. PTHrP heterozygous null mice develop a premature form of osteoporosis that is characterized by increased adipogenesis within the bone marrow. This is believed to be a result of reduced levels (haploinsufficiency) of PTHrP(9). In addition, overexpression of a secreted form of PTHrP was shown to inhibit adipocyte differentiation in 3T3-L1 pre-adipocytes as well as the pluripotent mesenchymal C3H 10T½ cell line, however, the mechanism of this inhibitory effect was shown to be mediated by stimulation of the PTH-1R receptor(51). PTHrP has been shown to be expressed in 3T3-L1 pre-adipocytes, however, following induction to undergo terminal differentiation, PTHrP mRNA decreases(51). In contrast to the expression profile of PTHrP, Rev ErbA mRNA levels have been shown to increase over the same period of time(52). Because both PTHrP and Rev ErbA are expressed in 3T3-L1 cells, we examined the effects of overexpression of nuclear PTHrP with Rev ErbA on the adipocytic differentiation of these pre-adipocytes and also on adipocytic differentiation in pluripotent mesenchymal C3H 10T½ cells.

Although no physiological effects could be demonstrated for Rev ErbA following its overexpression in differentiating 3T3-L1 pre-adipocytes, overexpression of nuclear PTHrP was found to inhibit the terminal differentiation of these cells. Overexpression of both proteins in 3T3-L1 demonstrated a phenotype similar to that of the PTHrP transfected cells suggesting that either nuclear PTHrP alone is sufficient for the inhibitory

effect observed or that PTHrP is the limiting factor in this inhibitory process involving Rev ErbA.

In C3H 10T½ pluripotent mesenchymal cells, the results were similar to that in 3T3-L1 cells, such that overexpression of Rev ErbA alone in these cells demonstrated no effect, whereas overexpression of nuclear PTHrP or Rev ErbA+PTHrP was able to disrupt adipogenesis as assessed by Oil O Red staining.

Although PTHrP is generally known to be a secreted peptide, PTHrP may gain access to the nucleus by one of at least three described mechanisms: secreted PTHrP has been reported to be internalized and to gain nuclear access by this route(1); nascent PTHrP can be reverse transported from the secretory system to the cytoplasm and gain nuclear access by this route(254); and alternate initiation of translation of PTHrP may occur, bypassing the functional leader sequence and preventing PTHrP from gaining access to the secretory apparatus(273). The latter mechanism has also been reported for other secreted proteins such as FGF. Thus, although the ability of nuclear PTHrP to inhibit adipogenesis was somewhat surprising in lieu of our previous results that demonstrated a similar inhibitory effect mediated by extracellular exogenous PTHrP (1-36) via the PTH-1R, it is possible that important physiological regulators such as PTHrP may use redundant processes, perhaps under differing conditions, to effect similar key functions.

The reciprocal relationship regarding bone marrow adiposity and bone volume is associated with many forms of bone loss, including that associated with reduced levels of circulating PTHrP due to haploinsufficiency in mice(9, 125). It has been suggested that this inverse relationship is a result of the inappropriate differentiation of pluripotent

mesenchymal precursors that are inadvertently directed towards the adipocytic lineage as opposed to the osteoblastic lineage(9, 125, 280). PTHrP has also been shown to play a role in osteoblast biology by enhancing differentiation and stimulating proliferation of osteoblast progenitors, however, these effects are attributed to the extracellular effects of PTHrP acting upon the PTH-1R(48, 258). We therefore examined whether nuclear PTHrP in combination with Rev ErbA is able to affect osteoblast differentiation of C3H 10T ½ cells induced to undergo adipogenesis with BMP2.

We found that nuclear PTHrP by itself had no effect on osteoblast differentiation. Rev ErbA alone demonstrated slight osteogenic potential in C3H 10T ½ cells. The introduction of both PTHrP and Rev ErbA was however, found to dramatically increase osteoblast differentiation. We employed a leaderless form of PTHrP in our studies which limits the secretion of PTHrP from cells and promotes its nuclear localization. Consequently, it is most likely that the effects we have observed are due to its action within the nucleus. Nevertheless, high levels of PTHrP production, as in overexpression, may result in the escape of PTHrP from cells and permit it to act upon the PTH-1R in an autocrine/paracrine manner. As PTHrP is able to stimulate protein kinase C signaling and MAPK activity(48, 51, 258), PTHrP signaling may affect Rev ErbA function by post-translational modification. Several consensus PKC phosphorylation sites (amino acids 14, 16, 428, 608) and one consensus MAPK phosphorylation (amino acid 55) site are present within Rev ErbA. As a result, it is possible that PTHrP stimulation of its receptor leads to phosphorylation of Rev ErbA thus altering its repressive function.

In conclusion, our findings demonstrate a novel role for the nuclear orphan receptor Rev ErbA in osteoblast biology and demonstrate its ability to enhance osteoblast

differentiation of the C3H 10T ½ cell line. *In vivo,* Rev ErbA deficient animals do not demonstrate any defects in fat tissue but instead, demonstrate abnormalities associated with the cerebellum. However, no *in vivo* studies to date have examined the effect of Rev ErbA in bone. We also demonstrate that nuclear PTHrP is able to inhibit adipogenesis of the 3T3-L1 and C3H 10T½ cell lines. Furthermore, we show that nuclear PTHrP may co-ordinate with Rev ErbA to promote osteogenesis.

FIGURES

Figure 1

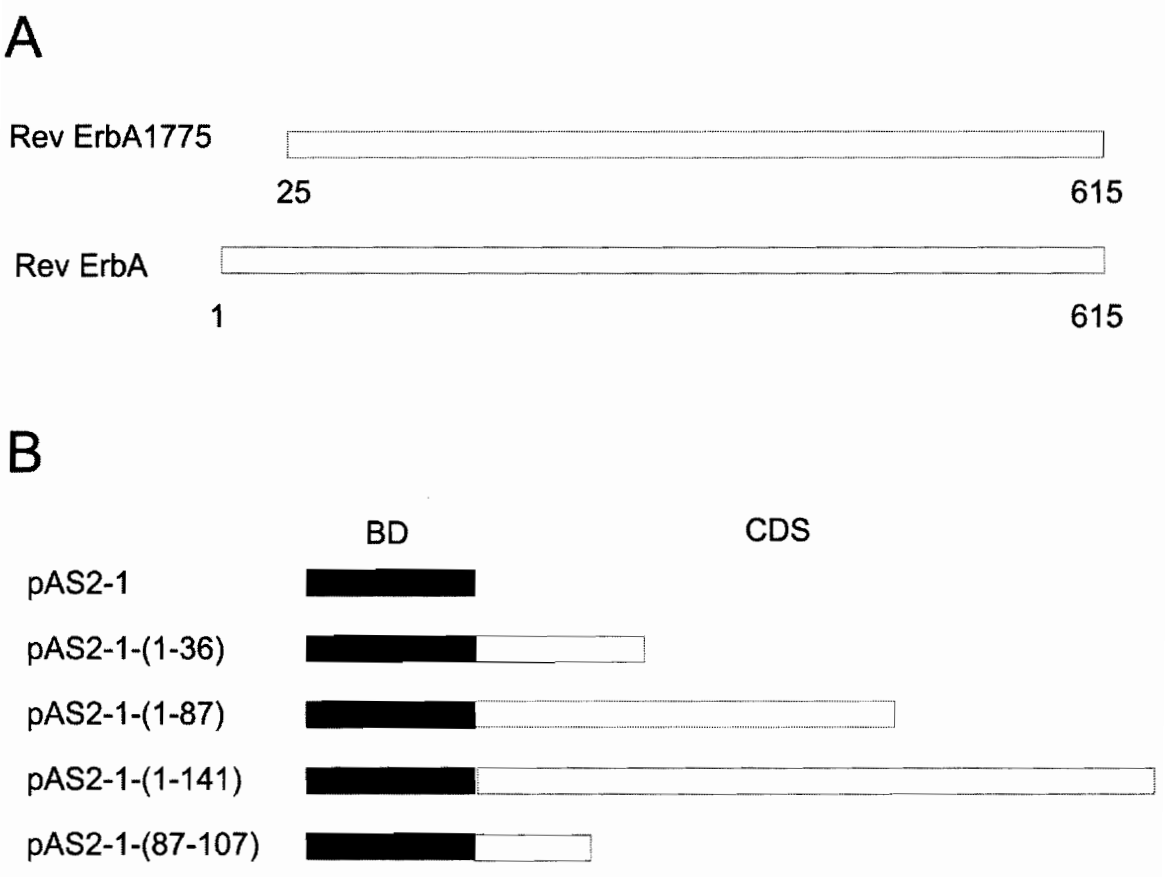

Fig 1. Rev ErbA and PTHrP Coordinate Expression of Yeast Selection Markers. A. A yeast two-hybrid osteosarcoma library was screened with the pAS2-1-(1-141) for interacting proteins. In total, $1x10^{-6}$ independent clones were screened. One positive clone was identified to be the partial cDNA of the nuclear Orphan Receptor Rev ErbA(1775). The cDNA represents 1775 nucleotides of the coding sequence (590 amino acids). B. PTHrP bait constructs were generated as described in MATERIALS AND METHODS and evaluated for their abilities to direct expression of yeast selection markers in combination with Rev ErbA(1775). The results are listed in Table 1.

Table 1

Bait	Adenine	Histidine	β-Gal
pAS2-1	-	-	-
1-36	+	+	+
1-87	+	+	+
1-141	+	+	+
87-107	-	-	-

Figure 2

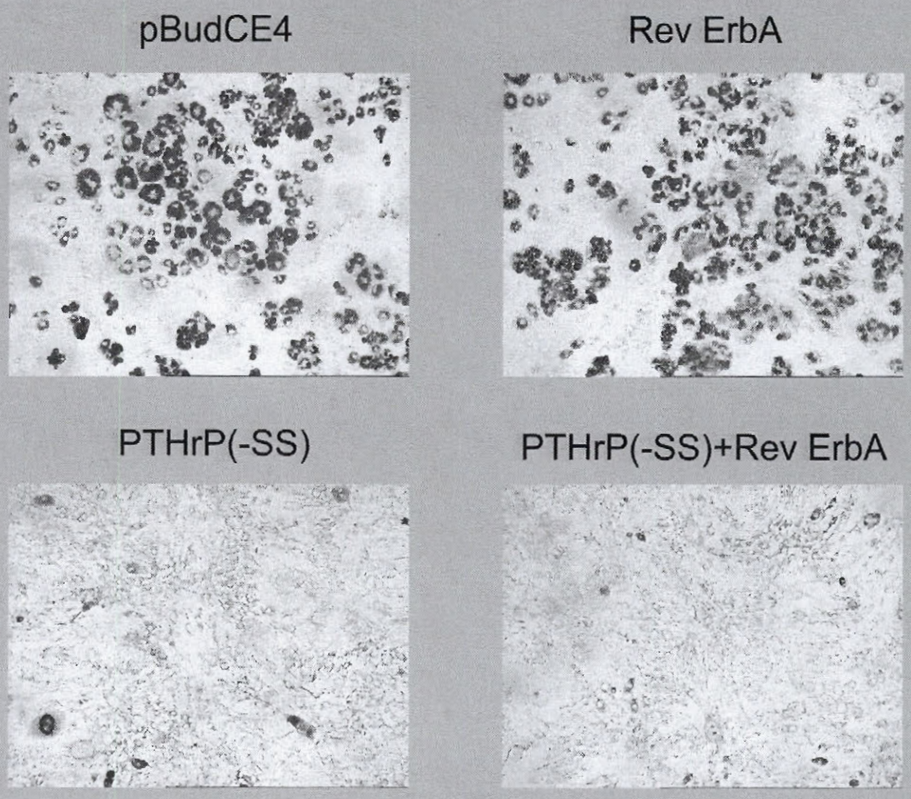

Fig. 2. Overexpression of Nuclear PTHrP Inhibits adipogenesis of 3T3-L1 Cells. 3T3-L1 cells overexpressing PTHrP(-SS), Rev ErbA or PTHrP(-SS) and Rev ErbA were induced to differentiate as described in MATERIALS AND METHODS. Following induction, cells were maintained in culture for 14 days then stained with Oil Red O as described in MATERIALS AND METHODS. Cells that stain red represent adipocytic uptake of triacylglycerol. Photomicrographs are representative of 3 random fields from 3 independent experiments.

Figure 3

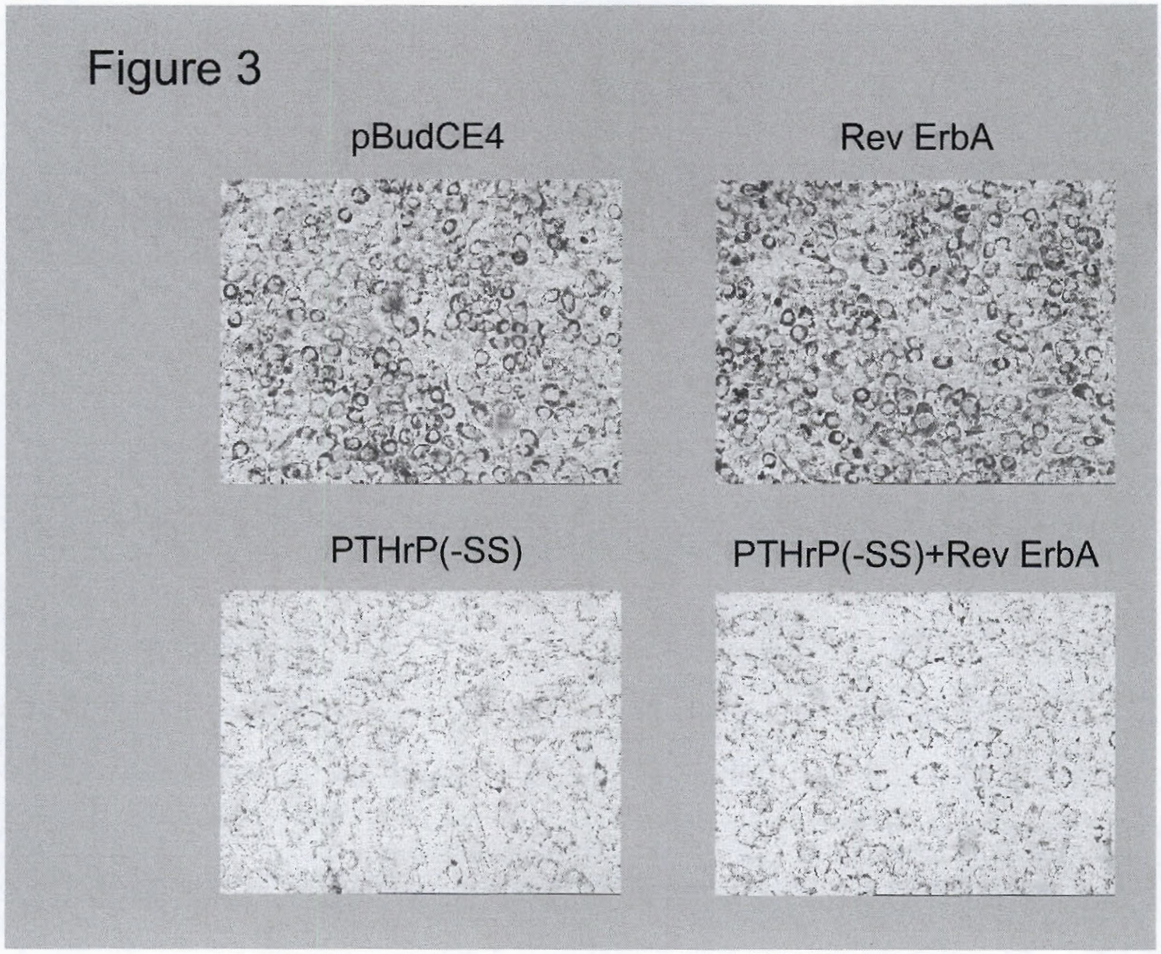

Fig. 3. Overexpression of Nuclear PTHrP Inhibits adipogenesis of C3H 10T½ Cells. Pluripotent mesenchymal C3H 10T½ cells stably transfected with PTHrP(-SS), Rev ErbA or PTHrP(-SS) and Rev ErbA were induced to undergo adipogenesis as described in MATERIALS AND METHODS. Following induction cells were maintained in culture for 14 days and afterwards stained with Oil Red O as described in MATERIALS AND METHODS. Red staining represents triacylglycerol uptake by fat cells. Photomicrographs are representative of 3 random fields from 3 different set of experiments.

Figure 4

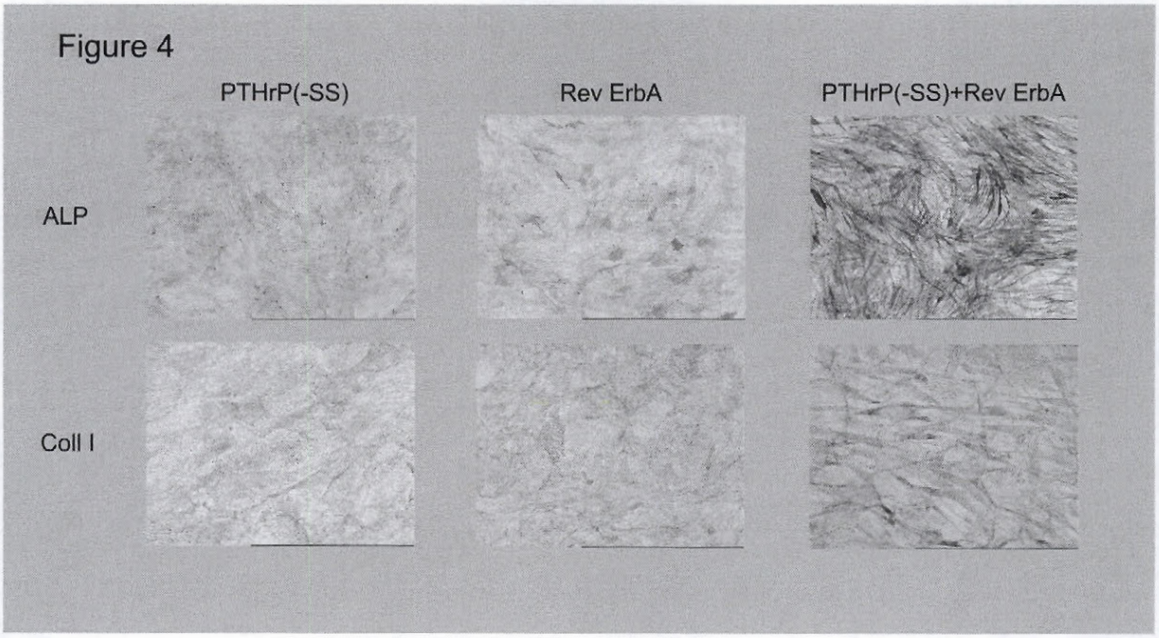

Fig. 4. Overexpression of Nuclear PTHrP and Rev ErbA Enhances Osteogenesis of C3H 10T½ Cells. Stable C3H 10T½ cell lines overexpressing PTHrP(-SS), Rev ErbA or PTHrP(-SS) and Rev ErbA were induced to undergo adipogenesis as described in MATERIALS AND METHODS. Cells were maintained in culture for 14 days then fixed and immunostaining was performed for collagen type I (Coll I) or stained for alkaline phosphatase activity (ALP). Photomicrographs are representative of 3 random fields from 3 different experiments.

PREFACE TO CHAPTER 5

Based on the findings from our previous work demonstrating a potential nuclear role for PTHrP in osteoblast differentiation, we proceeded to investigate the earliest gene regulatory events during osteoblast differentiation. To do so, we performed a genomic scan for CBFA1 regulatory elements in the proximal promoter region of every known gene.

Chapter 5

Genomic Scanning for CBFA1 Responsive Genes in the Mouse and Human Genome

ABSTRACT

The sequencing of the human and mouse genome was a landmark achievement that provides a human and mouse genomic template which can be used as a reference guide for every known gene. Asides from providing the core coding sequence for the mRNA of all proteins, the human genome also provides the proximal promoter sequences which are responsible for regulating gene expression. CBFA1 is the earliest known transcription factor associated with osteoblast differentiation and binds to responsive elements defined by the sequence PuACCPuCA (Pu=A or C). CBFA1 responsive elements have been identified in the proximal promoters of several genes associated with osteoblast function including collagen type I alpha, bone sialoprotein and osteocalcin. We therefore performed genomic scanning of the human and mouse genome for all genes with a CBFA1 responsive elements in their proximal promoter region, to evaluate the early regulatory events associated with osteoblast differentiation. Equivalent genes from the mouse and human genome with responsive elements within 100 nucleotides of one another relative to the mRNA initiation site was scored as positive. The compiled list of genes will then be categorized according to functionality. In total, the scanning has identified 379 unique genes with similar responsive elements in both the mouse and human genome.

INTRODUCTION

The commitment and differentiation process of osteoblastic cells involves the activation of transcription factors that are required for both specification and differentiation of the osteoblastic lineage. Core binding factor alpha 1 (CBFA1) is

currently the earliest known marker of the osteoblast lineage and is expressed as three different isoforms(20, 21, 139). Each isoform is characterized by similar functional domains including a 128 amino acid highly conserved DNA-binding domain known as the *runt domain*, a proline, serine threonine, rich domain referred to as a PST domain and a myc related nuclear localization signal(43). The central role that CBFA1 plays in osteoblast differentiation was demonstrated in mice lacking a functional CBFA1 gene. Absence of CBFA1 expression in these animals resulted in a lack of both endochondral and intramembranous bone with skeletons composed solely of chondrocytes and a cartilaginous matrix(89, 266, 292). As CBFA1 potentially initiates the osteoblast differentiation cascade, the identification of downstream genes that are activated or repressed by CBFA1 may serve to facilitate modeling of the mechanism for osteoblast commitment and differentiation.

CBFA1 is able to bind to specific response elements that are comprised of a core DNA sequence consisting of PuACCPuCA (Pu=A or C), whereupon it may act as either an activator or repressor of transcription based on the context of different cofactors (384). Genes that are directly regulated by CBFA1 will be referred to as 1st order genes, whereas genes that are affected by transcription factors induced directly by CBFA1 will be referred to as 2nd order. Efforts to define genes that are regulated 1st order by CBFA1 have been limited by the laborious nature of identifying the response elements in the promoter region of each gene followed by the characterizing of the response elements. Since the identification of the osteoblastic function of CBFA1 five years ago, only a handful of genes have been identified as being regulated 1st order by CBFA1(29, 77, 83, 120, 180, 183, 186, 197, 251, 296, 318, 345).

In an effort to identify genes that are regulated 1st order by CBFA1, we employed a strategy involving scanning of the human and mouse genomes for genes that harbour minimal CBFA1 response elements in their proximal promoter region. Equivalent genes identified in the mouse and human genome with these CBFA1 responsive elements were then subjected to a proximity filter where the distance of the CBFA1 response elements from the transcription initiation sites were compared to one another. Equivalent genes from mouse and human with CBFA1 response elements within 100 nucleotides of one another based on their position relative to their respective transcription initiation sites were scored as potential positives. The list of compiled genes will then be categorized by functionality.

MATERIALS AND METHODS

Genomic Scanning – Scanning of the human and mouse genomes was performed using the most recent human genome sequence (FASTA File April 28, 2003) and human GBS File (May 20,2003), as well as the mouse current mouse genome sequence (February 25, 2003) and mouse GBS file (March 11, 2003) from NCBI. Scanning was performed with the minimal CBFA1 responsive element (A/C)ACC(A/C)CA, as previously described(283, 386) that resides in the first 1.5 kb upstream of the transcription initiation site for all known and hypothetical genes. A gene that harbours a consensus CBFA1 response element in the promoter region of both the human and mouse genes within 100 nucleotides of their positions relative to the transcription initiation site was scored as a potential positive. The probability of CBFA1 responsive elements existing at a relative

position is determined according to the probability (P) of the response element sequence occurring naturally in the genome.

$$P = 0.25^5 \times 0.5^2 = 2.44 \times 10^{-4}$$

Probability of CBFA1 responsive elements being at a similar location relative to mRNA transcription initiation site within 100 nucleotides is according to the probability of both sequences occurring at similar positions which is the probability of the response element sequence occurring naturally in the genome multiplied by the probability of the response element sequence occurring naturally in the genome at the same location within 100 nucleotides can be represented by the formula,

$$P = \left(0.25^5 \times 0.5^2\right) \times \left(\left(0.25^5 \times 0.5^2\right) \times 100\right) = 6.00 \times 10^{-6}$$

RESULTS

Genome Scanning for CBFA1 Responsive Genes in Mouse and Human Genome – To our knowledge, no commercially available software can be used for scanning of the human genome for specific response elements relative to mRNA transcription initiation sites. The MATINSPECTOR program can scan for transcription factor responsive elements however the search is limited to less then 1000 bp. We therefore designed a software library using the publicly available NCBI data and designed code to scan the library for the sequence (A/C)ACC(A/C)CA within the first 1.5 kb upstream of all mRNA initiation sites. The compiled list from the mouse and human searches were then compared to identify genes with similar responsive elements in both the mouse and human genes. This similarity reflects a probability of approximately 1 in 6 000 000 that these elements should align. Based on our scan we identified 379 genes which harbor consensus

CBFA1 binding sequences in the proximal promoters of the mouse and human gene **(Table 1)**. In the case that more then one CBFA1 responsive element was found in the promoter region of a gene, they were all verified for a corresponding responsive element.

Functionality of Genes Harboring CBFA1 Responsive Elements – To facilitate our future micro-array analysis, we classified the genes according to their functionality based on previous reports. Certain genes have been reported to serve more than one function and are therefore represented more than once **(Table 2)**.

DISCUSSION

CBFA1 is currently the earliest known marker of the osteoblast lineage and is essential for osteoblast differentiation. As CBFA1 can affect the expression of numerous genes associated with osteoblast differentiation, the identification of genes that are directly regulated by CBFA1 may provide insight into the mechanism of osteoblast differentiation.

With the sequencing of the human and mouse genome, the proximal promoter sequences of all hypothetical and known genes are available for the identification of transcription factor response elements(217, 367, 380). We scanned the human and mouse genome for genes with conserved CBFA1 response elements in their proximal promoter regions. In total, we identified 379 genes with CBFA1 responsive elements. Certain genes that had been previously shown to be regulated by CBFA1 were identified such as osteocalcin(88) and collagenase 3(186), were identified by this scanning methodology. On the other hand, collagen type I alpha (CollIα) had also been reported to be regulated by CBFA1, and corresponding responsive elements have been identified in

both the mouse and human promoters(197). However this gene was omitted from our scan as the CollIα gene has yet to be assigned to the mouse genome. Thus, genes that have yet to be processed for either the mouse or human genome may be missed. This limitation will be corrected in time as the genomes become more complete.

Other limitations associated with genomic scanning include the range of the scan is determined empirically. This is due strictly to the lack of continuous sequence similarities amongst promoters(59). We therefore chose our range to be between 1.5 kb and 0 kb as genes that have been reported to be regulated by CBFA1 fall within this limit. It remains possible that other genes may be responsive to CBFA1 as a result of CBFA1 responsive elements residing outside of this limit, whether it be in an exon, intronic sequences, or beyond the 1.5 kb boundary. Furthermore, our scan relies on sequences which have been reported to interact with CBFA1. As a result, we are therefore subject to the faithfulness of these sequences. If the sequence requirements are not well defined then errors can occur in identifying 1st order responsive genes.

By itself, genomic scanning can identify functional response elements in the proximal promoter region of all hypothetical and known genes, however, the ability of a transcription factor to transactivate expression of the given gene requires further analysis due to the fact that the existence of a response element does not always confer trans-activation. For example, certain genes which possess CBFA1 responsive elements and which are associated with osteoblast function have been shown to function independently of CBFA1 (29, 147, 282). To address the responsiveness of our potential genes to CBFA1, we will employ DNA micro-array technology to identify genes that are affected following ectopic expression of CBFA1.

Scanning of the genome can also be applied to CBFA1 and different co-factors to determine the various combinations required for the activation of specific genes. For example, CBFA1 has been shown to interact with the AP1 transcriptional complex composed of c-fos and c-jun and is able to activate transcription from proximal promoters that harbor a CBFA1 response element and AP1 binding site on the condition that they are in close proximity to one another (within one nucleosome <200bp) and they are in the correct helical phase(70, 151). Within the collagenase-3 promoter, the two response elements are nonadjacent, with the CBFA1 binding site at positions -132 to-126, and the AP-1 binding site being located at -48 to -42. The insertion of 3 nucleotides between the two response elements is sufficient to disrupt their helical phasing, and consequently, basal collagenase-3 promoter activity. On the other hand, the insertion of 10 nucleotides, which maintained the helical phasing relationship, did not alter the activity of the promoter. Thus, preliminary scanning of genes with CBFA1 responsive elements as well as AP1 sites can be performed with proximity and helical phasing filters being applied to the output, thus limiting the number of potential genes regulated by a CBFA1 -AP1 transcriptional complex.

In conclusion, we applied a genomic scanning methodology to the mouse and human genome to identify genes that may be regulated 1st order by CBFA1. By examining the proximal promoter regions of every known gene, we were able to identify similar responsive elements in 379 genes. These genes may therefore represent true 1st order targets for CBFA1.

Table 1. Human and Mouse Genes Harboring CBFA1 Responsive Elements in Their Proximal Promoters Within 100 Nucleotides of Their Relative Positions.

Human Gene Name	Human Position	Mouse Gene Name	Mouse Position	Diff. in Nucleotides (Human Position-Mouse Position)
37865	-505	37865	-432	-73
ABCA3	-302	Abca3	-329	27
ACCN1	-216	Accn1	-178	-38
ACE	-1077	Ace	-984	-93
ADAM10	-1137	Adam10	-1228	91
ADAM19	-268	Adam19	-313	45
ADAM19	-249	Adam19	-313	64
ADAM2	-66	Adam2	-114	48
ADAMTS8	-1509	Adamts8	-1452	-57
ADSL	-1464	Adsl	-1420	-44
AGA	-1094	Aga	-1098	4
AIRE	-971	Aire	-946	-25
ALDH2	-1051	Aldh2	-1063	12
ALEX1	-171	3010033I09Rik	-233	62
ALPI	-497	Akp3	-576	79
ANGPTL3	-1239	Angptl3	-1192	-47
ANXA8	-638	Anxa8	-541	-97
AP1M2	-1315	Ap1m2	-1379	64
AP3D1	-667	Ap3d	-740	73
APRT	-610	Aprt	-640	30
AQP7	-823	Aqp7	-850	27
ARG2	-1414	Arg2	-1478	64
ASB10	-1423	Asb10	-1509	86
ATIC	-144	Atic	-190	46
ATP4A	-44	Atp4a	-6	-38
ATP5A1	-236	Atp5a1	-249	13
ATP5G2	-371	Atp5g2	-385	14
AUP1	-765	Aup1	-773	8
B4GALT2	-889	B4galt2	-857	-32
BACE	-332	Bace	-360	28
BATF	-5	Batf	-57	52
BCKDHB	-200	Bckdhb	-229	29
BCL10	-1324	Bcl10	-1365	41
BCL2	-1055	Bcl2	-1125	70
BECN1	-1033	Becn1	-1109	76
BGLAP	-194	Bglap2	-130	-64
BIRC6	-809	Birc6	-714	-95
BMPR2	-1226	Bmpr2	-1197	-29
BRF2	-179	Brf2	-154	-25
BTK	-1346	Btk	-1250	-96
C11orf10	-1400	1810006K21Rik	-1495	95
C14orf1	-762	ORF11	-705	-57
C3	-155	C3	-134	-21
CA14	-627	Car14	-574	-53
CA7	-430	Car7	-420	-10
CABP2	-65	Cabp2	-22	-43

CALML3	-71	2310068O22Rik	-111	40
CALR	-253	Calr	-250	-3
CAPN10	-657	Capn10	-625	-32
CAPN9	-829	Capn9	-801	-28
CASP2	-1344	Casp2	-1252	-92
CBL	-444	Cbl	-536	92
CCNT2	-832	Ccnt2	-884	52
CCT4	-1046	Cct4	-1030	-16
CD22	-1016	Cd22	-1086	70
CD69	-138	Cd69	-136	-2
CD74	-1157	Ii	-1165	8
CDA08	-921	2310047C21Rik	-894	-27
CDH18	-740	Cdh18	-762	22
CDK5	-425	Cdk5	-372	-53
CENPB	-1514	Cenpb	-1418	-96
CHAD	-1437	Chad	-1435	-2
CHRNA6	-310	Chrna6	-314	4
CITED2	-1050	Cited2	-1062	12
CKMT1	-1011	Ckmt1	-916	-95
CLDN1	-1125	Cldn1	-1125	0
CLDN14	-1268	Cldn14	-1190	-78
CLDN3	-1004	Cldn3	-1008	4
CLECSF5	-1015	Clecsf5	-1029	14
COL13A1	-211	Col13a1	-224	13
COMT	-479	Comt	-507	28
CORT	-163	Cort	-161	-2
COX10	-333	2410004F01Rik	-408	75
COX4I1	-665	Cox4a	-683	18
CRABP1	-1314	Crabp1	-1311	-3
CRIPT	-533	Cript-pending	-572	39
CRYBB3	-1267	Crybb3	-1269	2
CRYGD	-1495	Crygd	-1521	26
CS	-1332	Cs	-1398	66
CSF1	-39	Csf1	-70	31
CSTB	-1264	Cstb	-1259	-5
CTPS2	-603	Ctps2	-680	77
CTRL	-911	Ctrl	-816	-95
CTXL	-217	Ctxl-pending	-270	53
CYBA	-1182	Cyba	-1278	96
CYP1A1	-1112	Cyp1a1	-1126	14
CYP27B1	-782	Cyp40	-876	94
CYR61	-1327	Cyr61	-1323	-4
DAP3	-1411	Dap3	-1372	-39
DCI	-673	Dci	-586	-87
DDX8	-393	Ddx8	-440	47
DJ473B4	-143	1810018L05Rik	-153	10
DLG2	-404	Dlgh2	-463	59
DNM1	-209	Dnm	-224	15
DPYS	-1175	Dpys	-1222	47
DTX2	-401	Dtx2	-425	24
DUSP2	-1068	Dusp2	-1045	-23
EBAF	-48	Ebaf	-29	-19
EIF3S2	-448	Eif3s2	-516	68

ELF3	-277	Elf3	-278	1
ELK3	-698	Elk3	-707	9
EMP3	-213	Emp3	-205	-8
ENTPD2	-965	Entpd2	-948	-17
EPO	-1004	Epo	-948	-56
EPOR	-965	Epor	-882	-83
EVI2A	-385	Evi2	-378	-7
F2RL1	-838	F2rl1	-787	-51
FASN	-134	Fasn	-70	-64
FBXL8	-1477	Fbxl8	-1505	28
FDFT1	-168	Fdft1	-92	-76
FGD2	-644	Fgd2	-637	-7
FGD3	-92	Fgd3	-131	39
FGF10	-418	Fgf10	-413	-5
FGF14	-768	Fgf14	-779	11
FGFR1	-678	Fgfr1	-725	47
FLJ10634	-514	1700025B16Rik	-509	-5
FLJ11773	-1333	9430023L20Rik	-1419	86
FLJ14050	-273	2810043G13Rik	-342	69
FLJ20097	-947	1700034M03Rik	-959	12
FLJ20207	-1128	9130017L10Rik	-1108	-20
FLJ20671	-629	1700123O20Rik	-609	-20
FLJ23445	-852	1500006O09Rik	-922	70
FOXC1	-997	Foxc1	-909	-88
FOXC2	-1245	Foxc2	-1148	-97
FPGS	-827	Fpgs	-728	-99
FXC1	-1011	Fxc1	-1017	6
FXYD5	-631	Fxyd5	-629	-2
FZD3	-393	Fzd3	-357	-36
FZD7	-1282	Fzd7	-1305	23
GABRA3	-314	Gabra3	-304	-10
GABRQ	-1081	Gabrq	-1074	-7
GADD45G	-816	Gadd45g	-782	-34
GALR2	-433	Galr2	-401	-32
GDA	-148	Gda	-127	-21
GJA4	-1123	Gja4	-1199	76
GNAI2	-1312	Gnai2	-1376	64
GNAO1	-394	Gnao	-375	-19
GNAT1	-634	Gnat1	-552	-82
GPR12	-1273	Gpcr12	-1265	-8
GPR19	-850	Gpr19	-876	26
GPR65	-1485	Gpcr25	-1505	20
GZMA	-54	Gzma	-47	-7
GZMB	-85	Gzmb	-179	94
HES7	-1182	Hes7	-1259	77
HEYL	-522	Heyl	-511	-11
HK2	-1122	Hk2	-1137	15
HMBS	-1203	Hmbs	-1246	43
HNRPA1	-637	Hnrpa1	-714	77
HNRPC	-1180	Hnrpc	-1094	-86
HOXA11	-306	Hoxa11	-287	-19
HOXC13	-165	Hoxc13	-247	82
HPCL2	-541	Hpcl-pending	-467	-74

HPGD	-486	Hpgd	-534	48
HSPA9B	-8	Hspa9a	-29	21
HTF9C	-572	Htf9c	-505	-67
HTR6	-1019	Htr6	-965	-54
HYAL1	-1384	Hyal1	-1307	-77
ID2	-210	Idb2	-277	67
IFNG	-368	Ifng	-375	7
IGFBP7	-438	Igfbp7	-388	-50
IGSF4	-919	Igsf4	-877	-42
IL16	-1294	Il16	-1282	-12
IL9	-471	Il9	-437	-34
IMAGE3510317	-1150	1300018J18Rik	-1169	19
INHBC	-446	Inhbc	-468	22
INSL3	-894	Insl3	-884	-10
ISG20	-260	Isg20	-289	29
ITGA6	-294	Itga6	-330	36
ITGB5	-1106	Itgb5	-1183	77
JAG1	-1399	Jag1	-1475	76
JAM2	-105	Jcam2	-107	2
JAZ	-904	Zfp346	-902	-2
JUP	-577	Jup	-481	-96
KCNAB1	-806	Kcnab1	-887	81
KCNAB2	-486	Kcnab2	-448	-38
KCNJ12	-1430	Kcnj12	-1475	45
KCNJ5	-1157	Kcnj5	-1246	89
KCNK10	-1267	1700024D23Rik	-1175	-92
KCNN4	-1291	Kcnn4	-1288	-3
KCNQ5	-1026	Kcnq5	-998	-28
KIAA0052	-745	2610528A15Rik	-713	-32
KIAA0143	-371	C920006C10Rik	-301	-70
KIAA0540	-965	1110014F23Rik	-882	-83
KIAA0895	-117	9530077C05Rik	-187	70
KIAA0905	-580	1810024J13Rik	-509	-71
KIAA1173	-1161	1110011D13Rik	-1202	41
KIAA1497	-396	Lrrn1	-469	73
KIAA1536	-74	1810009B06Rik	-81	7
KIAA1543	-861	2310057J16Rik	-769	-92
KIP2	-1381	Kip2-pending	-1412	31
KLF1	-136	Klf1	-80	-56
KPNB3	-263	Kpnb3	-310	47
LGALS12	-1011	Lgals12	-1084	73
LGALS3	-1518	Lgals3	-1478	-40
LGP2	-1342	D11Lgp2e	-1380	38
LIF	-245	Lif	-161	-84
LIG1	-589	Lig1	-585	-4
LIPC	-480	Lipc	-492	12
LISCH7	-551	Lisch7-pending	-568	17
LNK	-868	Lnk	-960	92
LOC64180	-609	1700018F16Rik	-521	-88
LOXL1	-277	Loxl	-231	-46
LRP1	-808	Lrp1	-830	22
LSM2	-773	D17H6S56E-2	-754	-19
LSM2	-772	D17H6S56E-2	-754	-18

LSM2	-771	D17H6S56E-2	-754	-17
LTF	-1500	Ltf	-1498	-2
LUM	-77	Lum	-156	79
MADCAM1	-1240	Madcam1	-1228	-12
MAFB	-412	Mafb	-511	99
MAK	-1304	Mak	-1368	64
MAP2K2	-303	Map2k2	-282	-21
MAP2K3	-282	Map2k3	-342	60
MAP2K7	-796	Map2k7	-858	62
MAP3K8	-318	Map3k8	-371	53
MAPK6	-1244	Mapk6	-1169	-75
MASP2	-395	Masp2	-430	35
MBD2	-85	Mbd2	-158	73
MCRS1	-1030	Mcrs1	-1064	34
MGC10871	-718	4921506I22Rik	-727	9
MGC14793	-710	4930550B20Rik	-747	37
MGC3279	-218	1010001H16Rik	-293	75
MGC4276	-181	1810010A06Rik	-184	3
MHC2TA	-551	C2ta	-512	-39
MIP	-451	Mip	-431	-20
MKI67	-1324	Mki67	-1330	6
MLH1	-759	Mlh1	-743	-16
MMP13	-132	Mmp13	-150	18
MMP14	-177	Mmp14	-211	34
MOG	-1366	Mog	-1396	30
MPDZ	-631	Mpdz	-655	24
MRPS2	-610	Mrps2	-624	14
MRVI1	-852	Mrvi1	-860	8
MYCN	-860	Nmyc1	-879	19
NAP1L1	-985	Nap1l1	-1061	76
NEK2	-1483	Nek2	-1530	47
NEU2	-148	Neu2	-210	62
NFATC2	-697	Nfatc2	-759	62
NFKBIA	-338	Nfkbia	-327	-11
NIT1	-1544	Nit1	-1496	-48
NOS2A	-197	Nos2	-193	-4
NPR3	-506	Npr3	-566	60
NR1I2	-1332	Nr1i2	-1362	30
NR1I3	-67	Nr1i3	-101	34
NRBF-2	-843	Nrbf2	-872	29
NTSR1	-439	Ntsr	-498	59
NUP62	-624	Nup62	-535	-89
OCA2	-813	p	-878	65
OPTN	-1046	Optn	-1098	52
P38IP	-800	P38ip-pending	-820	20
PACSIN3	-1502	Pacsin3	-1478	-24
PDCD5	-1329	Pdcd5	-1408	79
PDE1B	-364	Pde1b	-420	56
PDGFRB	-546	Pdgfrb	-636	90
PEF	-298	2600002E23Rik	-287	-11
PFKP	-873	Pfkp	-857	-16
PGLYRP	-140	Pglyrp	-229	89
PHEMX	-608	Phemx	-644	36

PLA2R1	-117	Pla2g1br	-144	27
PLAT	-1217	Plat	-1143	-74
PLEK2	-650	Plek2	-639	-11
PLVAP	-1390	Plvap	-1458	68
POU3F3	-1047	Pou3f3	-1026	-21
PP35	-385	2310069P03Rik	-377	-8
PPIE	-1547	Ppie	-1499	-48
PRG2	-1122	Prg2	-1119	-3
PRODH2	-25	Prodh2	-122	97
PRPH	-263	Prph1	-338	75
PRSS21	-995	Prss21	-977	-18
PSMB4	-502	Psmb4	-503	1
PSMB9	-158	Psmb9	-167	9
PSME2	-387	Psme2	-338	-49
PSPN	-1225	Pspn	-1318	93
PTGIS	-282	Ptgis	-336	54
PTPN1	-950	Ptpn1	-976	26
PTPRK	-368	Ptprk	-321	-47
PTS	-1338	Pts	-1375	37
RAC1	-1119	Rac1	-1035	-84
RAD50	-242	Rad50	-334	92
RAGA	-208	1300010C19Rik	-167	-41
RAI2	-1423	Rai2	-1504	81
RASSF1	-428	Rassf1	-423	-5
RBBP2	-1190	Rbbp2	-1160	-30
RBM14	-579	Rbm14	-541	-38
RBM4	-1498	Rbm4	-1566	68
RBPSUHL	-968	Rbpsuhl	-983	15
RCN1	-227	Rcn	-292	65
REA	-431	Bcap37	-383	-48
RGN	-956	Rgn	-869	-87
RHCG	-1510	Rhcg	-1490	-20
ROS1	-1137	Ros1	-1091	-46
RPC32	-276	2310047G20Rik	-323	47
RPL18	-101	Rpl18	-166	65
RPL31	-873	Rpl31	-880	7
RPL36AL	-1272	2410038A03Rik	-1221	-51
RPS10	-68	2210402A09Rik	-74	6
RPS3A	-233	Rps3a	-266	33
RPS6KB2	-965	Rps6kb2	-992	27
RTKN	-322	Rtkn	-255	-67
SART1	-1181	Sart1	-1096	-85
SCAP2	-587	Scap2	-640	53
SCP2	-802	Scp2	-798	-4
SDFR1	-1410	Sdfr1	-1371	-39
SIRT6	-1361	Sirt6	-1332	-29
SLC15A2	-1143	Slc15a2	-1223	80
SLC25A11	-913	Slc25a11	-976	63
SLC25A19	-1220	Slc25a19	-1246	26
SLC29A2	-19	Slc29a2	-18	-1
SLC5A5	-409	Slc5a5	-331	-78
SLC7A11	-1379	Slc7a11	-1391	12
SLC7A3	-397	Slc7a3	-375	-22

SLC8A3	-1356	Slc8a3	-1332	-24
SNAPAP	-579	Snap25bp	-531	-48
SNK	-1327	Snk	-1312	-15
SNTB2	-498	Sntb2	-534	36
SNX12	-403	Snx12	-412	9
SOD2	-1365	Sod2	-1389	24
SP192	-1073	BC002292	-1077	4
SPAG9	-1436	Spag9	-1495	59
SPARC	-1009	Sparc	-970	-39
SPINK1	-235	Spink3	-225	-10
SPN	-1124	Spn	-1167	43
SPTA1	-1569	Spna1	-1485	-84
SREBF2	-1190	Srebf2	-1244	54
SRF	-409	Srf	-364	-45
SRP9	-914	Srp9	-996	82
STAG2	-1536	Stag2	-1457	-79
STAG3	-515	Stag3	-607	92
STAT6	-300	Stat6	-269	-31
STK23	-1023	Stk23	-1057	34
TAS1R3	-592	Tas1r3	-552	-40
TBX5	-1139	Tbx5	-1220	81
TCF2	-520	Tcf2	-569	49
TCF8	-244	Zfhx1a	-257	13
TDE1	-159	Tde1	-145	-14
TEM1	-182	Tem1-pending	-175	-7
TFAP2A	-842	Tcfap2a	-845	3
TGFB3	-480	Tgfb3	-549	69
THY1	-113	Thy1	-33	-80
TIAM2	-747	Tiam2	-801	54
TIMP2	-583	Timp2	-575	-8
TIMP4	-574	Timp4	-606	32
TKT	-1002	Tkt	-917	-85
TMSB10	-351	Tmsb10	-342	-9
TOM1	-930	Tom1	-913	-17
TPK1	-1266	Tpk1	-1278	12
TPM2	-1195	Tpm2	-1197	2
TPMT	-1273	Tpmt	-1258	-15
TPX1	-1324	Tpx1	-1317	-7
TRAF2	-651	Traf2	-552	-99
TRAF4	-529	Traf4	-521	-8
TRAF5	-1459	Traf5	-1522	63
TRHR	-1184	Trhr	-1115	-69
TRIM35	-559	0710005M05Rik	-501	-58
TSC1	-390	Tsc1	-318	-72
TSHR	-991	Tshr	-964	-27
TSSC3	-1131	Tssc3	-1079	-52
TSSC4	-432	Tssc4	-398	-34
TUBA8	-375	Tuba8	-369	-6
TUFT1	-1007	Tuft1	-1026	19
U5-100K	-192	4921506D17Rik	-170	-22
UBE1C	-474	Ube1c	-381	-93
UBL1	-1374	Ubl1	-1330	-44
UBL5	-980	Ubl5	-982	2

UNC5C	-901	Unc5h3	-924	23
UNC93B1	-159	Unc93b	-140	-19
VIPR1	-1369	Vipr1	-1439	70
VMD2	-508	Vmd2	-545	37
VPS29	-1215	Vps29	-1243	28
VRK1	-243	Vrk1	-256	13
WBP2	-1047	Wbp2	-1140	93
WDR10	-1078	Wdr10	-1097	19
WNT1	-453	Wnt1	-466	13
WNT3A	-319	Wnt3a	-274	-45
WNT4	-241	Wnt4	-219	-22
WRB	-1488	Wrb	-1444	-44
ZNF161	-283	Vezf1	-231	-52
ZNF179	-1283	Zfp179	-1323	40
ZNFN1A1	-207	Znfn1a1	-164	-43

Table 2. Human and Mouse Genes Harbouring CBFA1 Responsive Elements in Their Proximal Promoters From Table 1, Linked to Functionality.

Functions	Human Gene
Hs_20S core proteasome complex	PSMB4
Hs_20S core proteasome complex	PSMB9
Hs_20S core proteasome complex	PSME2
Hs_26S proteasome	PSMB4
Hs_26S proteasome	PSMB9
Hs_26S proteasome	PSME2
Hs_3',5'-cyclic-nucleotide phosphodiesterase	PDE1B
Hs_3'-5' exonuclease	RAD50
Hs_ABC-type efflux porter	OCA2
Hs_ABC-type uptake permease	ATP4A
Hs_acetylcholine receptor	CHRNA6
Hs_acid-D-amino acid ligase	FPGS
Hs_acidic amino acid transporter	SLC7A11
Hs_actin binding	FXYD5
Hs_actin binding	SPTA1
Hs_actin binding	TMSB10
Hs_actin binding	TPM2
Hs_actin cross-linking	SPTA1
Hs_actin cytoskeleton	TPM2
Hs_actin filament-based process	SPTA1
Hs_actin modulating	TMSB10
Hs_acyltransferase	AUP1
Hs_acyltransferase	FASN
Hs_adenosinetriphosphatase	DDX8
Hs_adenosinetriphosphatase	ENTPD2
Hs_adenosinetriphosphatase	MLH1
Hs_alcohol catabolism	HK2
Hs_alcohol catabolism	PFKP
Hs_alcohol dehydrogenase, zinc-dependent	FASN
Hs_alcohol metabolism	ALDH2
Hs_alcohol metabolism	COMT
Hs_alcohol metabolism	FDFT1
Hs_alcohol metabolism	HK2
Hs_alcohol metabolism	PFKP
Hs_alcohol metabolism	SREBF2
Hs_aldehyde dehydrogenase (NAD+)	ALDH2
Hs_alkaline phosphatase	ALPI
Hs_alpha-ketoglutarate dehydrogenase complex (sens	BCKDHB
Hs_amiloride-sensitive sodium channel	ACCN1
Hs_amine catabolism	ARG2
Hs_amine catabolism	PTS
Hs_amine metabolism	ARG2
Hs_amine metabolism	COMT
Hs_amine metabolism	FASN
Hs_amine metabolism	PTS
Hs_amine receptor	HTR6

Hs_amine/polyamine transporter	SLC7A11
Hs_amino acid catabolism	ARG2
Hs_amino acid catabolism	PTS
Hs_amino acid derivative metabolism	COMT
Hs_amino acid metabolism	FASN
Hs_amino acid metabolism	PTS
Hs_amino acid transport	SLC7A11
Hs_amino acid transporter	OCA2
Hs_amino acid-polyamine transporter	SLC7A11
Hs_ammonia-lyase	HMBS
Hs_ammonium transporter	RHCG
Hs_anion channel	ATP5A1
Hs_anion channel	GABRA3
Hs_anion channel	GABRQ
Hs_anion transport	GABRA3
Hs_anion transporter	OCA2
Hs_anion transporter	SLC5A5
Hs_anti-apoptosis	BCL2
Hs_anti-apoptosis	BECN1
Hs_anti-apoptosis	BIRC6
Hs_anticoagulant	ANXA8
Hs_antimicrobial humoral response	CD22
Hs_antimicrobial humoral response	CSF1
Hs_antimicrobial humoral response	MASP2
Hs_antimicrobial humoral response (sensu Invertebr	CD22
Hs_antimicrobial humoral response (sensu Invertebr	CSF1
Hs_antimicrobial humoral response (sensu Invertebr	MASP2
Hs_antimicrobial peptide	PGLYRP
Hs_antiporter	SLC25A11
Hs_antiporter	SLC7A11
Hs_antiporter	SLC8A3
Hs_apicolateral plasma membrane	CLDN1
Hs_apicolateral plasma membrane	CLDN14
Hs_apicolateral plasma membrane	CLDN3
Hs_apicolateral plasma membrane	GJA4
Hs_apicolateral plasma membrane	MIP
Hs_apoptosis	GADD45G
Hs_apoptosis	GZMA
Hs_apoptosis	GZMB
Hs_apoptosis	NFKBIA
Hs_apoptosis	PDE1B
Hs_apoptosis	PGLYRP
Hs_apoptosis inhibitor	BCL2
Hs_apoptosis inhibitor	BIRC6
Hs_apoptosis regulator	BCL2
Hs_apoptosis regulator	BIRC6
Hs_apoptosis regulator	CASP2
Hs_apoptotic program	CASP2
Hs_arginine metabolism	ARG2
Hs_aromatic compound catabolism	PTS
Hs_aromatic compound metabolism	COMT
Hs_aromatic compound metabolism	FPGS
Hs_aromatic compound metabolism	PTS

Hs_aspartic-type endopeptidase	BACE
Hs_astacin	TRAF2
Hs_astacin	TRAF4
Hs_ATP binding	ABCA3
Hs_ATP binding	ATP4A
Hs_ATP binding	ATP5A1
Hs_ATP binding	ATP5G2
Hs_ATP binding	BMPR2
Hs_ATP binding	BTK
Hs_ATP binding	CCT4
Hs_ATP binding	CDK5
Hs_ATP binding	DDX8
Hs_ATP binding	ENTPD2
Hs_ATP binding	FGFR1
Hs_ATP binding	FPGS
Hs_ATP binding	GNAI2
Hs_ATP binding	HK2
Hs_ATP binding	HSPA9B
Hs_ATP binding	LIG1
Hs_ATP binding	MAK
Hs_ATP binding	MAP2K2
Hs_ATP binding	MAP2K3
Hs_ATP binding	MAP2K7
Hs_ATP binding	MAP3K8
Hs_ATP binding	MAPK6
Hs_ATP binding	MHC2TA
Hs_ATP binding	MKI67
Hs_ATP binding	MLH1
Hs_ATP binding	NEK2
Hs_ATP binding	OCA2
Hs_ATP binding	PDGFRB
Hs_ATP binding	RAD50
Hs_ATP binding	ROS1
Hs_ATP binding	RPS6KB2
Hs_ATP binding	SNK
Hs_ATP binding	STK23
Hs_ATP binding	VRK1
Hs_ATP biosynthesis	ATP5A1
Hs_ATP dependent DNA helicase	ENTPD2
Hs_ATP dependent helicase	DDX8
Hs_ATP dependent helicase	ENTPD2
Hs_ATP dependent RNA helicase	DDX8
Hs_ATP dependent RNA helicase	ENTPD2
Hs_ATP-binding and phosphorylation-dependent chlor	ATP5A1
Hs_ATP-binding cassette (ABC) transporter	ABCA3
Hs_ATP-binding cassette (ABC) transporter	ATP4A
Hs_ATP-binding cassette (ABC) transporter	OCA2
Hs_ATP-binding cassette (ABC) transporter	RAD50
Hs_auxiliary transport protein	KCNAB1
Hs_auxiliary transport protein	KCNAB2
Hs_axis specification	EBAF
Hs_axon guidance	GNAO1
Hs_axon guidance	UNC5C

Hs_axonogenesis	GNAO1
Hs_axonogenesis	UNC5C
Hs_basement membrane	SPARC
Hs_behavior	GALR2
Hs_benzodiazepine receptor	GABRA3
Hs_binding	SLC25A11
Hs_binding	SPN
Hs_biogenic amine metabolism	COMT
Hs_biological_process unknown	RPL31
Hs_biological_process unknown	SIRT6
Hs_biological_process unknown	TEM1
Hs_biological_process unknown	TIMP4
Hs_biological_process unknown	UBL1
Hs_biological_process unknown	UBL5
Hs_biological_process unknown	ZNF179
Hs_biosynthesis	FASN
Hs_biosynthesis	FDFT1
Hs_biosynthesis	FPGS
Hs_blood coagulation	ANXA8
Hs_blood coagulation	F2RL1
Hs_blood coagulation	PLAT
Hs_bone remodeling	SPARC
Hs_brain development	UNC5C
Hs_calcium ion binding	ANXA8
Hs_calcium ion binding	BGLAP
Hs_calcium ion binding	CABP2
Hs_calcium ion binding	CALML3
Hs_calcium ion binding	CDH18
Hs_calcium ion binding	KIP2
Hs_calcium ion binding	LRP1
Hs_calcium ion binding	MASP2
Hs_calcium ion binding	NOS2A
Hs_calcium ion binding	RCN1
Hs_calcium ion binding	RGN
Hs_calcium ion binding	SPARC
Hs_calcium ion binding	SPTA1
Hs_calcium ion binding	TEM1
Hs_calcium ion homeostasis	CYP27B1
Hs_calcium ion storage	CALR
Hs_calcium:cation antiporter	SLC8A3
Hs_calcium:sodium antiporter	SLC8A3
Hs_calcium-activated potassium channel	KCNN4
Hs_calcium-dependent cell-cell adhesion	FXYD5
Hs_calcium-dependent phospholipid binding	ANXA8
Hs_calcium-dependent protein serine/threonine phos	DUSP2
Hs_calmodulin binding	KCNN4
Hs_calmodulin binding	NOS2A
Hs_calmodulin binding	PDE1B
Hs_calmodulin binding	SLC8A3
Hs_cAMP-dependent protein kinase	BMPR2
Hs_cAMP-dependent protein kinase	NEK2
Hs_cAMP-dependent protein kinase	STK23
Hs_cAMP-mediated signaling	CORT

Hs_cAMP-mediated signaling	GALR2
Hs_cAMP-mediated signaling	GNAI2
Hs_carbohydrate binding	CD22
Hs_carbohydrate binding	CD69
Hs_carbohydrate binding	LGALS3
Hs_carbohydrate binding	PRG2
Hs_carbohydrate binding	TEM1
Hs_carbohydrate catabolism	HK2
Hs_carbohydrate catabolism	PFKP
Hs_carbohydrate catabolism	PGLYRP
Hs_carbohydrate kinase	HK2
Hs_carbohydrate kinase	PFKP
Hs_carbohydrate metabolism	ALDH2
Hs_carbohydrate metabolism	CS
Hs_carbohydrate metabolism	HK2
Hs_carbohydrate metabolism	NEU2
Hs_carbohydrate metabolism	PFKP
Hs_carbohydrate metabolism	PGLYRP
Hs_carbonate dehydratase	CA14
Hs_carbonate dehydratase	CA7
Hs_carbon-carbon lyase	CS
Hs_carbon-nitrogen lyase	ADSL
Hs_carbon-nitrogen lyase	HMBS
Hs_carbon-oxygen lyase	CA14
Hs_carbon-oxygen lyase	CA7
Hs_carbon-oxygen lyase	PTS
Hs_carboxylic acid biosynthesis	FASN
Hs_carboxylic acid biosynthesis	PTGIS
Hs_carboxylic acid metabolism	ARG2
Hs_carboxylic acid metabolism	DCI
Hs_carboxylic acid metabolism	FASN
Hs_carboxylic acid metabolism	FPGS
Hs_carboxylic acid metabolism	HPGD
Hs_carboxylic acid metabolism	PTGIS
Hs_carboxylic acid metabolism	PTS
Hs_carboxylic ester hydrolase	LIPC
Hs_carboxypeptidase	ACE
Hs_cartilage condensation	LUM
Hs_casein kinase	BMPR2
Hs_casein kinase	NEK2
Hs_casein kinase	STK23
Hs_caspase	CASP2
Hs_catecholamine metabolism	COMT
Hs_cation channel	ACCN1
Hs_cation channel	CHRNA6
Hs_cation channel	KCNAB1
Hs_cation channel	KCNAB2
Hs_cation channel	KCNJ12
Hs_cation channel	KCNJ5
Hs_cation channel	KCNK10
Hs_cation channel	KCNN4
Hs_cation channel	KCNQ5
Hs_cation homeostasis	CYP27B1

Hs_cation homeostasis	LTF
Hs_cation transport	KCNQ5
Hs_cation transporter	ATP4A
Hs_cation transporter	ATP5A1
Hs_cation transporter	ATP5G2
Hs_cation transporter	RHCG
Hs_cation transporter	SLC25A11
Hs_cation-transporting ATPase	ATP4A
Hs_cell adhesion	CD22
Hs_cell adhesion	CDH18
Hs_cell adhesion	CLDN1
Hs_cell adhesion	JUP
Hs_cell adhesion	RAC1
Hs_cell adhesion	TSC1
Hs_cell adhesion molecule	ITGA6
Hs_cell adhesion receptor	ITGA6
Hs_cell adhesion receptor	ITGB5
Hs_cell communication	GJA4
Hs_cell cortex	SPTA1
Hs_cell cycle	37865
Hs_cell cycle	BCL2
Hs_cell cycle	CCNT2
Hs_cell cycle	CCT4
Hs_cell cycle	CDK5
Hs_cell cycle	CENPB
Hs_cell cycle	FGF10
Hs_cell cycle	HK2
Hs_cell cycle	LIG1
Hs_cell cycle	MAPK6
Hs_cell cycle	MKI67
Hs_cell cycle	MLH1
Hs_cell cycle	NAP1L1
Hs_cell cycle	NEK2
Hs_cell cycle	RAD50
Hs_cell cycle	RPS6KB2
Hs_cell cycle	SNK
Hs_cell cycle	STAG3
Hs_cell cycle	TGFB3
Hs_cell cycle	TSC1
Hs_cell death	EMP3
Hs_cell differentiation	CSF1
Hs_cell differentiation	GADD45G
Hs_cell growth	CYR61
Hs_cell growth	EBAF
Hs_cell growth	EMP3
Hs_cell growth	IFNG
Hs_cell growth	TGFB3
Hs_cell growth and/or maintenance	BCL2
Hs_cell growth and/or maintenance	CBL
Hs_cell growth and/or maintenance	INHBC
Hs_cell growth and/or maintenance	MAP3K8
Hs_cell growth and/or maintenance	MLH1
Hs_cell growth and/or maintenance	MYCN

Hs_cell growth and/or maintenance	PDGFRB
Hs_cell growth and/or maintenance	ROS1
Hs_cell growth and/or maintenance	TSC1
Hs_cell growth and/or maintenance	WNT1
Hs_cell junction	CLDN1
Hs_cell junction	CLDN14
Hs_cell junction	CLDN3
Hs_cell junction	GJA4
Hs_cell junction	MIP
Hs_cell motility	IFNG
Hs_cell motility	RAC1
Hs_cell organization and biogenesis	CENPB
Hs_cell organization and biogenesis	DNM1
Hs_cell organization and biogenesis	FXC1
Hs_cell organization and biogenesis	FXYD5
Hs_cell organization and biogenesis	GJA4
Hs_cell organization and biogenesis	ITGA6
Hs_cell organization and biogenesis	NAP1L1
Hs_cell organization and biogenesis	RAC1
Hs_cell organization and biogenesis	RAD50
Hs_cell organization and biogenesis	SIRT6
Hs_cell organization and biogenesis	SPN
Hs_cell organization and biogenesis	SPTA1
Hs_cell organization and biogenesis	TUBA8
Hs_cell proliferation	ADAMTS8
Hs_cell proliferation	BCL2
Hs_cell proliferation	CDK5
Hs_cell proliferation	CSF1
Hs_cell proliferation	CYR61
Hs_cell proliferation	EMP3
Hs_cell proliferation	EVI2A
Hs_cell proliferation	FZD3
Hs_cell proliferation	IL9
Hs_cell proliferation	ISG20
Hs_cell proliferation	LIF
Hs_cell proliferation	LRP1
Hs_cell proliferation	MKI67
Hs_cell proliferation	NAP1L1
Hs_cell proliferation	SLC29A2
Hs_cell proliferation	TCF8
Hs_cell proliferation	TGFB3
Hs_cell proliferation	TSHR
Hs_cell proliferation	VIPR1
Hs_cell recognition	GNAO1
Hs_cell recognition	UNC5C
Hs_cell surface receptor linked signal transduction	BMPR2
Hs_cell surface receptor linked signal transduction	C3
Hs_cell surface receptor linked signal transduction	CBL
Hs_cell surface receptor linked signal transduction	CD69
Hs_cell surface receptor linked signal transduction	CORT
Hs_cell surface receptor linked signal transduction	EBAF
Hs_cell surface receptor linked signal transduction	F2RL1
Hs_cell surface receptor linked signal transduction	FGFR1

Hs_cell surface receptor linked signal transduction	FZD3
Hs_cell surface receptor linked signal transduction	FZD7
Hs_cell surface receptor linked signal transduction	GABRA3
Hs_cell surface receptor linked signal transduction	GALR2
Hs_cell surface receptor linked signal transduction	GNAI2
Hs_cell surface receptor linked signal transduction	GNAO1
Hs_cell surface receptor linked signal transduction	GNAT1
Hs_cell surface receptor linked signal transduction	HTR6
Hs_cell surface receptor linked signal transduction	IFNG
Hs_cell surface receptor linked signal transduction	ITGA6
Hs_cell surface receptor linked signal transduction	ITGB5
Hs_cell surface receptor linked signal transduction	LIF
Hs_cell surface receptor linked signal transduction	NTSR1
Hs_cell surface receptor linked signal transduction	PDGFRB
Hs_cell surface receptor linked signal transduction	RASSF1
Hs_cell surface receptor linked signal transduction	ROS1
Hs_cell surface receptor linked signal transduction	TRHR
Hs_cell surface receptor linked signal transduction	TSHR
Hs_cell surface receptor linked signal transduction	VIPR1
Hs_cell surface receptor linked signal transduction	WNT1
Hs_cell surface receptor linked signal transduction	WNT3A
Hs_cell surface receptor linked signal transduction	WNT4
Hs_cell-cell adhesion	CDH18
Hs_cell-cell adhesion	FXYD5
Hs_cell-cell adhesion	JAM2
Hs_cell-cell signaling	ACCN1
Hs_cell-cell signaling	ADAM10
Hs_cell-cell signaling	CHRNA6
Hs_cell-cell signaling	COMT
Hs_cell-cell signaling	CORT
Hs_cell-cell signaling	DNM1
Hs_cell-cell signaling	EBAF
Hs_cell-cell signaling	EPO
Hs_cell-cell signaling	FGF10
Hs_cell-cell signaling	FGF14
Hs_cell-cell signaling	GABRA3
Hs_cell-cell signaling	GABRQ
Hs_cell-cell signaling	GALR2
Hs_cell-cell signaling	HTR6
Hs_cell-cell signaling	IFNG
Hs_cell-cell signaling	IL9
Hs_cell-cell signaling	INSL3
Hs_cell-cell signaling	KCNQ5
Hs_cell-cell signaling	LIF
Hs_cell-cell signaling	MIP
Hs_cell-cell signaling	MOG
Hs_cell-cell signaling	NTSR1
Hs_cell-cell signaling	TGFB3
Hs_cell-cell signaling	TSHR
Hs_cell-cell signaling	VIPR1
Hs_cell-cell signaling	WNT3A
Hs_cell-cell signaling	WNT4
Hs_cell-matrix adhesion	FXC1

Hs_cell-matrix adhesion	ITGA6
Hs_cell-matrix adhesion	ITGB5
Hs_cellular defense response	BECN1
Hs_cellular defense response	SPN
Hs_cellular defense response	ZNF161
Hs_cellular_component unknown	37865
Hs_cellular_component unknown	BIRC6
Hs_cellular_component unknown	CSF1
Hs_cellular_component unknown	RAI2
Hs_cellular_component unknown	RPL31
Hs_cellular_component unknown	SIRT6
Hs_cellular_component unknown	SOD2
Hs_cellular_component unknown	STK23
Hs_cellular_component unknown	UBL1
Hs_cellular_component unknown	ZNF179
Hs_central nervous system development	ACCN1
Hs_central nervous system development	MOG
Hs_central nervous system development	POU3F3
Hs_central nervous system development	PSPN
Hs_central nervous system development	PTS
Hs_central nervous system development	UNC5C
Hs_central nervous system development	WNT1
Hs_centromere	CENPB
Hs_centrosome	NEK2
Hs_channel regulator	KCNAB1
Hs_channel regulator	KCNAB2
Hs_chaperone	CCT4
Hs_chaperone	CD74
Hs_chemotaxis	IL16
Hs_chemotaxis	SPN
Hs_chloride channel	ATP5A1
Hs_chloride channel	GABRQ
Hs_chloride transport	GABRA3
Hs_cholesterol metabolism	FDFT1
Hs_cholesterol metabolism	SREBF2
Hs_chromatin	MYCN
Hs_chromatin assembly/disassembly	NAP1L1
Hs_chromatin binding	CENPB
Hs_chromatin modification	SIRT6
Hs_chromatin remodeling complex	NAP1L1
Hs_chromatin silencing complex	SIRT6
Hs_chromosome	CENPB
Hs_chromosome	MYCN
Hs_chromosome	STAG3
Hs_chromosome organization and biogenesis	CENPB
Hs_chymotrypsin	CTRL
Hs_chymotrypsin	GZMA
Hs_chymotrypsin	GZMB
Hs_chymotrypsin	MASP2
Hs_chymotrypsin	PLAT
Hs_chymotrypsin	PRSS21
Hs_circulation	ACE
Hs_circulation	EPO

Hs_circulation	KCNJ12
Hs_cis-trans isomerase	PPIE
Hs_class II major histocompatibility complex antigen	CD74
Hs_clathrin-coated vesicle	AP1M2
Hs_coated pit	AP1M2
Hs_coated pit	DNM1
Hs_coated pit	LRP1
Hs_coated vesicle	AP1M2
Hs_coated vesicle	KIAA0905
Hs_coenzyme biosynthesis	ATP5A1
Hs_coenzyme biosynthesis	FPGS
Hs_coenzyme metabolism	ATP5A1
Hs_coenzyme metabolism	FPGS
Hs_coenzymes and prosthetic group biosynthesis	ATP5A1
Hs_coenzymes and prosthetic group biosynthesis	COX10
Hs_coenzymes and prosthetic group biosynthesis	FPGS
Hs_coenzymes and prosthetic group biosynthesis	HMBS
Hs_collagen binding	SPARC
Hs_collagenase	MMP13
Hs_complement activation	MASP2
Hs_complement activation, alternative pathway	C3
Hs_complement activation, classical pathway	MASP2
Hs_complement activity	C3
Hs_connexon complex	GJA4
Hs_copper binding	LOXL1
Hs_copper, zinc superoxide dismutase	SOD2
Hs_CTD phosphatase	DUSP2
Hs_cyclic-nucleotide dependent protein kinase	BMPR2
Hs_cyclic-nucleotide dependent protein kinase	NEK2
Hs_cyclic-nucleotide dependent protein kinase	STK23
Hs_cyclic-nucleotide phosphodiesterase	PDE1B
Hs_cyclic-nucleotide-mediated signaling	CORT
Hs_cyclic-nucleotide-mediated signaling	GALR2
Hs_cyclic-nucleotide-mediated signaling	GNAI2
Hs_cyclic-nucleotide-mediated signaling	HTR6
Hs_cyclic-nucleotide-mediated signaling	TSHR
Hs_cyclic-nucleotide-mediated signaling	VIPR1
Hs_cyclin-dependent protein kinase	CCNT2
Hs_cyclin-dependent protein kinase	CDK5
Hs_cyclin-dependent protein kinase, regulator	CCNT2
Hs_cyclophilin-type peptidy-prolyl cis-trans isomer	PPIE
Hs_cysteine protease inhibitor	BIRC6
Hs_cysteine protease inhibitor	CSTB
Hs_cysteine-type peptidase	CASP2
Hs_cytochrome P450	CYP1A1
Hs_cytochrome P450	CYP27B1
Hs_cytochrome P450	PTGIS
Hs_cytokine	CSF1
Hs_cytokine	EBAF
Hs_cytokine	EPO
Hs_cytokine	IFNG
Hs_cytokine	IL16
Hs_cytokine	IL9

Hs_cytokine	INHBC
Hs_cytokine	LIF
Hs_cytokine	PGLYRP
Hs_cytokine	TGFB3
Hs_cytokinesis	37865
Hs_cytokinesis	CCNT2
Hs_cytokinesis	CDK5
Hs_cytokinesis	LIG1
Hs_cytokinesis	NEK2
Hs_cytolysis	GZMA
Hs_cytolysis	GZMB
Hs_cytoplasm	AGA
Hs_cytoplasm	ALDH2
Hs_cytoplasm	AP1M2
Hs_cytoplasm	ARG2
Hs_cytoplasm	ATP5A1
Hs_cytoplasm	ATP5G2
Hs_cytoplasm	B4GALT2
Hs_cytoplasm	BCKDHB
Hs_cytoplasm	BCL2
Hs_cytoplasm	BTK
Hs_cytoplasm	CALR
Hs_cytoplasm	CCT4
Hs_cytoplasm	CKMT1
Hs_cytoplasm	COX10
Hs_cytoplasm	CRABP1
Hs_cytoplasm	CS
Hs_cytoplasm	CYBA
Hs_cytoplasm	CYP1A1
Hs_cytoplasm	CYP27B1
Hs_cytoplasm	DCI
Hs_cytoplasm	EIF3S2
Hs_cytoplasm	F2RL1
Hs_cytoplasm	FDFT1
Hs_cytoplasm	FPGS
Hs_cytoplasm	FXC1
Hs_cytoplasm	GADD45G
Hs_cytoplasm	GZMB
Hs_cytoplasm	HK2
Hs_cytoplasm	HNRPA1
Hs_cytoplasm	HSPA9B
Hs_cytoplasm	JUP
Hs_cytoplasm	KCNAB1
Hs_cytoplasm	KCNAB2
Hs_cytoplasm	KIAA0905
Hs_cytoplasm	KPNB3
Hs_cytoplasm	MAP3K8
Hs_cytoplasm	MRVI1
Hs_cytoplasm	NEK2
Hs_cytoplasm	NEU2
Hs_cytoplasm	NFATC2
Hs_cytoplasm	NFKBIA
Hs_cytoplasm	NOS2A

Hs_cytoplasm	NTSR1
Hs_cytoplasm	OCA2
Hs_cytoplasm	PFKP
Hs_cytoplasm	PRG2
Hs_cytoplasm	PRPH
Hs_cytoplasm	PRSS21
Hs_cytoplasm	PSMB4
Hs_cytoplasm	PSMB9
Hs_cytoplasm	PSME2
Hs_cytoplasm	PTGIS
Hs_cytoplasm	PTPN1
Hs_cytoplasm	RAC1
Hs_cytoplasm	RCN1
Hs_cytoplasm	RPL18
Hs_cytoplasm	RPL31
Hs_cytoplasm	RPS10
Hs_cytoplasm	RPS3A
Hs_cytoplasm	SCP2
Hs_cytoplasm	SLC25A11
Hs_cytoplasm	SOD2
Hs_cytoplasm	SPTA1
Hs_cytoplasm	SREBF2
Hs_cytoplasm	SRP9
Hs_cytoplasm	TMSB10
Hs_cytoplasm	TPM2
Hs_cytoplasm	TUBA8
Hs_cytoplasm	UBL5
Hs_cytoplasm organization and biogenesis	DNM1
Hs_cytoplasm organization and biogenesis	FXC1
Hs_cytoplasm organization and biogenesis	GJA4
Hs_cytoplasm organization and biogenesis	ITGA6
Hs_cytoplasm organization and biogenesis	SPN
Hs_cytoplasm organization and biogenesis	SPTA1
Hs_cytoplasm organization and biogenesis	TUBA8
Hs_cytoplasmic transport	HNRPA1
Hs_cytoplasmic transport	KPNB3
Hs_cytoplasmic transport	NFKBIA
Hs_cytoplasmic vesicle	AP1M2
Hs_cytoplasmic vesicle	KIAA0905
Hs_cytoskeletal protein binding	JUP
Hs_cytoskeleton	JUP
Hs_cytoskeleton	SPTA1
Hs_cytoskeleton	TMSB10
Hs_cytoskeleton	TPM2
Hs_cytoskeleton	TUBA8
Hs_cytoskeleton organization and biogenesis	DNM1
Hs_cytoskeleton organization and biogenesis	SPN
Hs_cytoskeleton organization and biogenesis	SPTA1
Hs_cytoskeleton organization and biogenesis	TUBA8
Hs_cytosol	FPGS
Hs_cytosol	MAP3K8
Hs_cytosol	PFKP
Hs_cytosol	PSMB4

Hs_cytosol	PSMB9
Hs_cytosol	PSME2
Hs_cytosol	RPL18
Hs_cytosol	RPL31
Hs_cytosol	RPS10
Hs_cytosol	RPS3A
Hs_cytosolic calcium ion concentration elevation	F2RL1
Hs_cytosolic calcium ion concentration elevation	GALR2
Hs_cytosolic large ribosomal subunit (sensu Eukary	RPL18
Hs_cytosolic large ribosomal subunit (sensu Eukary	RPL31
Hs_cytosolic ribosome (sensu Eukarya)	RPL18
Hs_cytosolic ribosome (sensu Eukarya)	RPL31
Hs_cytosolic ribosome (sensu Eukarya)	RPS10
Hs_cytosolic ribosome (sensu Eukarya)	RPS3A
Hs_cytosolic small ribosomal subunit (sensu Eukary	RPS10
Hs_cytosolic small ribosomal subunit (sensu Eukary	RPS3A
Hs_cytotoxin	PRG2
Hs_deaminase	GDA
Hs_defense response	AIRE
Hs_defense response	BCL2
Hs_defense response	BECN1
Hs_defense response	C3
Hs_defense response	CD22
Hs_defense response	CD69
Hs_defense response	CD74
Hs_defense response	CSF1
Hs_defense response	GZMA
Hs_defense response	IFNG
Hs_defense response	IL16
Hs_defense response	IL9
Hs_defense response	KCNN4
Hs_defense response	LIF
Hs_defense response	LTF
Hs_defense response	MASP2
Hs_defense response	MHC2TA
Hs_defense response	PGLYRP
Hs_defense response	PRG2
Hs_defense response	PSMB9
Hs_defense response	PSME2
Hs_defense response	RAC1
Hs_defense response	SPN
Hs_defense response	TCF8
Hs_defense response	VIPR1
Hs_defense response	ZNF161
Hs_defense/immunity protein	CD22
Hs_defense/immunity protein	MHC2TA
Hs_deoxyribonuclease	RAD50
Hs_dephosphorylation	DUSP2
Hs_dephosphorylation	PTPN1
Hs_dephosphorylation	PTPRK
Hs_development	ACCN1
Hs_development	ADAM2
Hs_development	BGLAP

Hs_development	BMPR2
Hs_development	BTK
Hs_development	CRABP1
Hs_development	CSF1
Hs_development	EBAF
Hs_development	EMP3
Hs_development	EPO
Hs_development	FGF14
Hs_development	FGFR1
Hs_development	FOXC2
Hs_development	FZD3
Hs_development	FZD7
Hs_development	GADD45G
Hs_development	GALR2
Hs_development	GDA
Hs_development	GNAO1
Hs_development	HES7
Hs_development	HEYL
Hs_development	HOXA11
Hs_development	HOXC13
Hs_development	ID2
Hs_development	INSL3
Hs_development	ITGB5
Hs_development	LIF
Hs_development	LUM
Hs_development	MAK
Hs_development	MOG
Hs_development	NPR3
Hs_development	POU3F3
Hs_development	PSPN
Hs_development	PTS
Hs_development	RAC1
Hs_development	RAI2
Hs_development	SPARC
Hs_development	TBX5
Hs_development	TFAP2A
Hs_development	TMSB10
Hs_development	TPM2
Hs_development	UNC5C
Hs_development	WNT1
Hs_development	WNT3A
Hs_development	WNT4
Hs_development	ZNFN1A1
Hs_di-, tri-valent inorganic cation homeostasis	CYP27B1
Hs_di-, tri-valent inorganic cation transport	LTF
Hs_di-, tri-valent inorganic cation transport	SLC8A3
Hs_digestion	CTRL
Hs_digestion	GALR2
Hs_digestion	VIPR1
Hs_dipeptidase	ACE
Hs_DNA binding	AIRE
Hs_DNA binding	CBL
Hs_DNA binding	CENPB

Hs_DNA binding	ELK3
Hs_DNA binding	ENTPD2
Hs_DNA binding	FOXC1
Hs_DNA binding	FOXC2
Hs_DNA binding	HES7
Hs_DNA binding	HEYL
Hs_DNA binding	HOXA11
Hs_DNA binding	HOXC13
Hs_DNA binding	KLF1
Hs_DNA binding	LIG1
Hs_DNA binding	MBD2
Hs_DNA binding	MHC2TA
Hs_DNA binding	MYCN
Hs_DNA binding	NAP1L1
Hs_DNA binding	NFATC2
Hs_DNA binding	NR1I2
Hs_DNA binding	NR1I3
Hs_DNA binding	POU3F3
Hs_DNA binding	RAD50
Hs_DNA binding	RBBP2
Hs_DNA binding	SIRT6
Hs_DNA binding	SREBF2
Hs_DNA binding	SRF
Hs_DNA binding	STAT6
Hs_DNA binding	TBX5
Hs_DNA binding	TCF2
Hs_DNA binding	TCF8
Hs_DNA binding	TFAP2A
Hs_DNA binding	ZNF161
Hs_DNA binding	ZNFN1A1
Hs_DNA dependent adenosinetriphosphatase	ENTPD2
Hs_DNA dependent DNA replication	MLH1
Hs_DNA helicase	ENTPD2
Hs_DNA ligase (ATP)	LIG1
Hs_DNA packaging	NAP1L1
Hs_DNA packaging	SIRT6
Hs_DNA recombination	LIG1
Hs_DNA repair	GADD45G
Hs_DNA repair	LIG1
Hs_DNA repair	MLH1
Hs_DNA repair	RAD50
Hs_DNA repair	UBL1
Hs_DNA replication	LIG1
Hs_DNA replication	MLH1
Hs_DNA replication	NAP1L1
Hs_DNA replication and chromosome cycle	CENPB
Hs_DNA replication and chromosome cycle	LIG1
Hs_DNA replication and chromosome cycle	MLH1
Hs_DNA replication and chromosome cycle	NAP1L1
Hs_DNA-directed RNA polymerase III	RPC32
Hs_double-strand break repair	RAD50
Hs_drug binding	PPIE
Hs_drug resistance	ABCA3

Hs_ectoderm development	TFAP2A
Hs_eicosanoid biosynthesis	PTGIS
Hs_eicosanoid metabolism	HPGD
Hs_eicosanoid metabolism	PTGIS
Hs_electrochemical potential-driven transporter	SLC15A2
Hs_electrochemical potential-driven transporter	SLC25A11
Hs_electrochemical potential-driven transporter	SLC5A5
Hs_electrochemical potential-driven transporter	SLC7A11
Hs_electrochemical potential-driven transporter	SLC8A3
Hs_electron transfer flavoprotein	NOS2A
Hs_electron transport	CYBA
Hs_electron transport	CYP1A1
Hs_electron transport	CYP27B1
Hs_electron transport	NOS2A
Hs_electron transport	PTGIS
Hs_electron transporter	ALDH2
Hs_electron transporter	HPGD
Hs_electron transporter	LOXL1
Hs_electron transporter	NOS2A
Hs_embryogenesis and morphogenesis	ADAMTS8
Hs_embryogenesis and morphogenesis	CYR61
Hs_embryogenesis and morphogenesis	FOXC1
Hs_embryogenesis and morphogenesis	HEYL
Hs_embryogenesis and morphogenesis	HOXA11
Hs_embryogenesis and morphogenesis	HOXC13
Hs_embryogenesis and morphogenesis	LIG1
Hs_embryogenesis and morphogenesis	RAC1
Hs_embryogenesis and morphogenesis	TBX5
Hs_embryogenesis and morphogenesis	WNT1
Hs_embryonic development	RAI2
Hs_endocytosis	AP1M2
Hs_endocytosis	DNM1
Hs_endocytosis	LRP1
Hs_endodeoxyribonuclease	RAD50
Hs_endomembrane system	KPNB3
Hs_endomembrane system	MRVI1
Hs_endomembrane system	NUP62
Hs_endomembrane system	UBL1
Hs_endonuclease	RAD50
Hs_endopeptidase	ADAM10
Hs_endopeptidase	ADAM2
Hs_endopeptidase	ADAMTS8
Hs_endopeptidase	MMP13
Hs_endopeptidase	MMP14
Hs_endopeptidase	TRAF2
Hs_endopeptidase	TRAF4
Hs_endopeptidase inhibitor	BIRC6
Hs_endopeptidase inhibitor	C3
Hs_endopeptidase inhibitor	CSTB
Hs_endopeptidase inhibitor	SPINK1
Hs_endopeptidase inhibitor	TIMP2
Hs_endopeptidase inhibitor	TIMP4
Hs_endoplasmic reticulum	CALR

Hs_endoplasmic reticulum	CYP1A1
Hs_endoplasmic reticulum	FDFT1
Hs_endoplasmic reticulum	MRVI1
Hs_endoplasmic reticulum	NTSR1
Hs_endoplasmic reticulum	PTGIS
Hs_endoplasmic reticulum	RCN1
Hs_endoplasmic reticulum	SREBF2
Hs_endoplasmic reticulum	SRP9
Hs_endoplasmic reticulum lumen	CALR
Hs_endoplasmic reticulum lumen	RCN1
Hs_endoplasmic reticulum membrane	MRVI1
Hs_endoplasmic reticulum receptor	SRP9
Hs_energy derivation by oxidation of organic compo	CS
Hs_energy derivation by oxidation of organic compo	HK2
Hs_energy derivation by oxidation of organic compo	PFKP
Hs_energy pathways	AQP7
Hs_energy pathways	CS
Hs_energy pathways	HK2
Hs_energy pathways	PFKP
Hs_enzyme activator	PSME2
Hs_enzyme binding	CASP2
Hs_enzyme inhibitor	BIRC6
Hs_enzyme inhibitor	C3
Hs_enzyme inhibitor	CSTB
Hs_enzyme inhibitor	KPNB3
Hs_enzyme inhibitor	SPINK1
Hs_enzyme inhibitor	TIMP2
Hs_enzyme inhibitor	TIMP4
Hs_enzyme linked receptor protein signaling pathwa	BMPR2
Hs_enzyme linked receptor protein signaling pathwa	EBAF
Hs_enzyme linked receptor protein signaling pathwa	FGFR1
Hs_enzyme linked receptor protein signaling pathwa	PDGFRB
Hs_enzyme linked receptor protein signaling pathwa	ROS1
Hs_ER to Golgi transport	KIAA0905
Hs_establishment and/or maintenance of cell polari	SPN
Hs_establishment and/or maintenance of chromatin a	NAP1L1
Hs_establishment and/or maintenance of chromatin a	SIRT6
Hs_eukaryotic 43S pre-initiation complex	EIF3S2
Hs_eukaryotic 43S pre-initiation complex	RPS10
Hs_eukaryotic 43S pre-initiation complex	RPS3A
Hs_eukaryotic 48S initiation complex	RPS10
Hs_eukaryotic 48S initiation complex	RPS3A
Hs_eukaryotic translation initiation factor 3 comp	EIF3S2
Hs_excitatory extracellular ligand-gated ion chann	CHRNA6
Hs_excretion	AQP7
Hs_exocytosis	AP1M2
Hs_exonuclease	ISG20
Hs_exonuclease	RAD50
Hs_exopeptidase	ACE
Hs_exotoxin	PRG2
Hs_extracellular	ADAMTS8
Hs_extracellular	BGLAP
Hs_extracellular	C3

Hs_extracellular	CORT
Hs_extracellular	CTRL
Hs_extracellular	CYR61
Hs_extracellular	EPO
Hs_extracellular	FGF10
Hs_extracellular	FGF14
Hs_extracellular	IFNG
Hs_extracellular	IL16
Hs_extracellular	IL9
Hs_extracellular	INHBC
Hs_extracellular	INSL3
Hs_extracellular	LGALS3
Hs_extracellular	LIF
Hs_extracellular	LOXL1
Hs_extracellular	LTF
Hs_extracellular	LUM
Hs_extracellular	MAP2K2
Hs_extracellular	MMP13
Hs_extracellular	MMP14
Hs_extracellular	PLAT
Hs_extracellular	PRG2
Hs_extracellular	SPARC
Hs_extracellular	TEM1
Hs_extracellular	TIMP2
Hs_extracellular	TIMP4
Hs_extracellular	TPX1
Hs_extracellular	WNT1
Hs_extracellular	WNT3A
Hs_extracellular	WNT4
Hs_extracellular ligand-gated ion channel	CHRNA6
Hs_extracellular ligand-gated ion channel	GABRA3
Hs_extracellular ligand-gated ion channel	GABRQ
Hs_extracellular matrix	ADAMTS8
Hs_extracellular matrix	LUM
Hs_extracellular matrix	MMP13
Hs_extracellular matrix	MMP14
Hs_extracellular matrix	TEM1
Hs_extracellular matrix	TIMP2
Hs_extracellular matrix	TIMP4
Hs_extracellular matrix	WNT3A
Hs_extracellular matrix glycoprotein	LUM
Hs_extracellular matrix structural constituent	WNT3A
Hs_extracellular matrix structural constituent	WNT4
Hs_extracellular space	CTRL
Hs_extracellular space	EPO
Hs_extracellular space	FGF10
Hs_extracellular space	FGF14
Hs_extracellular space	IL16
Hs_extracellular space	LIF
Hs_extracellular space	LTF
Hs_extracellular space	MMP13
Hs_extracellular space	TPX1
Hs_fatty acid biosynthesis	FASN

Hs_fatty acid biosynthesis	PTGIS
Hs_fatty acid metabolism	DCI
Hs_fatty acid metabolism	FASN
Hs_fatty acid metabolism	HPGD
Hs_fatty acid metabolism	PTGIS
Hs_fatty-acid synthase	FASN
Hs_feeding behavior	GALR2
Hs_female meiosis I	RAD50
Hs_ferric iron binding	LTF
Hs_fertilization (sensu Animalia)	ADAM2
Hs_fibroblast growth factor receptor	FGFR1
Hs_FK506-sensitive peptidyl-prolyl cis-trans isome	PPIE
Hs_flavodoxin	NOS2A
Hs_fluid transport	AQP7
Hs_folic acid and derivative metabolism	FPGS
Hs_frizzled receptor	FZD3
Hs_frizzled receptor	FZD7
Hs_frizzled receptor signaling pathway	FZD3
Hs_frizzled receptor signaling pathway	FZD7
Hs_frizzled-2 receptor signaling pathway	WNT1
Hs_frizzled-2 receptor signaling pathway	WNT3A
Hs_frizzled-2 receptor signaling pathway	WNT4
Hs_G2/M transition of mitotic cell cycle	CCNT2
Hs_GABA receptor	GABRA3
Hs_GABA receptor	GABRQ
Hs_GABA-A receptor	GABRA3
Hs_GABA-A receptor	GABRQ
Hs_galactose binding lectin	LGALS3
Hs_galactosyltransferase	B4GALT2
Hs_galanin receptor	GALR2
Hs_gametogenesis	EBAF
Hs_gametogenesis	INSL3
Hs_gametogenesis	MAK
Hs_gametogenesis	TMSB10
Hs_gametogenesis	WNT1
Hs_gamma-amino butyric acid signaling pathway	GABRA3
Hs_gap junction	MIP
Hs_gap-junction forming channel	GJA4
Hs_gene silencing	SIRT6
Hs_glucose catabolism	HK2
Hs_glucose catabolism	PFKP
Hs_glucose metabolism	HK2
Hs_glucose metabolism	PFKP
Hs_glutamate transporter	SLC7A11
Hs_glutamine family amino acid metabolism	ARG2
Hs_glycine hydroxymethyltransferase	ATIC
Hs_glycolysis	HK2
Hs_glycolysis	PFKP
Hs_glycopeptide hormone	EPO
Hs_glycopeptide hormone	INHBC
Hs_glycoprotein biosynthesis	LIPC
Hs_glycoprotein catabolism	AGA
Hs_glycoprotein metabolism	AGA

Hs_glycoprotein metabolism	LIPC
Hs_glycosaminoglycan binding	ADAMTS8
Hs_glycosaminoglycan binding	LIPC
Hs_glycosaminoglycan binding	PRG2
Hs_Golgi apparatus	AP1M2
Hs_Golgi apparatus	B4GALT2
Hs_Golgi apparatus	F2RL1
Hs_Golgi apparatus	NTSR1
Hs_Golgi apparatus	SREBF2
Hs_G-protein coupled receptor	FZD3
Hs_G-protein coupled receptor	FZD7
Hs_G-protein coupled receptor	VIPR1
Hs_G-protein coupled receptor protein signaling pa	C3
Hs_G-protein coupled receptor protein signaling pa	CORT
Hs_G-protein coupled receptor protein signaling pa	F2RL1
Hs_G-protein coupled receptor protein signaling pa	FZD3
Hs_G-protein coupled receptor protein signaling pa	FZD7
Hs_G-protein coupled receptor protein signaling pa	GABRA3
Hs_G-protein coupled receptor protein signaling pa	GALR2
Hs_G-protein coupled receptor protein signaling pa	GNAI2
Hs_G-protein coupled receptor protein signaling pa	GNAO1
Hs_G-protein coupled receptor protein signaling pa	GNAT1
Hs_G-protein coupled receptor protein signaling pa	HTR6
Hs_G-protein coupled receptor protein signaling pa	NTSR1
Hs_G-protein coupled receptor protein signaling pa	RASSF1
Hs_G-protein coupled receptor protein signaling pa	TRHR
Hs_G-protein coupled receptor protein signaling pa	TSHR
Hs_G-protein coupled receptor protein signaling pa	VIPR1
Hs_G-protein signaling, adenylate cyclase inhibiti	CORT
Hs_G-protein signaling, adenylate cyclase inhibiti	GNAI2
Hs_G-protein signaling, coupled to cAMP nucleotide	CORT
Hs_G-protein signaling, coupled to cAMP nucleotide	GALR2
Hs_G-protein signaling, coupled to cAMP nucleotide	GNAI2
Hs_G-protein signaling, coupled to cyclic nucleoti	CORT
Hs_G-protein signaling, coupled to cyclic nucleoti	GALR2
Hs_G-protein signaling, coupled to cyclic nucleoti	GNAI2
Hs_G-protein signaling, coupled to cyclic nucleoti	HTR6
Hs_G-protein signaling, coupled to cyclic nucleoti	TSHR
Hs_G-protein signaling, coupled to cyclic nucleoti	VIPR1
Hs_G-protein signaling, coupled to IP3 second mess	F2RL1
Hs_G-protein signaling, coupled to IP3 second mess	GALR2
Hs_group transfer coenzyme metabolism	ATP5A1
Hs_group transfer coenzyme metabolism	FPGS
Hs_growth factor	CSF1
Hs_growth factor	EBAF
Hs_growth factor	FGF10
Hs_growth factor	FGF14
Hs_growth factor	IL9
Hs_growth factor	INHBC
Hs_growth factor	LIF
Hs_growth factor	PSPN
Hs_growth factor	TGFB3
Hs_growth factor binding	CYR61

Hs_GTP binding	37865
Hs_GTP binding	GNAI2
Hs_GTP binding	GNAO1
Hs_GTP binding	GNAT1
Hs_GTP binding	RAC1
Hs_GTP binding	TUBA8
Hs_GTPase	DNM1
Hs_GTPase	RAC1
Hs_GTPase inhibitor	KPNB3
Hs_guanyl nucleotide binding	37865
Hs_guanyl nucleotide binding	DNM1
Hs_guanyl nucleotide binding	GNAI2
Hs_guanyl nucleotide binding	GNAO1
Hs_guanyl nucleotide binding	GNAT1
Hs_guanyl nucleotide binding	RAC1
Hs_guanyl nucleotide binding	TIAM2
Hs_guanyl nucleotide binding	TUBA8
Hs_guanylate kinase	DLG2
Hs_guanyl-nucleotide exchange factor	TIAM2
Hs_hearing	CLDN14
Hs_heart development	TBX5
Hs_heavy metal ion transport	LTF
Hs_helicase	DDX8
Hs_helicase	ENTPD2
Hs_hematopoietin/interferon-class (D200-domain) cy	EPO
Hs_hematopoietin/interferon-class (D200-domain) cy	EPOR
Hs_hematopoietin/interferon-class (D200-domain) cy	IFNG
Hs_hematopoietin/interferon-class (D200-domain) cy	IL9
Hs_hematopoietin/interferon-class (D200-domain) cy	LIF
Hs_heme biosynthesis	COX10
Hs_heme biosynthesis	HMBS
Hs_heme metabolism	COX10
Hs_heme metabolism	HMBS
Hs_hemopoiesis	CSF1
Hs_hemostasis	ANXA8
Hs_hemostasis	F2RL1
Hs_hemostasis	PLAT
Hs_heparin binding	ADAMTS8
Hs_heparin binding	LIPC
Hs_heparin binding	PRG2
Hs_heterocycle metabolism	COX10
Hs_heterocycle metabolism	FPGS
Hs_heterocycle metabolism	HMBS
Hs_heterogeneous nuclear ribonucleoprotein	HNRPA1
Hs_heterogeneous nuclear ribonucleoprotein	HNRPC
Hs_heterotrimeric G-protein GTPase, alpha-subunit	GNAI2
Hs_heterotrimeric G-protein GTPase, alpha-subunit	GNAO1
Hs_heterotrimeric G-protein GTPase, alpha-subunit	GNAT1
Hs_hexose catabolism	HK2
Hs_hexose metabolism	HK2
Hs_hexose metabolism	PFKP
Hs_histamine receptor	HTR6
Hs_histogenesis	BTK

Hs_histogenesis	FOXC2
Hs_histogenesis	HES7
Hs_histogenesis	TFAP2A
Hs_histogenesis	ZNFN1A1
Hs_histogenesis and organogenesis	FGF10
Hs_histogenesis and organogenesis	HEYL
Hs_histogenesis and organogenesis	KLF1
Hs_histogenesis and organogenesis	TGFB3
Hs_homeostasis	CYP27B1
Hs_homeostasis	LTF
Hs_homophilic cell adhesion	CDH18
Hs_hormone	CORT
Hs_hormone	EPO
Hs_hormone	INHBC
Hs_hormone	INSL3
Hs_humoral defense mechanism (sensu Invertebrata)	CD22
Hs_humoral defense mechanism (sensu Invertebrata)	CSF1
Hs_humoral defense mechanism (sensu Invertebrata)	MASP2
Hs_humoral defense mechanism (sensu Vertebrata)	C3
Hs_humoral defense mechanism (sensu Vertebrata)	MASP2
Hs_humoral immune response	BCL2
Hs_humoral immune response	C3
Hs_humoral immune response	CD22
Hs_humoral immune response	CSF1
Hs_humoral immune response	LTF
Hs_humoral immune response	MASP2
Hs_hydrogen ion transporter	ATP4A
Hs_hydrogen ion transporter	ATP5A1
Hs_hydrogen ion transporter	ATP5G2
Hs_hydrogen transport	ATP4A
Hs_hydrogen transport	ATP5A1
Hs_hydrogen transport	ATP5G2
Hs_hydrogen transport	SLC15A2
Hs_hydrogen transport	SLC5A5
Hs_hydrogen transport	SLC8A3
Hs_hydrogen-/sodium-translocating ATPase	ATP5A1
Hs_hydrogen-/sodium-translocating ATPase	ATP5G2
Hs_hydrogen-transporting two-sector ATPase	ATP5A1
Hs_hydrogen-transporting two-sector ATPase	ATP5G2
Hs_hydrogen-transporting two-sector ATPase complex	ATP5A1
Hs_hydrolase	ABCA3
Hs_hydrolase	ACE
Hs_hydrolase	ADAM10
Hs_hydrolase	ADAM2
Hs_hydrolase	ADAMTS8
Hs_hydrolase	AGA
Hs_hydrolase	ALPI
Hs_hydrolase	ARG2
Hs_hydrolase	ATIC
Hs_hydrolase	ATP4A
Hs_hydrolase	ATP5A1
Hs_hydrolase	ATP5G2
Hs_hydrolase	BACE

Hs_hydrolase	CASP2
Hs_hydrolase	CTRL
Hs_hydrolase	DDX8
Hs_hydrolase	DNM1
Hs_hydrolase	DPYS
Hs_hydrolase	DUSP2
Hs_hydrolase	ENTPD2
Hs_hydrolase	FASN
Hs_hydrolase	GDA
Hs_hydrolase	GZMA
Hs_hydrolase	GZMB
Hs_hydrolase	ISG20
Hs_hydrolase	LIPC
Hs_hydrolase	LTF
Hs_hydrolase	MASP2
Hs_hydrolase	MLH1
Hs_hydrolase	MMP13
Hs_hydrolase	MMP14
Hs_hydrolase	NEU2
Hs_hydrolase	NIT1
Hs_hydrolase	OCA2
Hs_hydrolase	PDE1B
Hs_hydrolase	PGLYRP
Hs_hydrolase	PLAT
Hs_hydrolase	PRSS21
Hs_hydrolase	PSMB4
Hs_hydrolase	PSMB9
Hs_hydrolase	PTPN1
Hs_hydrolase	PTPRK
Hs_hydrolase	RAC1
Hs_hydrolase	RAD50
Hs_hydrolase	TRAF2
Hs_hydrolase	TRAF4
Hs_hydrolase, acting on acid anhydrides, catalyzin	ABCA3
Hs_hydrolase, acting on acid anhydrides, catalyzin	ATP4A
Hs_hydrolase, acting on acid anhydrides, catalyzin	ATP5A1
Hs_hydrolase, acting on acid anhydrides, catalyzin	ATP5G2
Hs_hydrolase, acting on acid anhydrides, catalyzin	OCA2
Hs_hydrolase, acting on acid anhydrides, catalyzin	RAD50
Hs_hydrolase, acting on acid anhydrides, in phosph	ABCA3
Hs_hydrolase, acting on acid anhydrides, in phosph	ATP4A
Hs_hydrolase, acting on acid anhydrides, in phosph	ATP5A1
Hs_hydrolase, acting on acid anhydrides, in phosph	ATP5G2
Hs_hydrolase, acting on acid anhydrides, in phosph	DDX8
Hs_hydrolase, acting on acid anhydrides, in phosph	DNM1
Hs_hydrolase, acting on acid anhydrides, in phosph	ENTPD2
Hs_hydrolase, acting on acid anhydrides, in phosph	MLH1
Hs_hydrolase, acting on acid anhydrides, in phosph	OCA2
Hs_hydrolase, acting on acid anhydrides, in phosph	RAC1
Hs_hydrolase, acting on acid anhydrides, in phosph	RAD50
Hs_hydrolase, acting on carbon-nitrogen (but not p	AGA
Hs_hydrolase, acting on carbon-nitrogen (but not p	ARG2
Hs_hydrolase, acting on carbon-nitrogen (but not p	ATIC

Hs_hydrolase, acting on carbon-nitrogen (but not p	DPYS
Hs_hydrolase, acting on carbon-nitrogen (but not p	GDA
Hs_hydrolase, acting on carbon-nitrogen (but not p	NIT1
Hs_hydrolase, acting on carbon-nitrogen (but not p	PGLYRP
Hs_hydrolase, acting on ester bonds	ALPI
Hs_hydrolase, acting on ester bonds	DUSP2
Hs_hydrolase, acting on ester bonds	FASN
Hs_hydrolase, acting on ester bonds	ISG20
Hs_hydrolase, acting on ester bonds	LIPC
Hs_hydrolase, acting on ester bonds	PDE1B
Hs_hydrolase, acting on ester bonds	PTPN1
Hs_hydrolase, acting on ester bonds	PTPRK
Hs_hydrolase, acting on ester bonds	RAD50
Hs_hydrolase, hydrolyzing O-glycosyl compounds	NEU2
Hs_hydro-lyase	CA14
Hs_hydro-lyase	CA7
Hs_hydroxymethyl-, formyl- and related transferase	ATIC
Hs_IgE binding	LGALS3
Hs_immune response	AIRE
Hs_immune response	C3
Hs_immune response	CD22
Hs_immune response	CD74
Hs_immune response	GZMA
Hs_immune response	IFNG
Hs_immune response	IL16
Hs_immune response	IL9
Hs_immune response	LIF
Hs_immune response	MHC2TA
Hs_immune response	PGLYRP
Hs_immune response	PRG2
Hs_immune response	PSMB9
Hs_immune response	PSME2
Hs_immune response	TCF8
Hs_immune response	VIPR1
Hs_immunoglobulin binding	LGALS3
Hs_inactivation of MAPK	DUSP2
Hs_induction of apoptosis	PDCD5
Hs_induction of apoptosis by extracellular signals	BTK
Hs_induction of programmed cell death	BTK
Hs_induction of programmed cell death	PDCD5
Hs_inflammatory response	C3
Hs_inflammatory response	IL9
Hs_inflammatory response	PRG2
Hs_inflammatory response	RAC1
Hs_inhibitory extracellular ligand-gated ion chann	GABRA3
Hs_inhibitory extracellular ligand-gated ion chann	GABRQ
Hs_innate immune response	C3
Hs_innate immune response	IL9
Hs_innate immune response	PRG2
Hs_innate immune response	RAC1
Hs_inner membrane	ATP5A1
Hs_inner membrane	ATP5G2
Hs_inner membrane	BCL2

Hs_inner membrane	FXC1
Hs_inner membrane	SLC25A11
Hs_inorganic anion transport	GABRA3
Hs_inorganic anion transporter	OCA2
Hs_inorganic anion transporter	SLC5A5
Hs_insulin receptor binding	INSL3
Hs_insulin-like growth factor binding	CYR61
Hs_integral to membrane	ABCA3
Hs_integral to membrane	ACE
Hs_integral to membrane	ALPI
Hs_integral to membrane	ATP5G2
Hs_integral to membrane	B4GALT2
Hs_integral to membrane	BCL2
Hs_integral to membrane	CA14
Hs_integral to membrane	CD22
Hs_integral to membrane	CD74
Hs_integral to membrane	CDH18
Hs_integral to membrane	CHRNA6
Hs_integral to membrane	CLDN14
Hs_integral to membrane	COMT
Hs_integral to membrane	COX10
Hs_integral to membrane	EMP3
Hs_integral to membrane	ENTPD2
Hs_integral to membrane	EVI2A
Hs_integral to membrane	FDFT1
Hs_integral to membrane	FXYD5
Hs_integral to membrane	FZD7
Hs_integral to membrane	GALR2
Hs_integral to membrane	ITGA6
Hs_integral to membrane	ITGB5
Hs_integral to membrane	KCNAB1
Hs_integral to membrane	KCNAB2
Hs_integral to membrane	KCNJ5
Hs_integral to membrane	KCNN4
Hs_integral to membrane	KCNQ5
Hs_integral to membrane	KIAA0143
Hs_integral to membrane	NPR3
Hs_integral to membrane	OCA2
Hs_integral to membrane	PDGFRB
Hs_integral to membrane	PTGIS
Hs_integral to membrane	RHCG
Hs_integral to membrane	ROS1
Hs_integral to membrane	SLC5A5
Hs_integral to membrane	SLC7A11
Hs_integral to membrane	SLC8A3
Hs_integral to membrane	SREBF2
Hs_integral to membrane	VMD2
Hs_integral to plasma membrane	ACCN1
Hs_integral to plasma membrane	ADAM10
Hs_integral to plasma membrane	ADAM2
Hs_integral to plasma membrane	AQP7
Hs_integral to plasma membrane	ATP4A
Hs_integral to plasma membrane	BACE

Hs_integral to plasma membrane	BMPR2
Hs_integral to plasma membrane	CD22
Hs_integral to plasma membrane	CD69
Hs_integral to plasma membrane	CHRNA6
Hs_integral to plasma membrane	CLDN1
Hs_integral to plasma membrane	CLDN3
Hs_integral to plasma membrane	CSF1
Hs_integral to plasma membrane	EPOR
Hs_integral to plasma membrane	F2RL1
Hs_integral to plasma membrane	FGFR1
Hs_integral to plasma membrane	FZD3
Hs_integral to plasma membrane	GABRA3
Hs_integral to plasma membrane	GABRQ
Hs_integral to plasma membrane	GJA4
Hs_integral to plasma membrane	HTR6
Hs_integral to plasma membrane	ITGA6
Hs_integral to plasma membrane	ITGB5
Hs_integral to plasma membrane	JAM2
Hs_integral to plasma membrane	KCNJ12
Hs_integral to plasma membrane	KCNJ5
Hs_integral to plasma membrane	KCNK10
Hs_integral to plasma membrane	KCNN4
Hs_integral to plasma membrane	KCNQ5
Hs_integral to plasma membrane	LRP1
Hs_integral to plasma membrane	MIP
Hs_integral to plasma membrane	MMP14
Hs_integral to plasma membrane	MOG
Hs_integral to plasma membrane	NTSR1
Hs_integral to plasma membrane	PTPRK
Hs_integral to plasma membrane	ROS1
Hs_integral to plasma membrane	SLC15A2
Hs_integral to plasma membrane	SLC25A11
Hs_integral to plasma membrane	SLC29A2
Hs_integral to plasma membrane	SPN
Hs_integral to plasma membrane	THY1
Hs_integral to plasma membrane	TRHR
Hs_integral to plasma membrane	TSHR
Hs_integral to plasma membrane	VIPR1
Hs_integrin binding	ADAM2
Hs_integrin binding	ADAMTS8
Hs_integrin complex	ITGA6
Hs_integrin complex	ITGB5
Hs_integrin-mediated signaling pathway	ITGA6
Hs_integrin-mediated signaling pathway	ITGB5
Hs_intercellular junction	CLDN1
Hs_intercellular junction	CLDN14
Hs_intercellular junction	CLDN3
Hs_intercellular junction	GJA4
Hs_intercellular junction	MIP
Hs_intercellular junction assembly and/or maintena	GJA4
Hs_intermediate filament cytoskeleton	PRPH
Hs_intracellular	BIRC6
Hs_intracellular	CASP2

Hs_intracellular	GDA
Hs_intracellular	ISG20
Hs_intracellular	RPL18
Hs_intracellular	RPL31
Hs_intracellular	RPS3A
Hs_intracellular ligand-gated ion channel	ATP5A1
Hs_intracellular protein transport	KPNB3
Hs_intracellular protein transport	SNX12
Hs_intracellular signaling cascade	BTK
Hs_intracellular signaling cascade	CORT
Hs_intracellular signaling cascade	DLG2
Hs_intracellular signaling cascade	DUSP2
Hs_intracellular signaling cascade	FGFR1
Hs_intracellular signaling cascade	GADD45G
Hs_intracellular signaling cascade	GALR2
Hs_intracellular signaling cascade	GNAI2
Hs_intracellular signaling cascade	HTR6
Hs_intracellular signaling cascade	IL16
Hs_intracellular signaling cascade	LNK
Hs_intracellular signaling cascade	NFKBIA
Hs_intracellular signaling cascade	RAC1
Hs_intracellular signaling cascade	RASSF1
Hs_intracellular signaling cascade	SNX12
Hs_intracellular signaling cascade	STAT6
Hs_intracellular signaling cascade	TIAM2
Hs_intracellular signaling cascade	TSC1
Hs_intracellular signaling cascade	TSHR
Hs_intracellular signaling cascade	VIPR1
Hs_intramolecular isomerase	DCI
Hs_intramolecular isomerase	HPGD
Hs_intramolecular isomerase	PTGIS
Hs_intramolecular isomerase, other intramolecular	HPGD
Hs_intramolecular isomerase, other intramolecular	PTGIS
Hs_intramolecular isomerase, transposing C=C bonds	DCI
Hs_inward rectifier potassium channel	KCNJ12
Hs_inward rectifier potassium channel	KCNJ5
Hs_inward rectifier potassium channel	KCNQ5
Hs_ion channel	ACCN1
Hs_ion channel	AQP7
Hs_ion channel	ATP5A1
Hs_ion channel	CHRNA6
Hs_ion channel	FXYD5
Hs_ion channel	GABRA3
Hs_ion channel	GABRQ
Hs_ion channel	KCNAB1
Hs_ion channel	KCNAB2
Hs_ion channel	KCNJ12
Hs_ion channel	KCNJ5
Hs_ion channel	KCNK10
Hs_ion channel	KCNN4
Hs_ion channel	KCNQ5
Hs_ion homeostasis	CYP27B1
Hs_ion homeostasis	LTF

Hs_ion transport	ACCN1
Hs_ion transport	CHRNA6
Hs_ion transport	FXYD5
Hs_ion transport	GABRA3
Hs_ion transport	GABRQ
Hs_ion transport	KCNAB1
Hs_ion transport	KCNAB2
Hs_ion transport	KCNJ12
Hs_ion transport	KCNJ5
Hs_ion transport	KCNK10
Hs_ion transport	KCNN4
Hs_iron homeostasis	LTF
Hs_iron superoxide dismutase	SOD2
Hs_iron transport	LTF
Hs_isomerase	DCI
Hs_isomerase	HPGD
Hs_isomerase	PPIE
Hs_isomerase	PTGIS
Hs_isoprenoid binding	CRABP1
Hs_isoprenoid metabolism	FDFT1
Hs_kinase	BMPR2
Hs_kinase	BTK
Hs_kinase	CCNT2
Hs_kinase	CDK5
Hs_kinase	CKMT1
Hs_kinase	DLG2
Hs_kinase	FGFR1
Hs_kinase	HK2
Hs_kinase	MAK
Hs_kinase	MAP2K2
Hs_kinase	MAP2K3
Hs_kinase	MAP2K7
Hs_kinase	MAP3K8
Hs_kinase	MAPK6
Hs_kinase	NEK2
Hs_kinase	PDGFRB
Hs_kinase	PFKP
Hs_kinase	ROS1
Hs_kinase	RPS6KB2
Hs_kinase	SNK
Hs_kinase	STK23
Hs_kinase	VRK1
Hs_kinase regulator	CCNT2
Hs_L-amino acid transporter	OCA2
Hs_L-amino acid transporter	SLC7A11
Hs_large ribosomal subunit	RPL18
Hs_large ribosomal subunit	RPL31
Hs_learning and/or memory	GALR2
Hs_lectin	CD22
Hs_lectin	CD69
Hs_lectin	LGALS3
Hs_lectin	PRG2
Hs_ligand-dependent nuclear receptor	NR1I2

Hs_ligand-dependent nuclear receptor	NR1I3
Hs_ligand-gated ion channel	ACCN1
Hs_ligand-gated ion channel	ATP5A1
Hs_ligand-gated ion channel	CHRNA6
Hs_ligand-gated ion channel	GABRA3
Hs_ligand-gated ion channel	GABRQ
Hs_ligand-regulated transcription factor	NR1I2
Hs_ligand-regulated transcription factor	NR1I3
Hs_ligase	BIRC6
Hs_ligase	FPGS
Hs_ligase	LIG1
Hs_ligase, forming carbon-nitrogen bonds	FPGS
Hs_ligase, forming phosphoric ester bonds	LIG1
Hs_lipase	LIPC
Hs_lipid binding	ANXA8
Hs_lipid binding	ATP5G2
Hs_lipid binding	CRABP1
Hs_lipid binding	SCP2
Hs_lipid biosynthesis	FASN
Hs_lipid biosynthesis	FDFT1
Hs_lipid biosynthesis	PTGIS
Hs_lipid biosynthesis	SCP2
Hs_lipid catabolism	LIPC
Hs_lipid metabolism	CYP27B1
Hs_lipid metabolism	LRP1
Hs_lipid metabolism	PTGIS
Hs_lipid metabolism	SREBF2
Hs_lipid transporter	LIPC
Hs_lipoprotein binding	LRP1
Hs_lyase	ADSL
Hs_lyase	CA14
Hs_lyase	CA7
Hs_lyase	CS
Hs_lyase	FASN
Hs_lyase	HMBS
Hs_lyase	PTS
Hs_lytic vacuole	AGA
Hs_M phase of mitotic cell cycle	NEK2
Hs_macrolide binding	PPIE
Hs_macromolecule biosynthesis	EIF3S2
Hs_macromolecule biosynthesis	GADD45G
Hs_macromolecule biosynthesis	LIPC
Hs_macromolecule biosynthesis	RPL18
Hs_macromolecule biosynthesis	RPL31
Hs_macromolecule biosynthesis	RPS10
Hs_macromolecule biosynthesis	RPS3A
Hs_macromolecule biosynthesis	RPS6KB2
Hs_macromolecule biosynthesis	SRP9
Hs_macromolecule catabolism	ACE
Hs_macromolecule catabolism	ADAM10
Hs_macromolecule catabolism	ADAM2
Hs_macromolecule catabolism	ADAMTS8
Hs_macromolecule catabolism	AGA

Hs_macromolecule catabolism	BACE
Hs_macromolecule catabolism	BIRC6
Hs_macromolecule catabolism	CASP2
Hs_macromolecule catabolism	CTRL
Hs_macromolecule catabolism	GZMA
Hs_macromolecule catabolism	GZMB
Hs_macromolecule catabolism	MASP2
Hs_macromolecule catabolism	MMP13
Hs_macromolecule catabolism	MMP14
Hs_macromolecule catabolism	PLAT
Hs_macromolecule catabolism	PRSS21
Hs_macromolecule catabolism	PSMB4
Hs_macromolecule catabolism	PSMB9
Hs_macromolecule catabolism	TRAF2
Hs_macromolecule catabolism	TRAF4
Hs_magnesium binding	ALPI
Hs_magnesium binding	ATP4A
Hs_magnesium binding	COMT
Hs_magnesium binding	ENTPD2
Hs_magnesium binding	FDFT1
Hs_magnesium binding	PFKP
Hs_magnesium binding	PTS
Hs_main pathways of carbohydrate metabolism	CS
Hs_main pathways of carbohydrate metabolism	HK2
Hs_main pathways of carbohydrate metabolism	PFKP
Hs_maintenance of fidelity during DNA dependent DN	MLH1
Hs_manganese binding	ARG2
Hs_manganese binding	SOD2
Hs_manganese superoxide dismutase	SOD2
Hs_MAP kinase kinase	MAP2K3
Hs_MAP kinase kinase	MAP2K7
Hs_MAPKKK cascade	DUSP2
Hs_MAPKKK cascade	FGFR1
Hs_MAPKKK cascade	GADD45G
Hs_meiosis	NEK2
Hs_meiosis	RAD50
Hs_meiosis	STAG3
Hs_meiosis I	RAD50
Hs_meiotic chromosome	STAG3
Hs_meiotic prophase I	RAD50
Hs_meiotic prophase I	STAG3
Hs_meiotic recombination	RAD50
Hs_membrane	CYBA
Hs_membrane	CYP1A1
Hs_membrane	CYP27B1
Hs_membrane	HK2
Hs_membrane	RAD50
Hs_membrane	SPTA1
Hs_membrane coat	AP1M2
Hs_membrane coat	KIAA0905
Hs_membrane fraction	ABCA3
Hs_membrane fraction	ACCN1
Hs_membrane fraction	ACE

Hs_membrane fraction	ATP5A1
Hs_membrane fraction	ATP5G2
Hs_membrane fraction	BACE
Hs_membrane fraction	COMT
Hs_membrane fraction	CYP1A1
Hs_membrane fraction	EMP3
Hs_membrane fraction	JUP
Hs_membrane fraction	KCNJ12
Hs_membrane fraction	KCNK10
Hs_membrane fraction	KCNN4
Hs_membrane fraction	LRP1
Hs_membrane fraction	MIP
Hs_membrane fraction	PRSS21
Hs_membrane fraction	ROS1
Hs_membrane fraction	SPTA1
Hs_membrane fraction	VMD2
Hs_membrane fusion	ADAM2
Hs_mesoderm development	BTK
Hs_mesoderm development	FOXC2
Hs_mesoderm development	HES7
Hs_mesoderm development	ZNFN1A1
Hs_metabolism	ALDH2
Hs_metabolism	ALPI
Hs_metabolism	ATP4A
Hs_metabolism	AUP1
Hs_metabolism	DCI
Hs_metabolism	HPGD
Hs_metabolism	SIRT6
Hs_metal ion homeostasis	CYP27B1
Hs_metal ion homeostasis	LTF
Hs_metal ion transport	ACCN1
Hs_metal ion transport	ATP4A
Hs_metal ion transport	KCNAB1
Hs_metal ion transport	KCNAB2
Hs_metal ion transport	KCNJ12
Hs_metal ion transport	KCNJ5
Hs_metal ion transport	KCNK10
Hs_metal ion transport	KCNN4
Hs_metal ion transport	KCNQ5
Hs_metal ion transport	LTF
Hs_metal ion transport	SLC5A5
Hs_metal ion transport	SLC8A3
Hs_metalloendopeptidase	ADAM10
Hs_metalloendopeptidase	ADAM2
Hs_metalloendopeptidase	ADAMTS8
Hs_metalloendopeptidase	MMP13
Hs_metalloendopeptidase	MMP14
Hs_metalloendopeptidase	TRAF2
Hs_metalloendopeptidase	TRAF4
Hs_metalloendopeptidase inhibitor	TIMP2
Hs_metalloendopeptidase inhibitor	TIMP4
Hs_methyltransferase	ATIC
Hs_methyltransferase	COMT

Hs_methyltransferase	FASN
Hs_methyltransferase	TPMT
Hs_microsome	COMT
Hs_microsome	CYP1A1
Hs_microtubule cytoskeleton	TUBA8
Hs_microtubule organizing center	NEK2
Hs_microtubule-based movement	TUBA8
Hs_microtubule-based process	DNM1
Hs_microtubule-based process	TUBA8
Hs_mismatch repair	MLH1
Hs_mitochondrial inner membrane	ATP5A1
Hs_mitochondrial inner membrane	ATP5G2
Hs_mitochondrial inner membrane	BCL2
Hs_mitochondrial inner membrane	FXC1
Hs_mitochondrial inner membrane	SLC25A11
Hs_mitochondrial matrix	BCKDHB
Hs_mitochondrial membrane	ATP5A1
Hs_mitochondrial membrane	ATP5G2
Hs_mitochondrial membrane	BCL2
Hs_mitochondrial membrane	FXC1
Hs_mitochondrial membrane	SLC25A11
Hs_mitochondrial outer membrane	HK2
Hs_mitochondrial translocation	FXC1
Hs_mitochondrion	ALDH2
Hs_mitochondrion	ARG2
Hs_mitochondrion	ATP5A1
Hs_mitochondrion	ATP5G2
Hs_mitochondrion	BCKDHB
Hs_mitochondrion	BCL2
Hs_mitochondrion	CKMT1
Hs_mitochondrion	COX10
Hs_mitochondrion	CS
Hs_mitochondrion	CYBA
Hs_mitochondrion	CYP27B1
Hs_mitochondrion	DCI
Hs_mitochondrion	FPGS
Hs_mitochondrion	FXC1
Hs_mitochondrion	HK2
Hs_mitochondrion	HSPA9B
Hs_mitochondrion	SCP2
Hs_mitochondrion	SLC25A11
Hs_mitochondrion	SOD2
Hs_mitochondrion organization and biogenesis	FXC1
Hs_mitosis	NEK2
Hs_mitotic recombination	RAD50
Hs_molecular_function unknown	37865
Hs_molecular_function unknown	AIRE
Hs_molecular_function unknown	AUP1
Hs_molecular_function unknown	KIAA0905
Hs_molecular_function unknown	NIT1
Hs_molecular_function unknown	RAI2
Hs_molecular_function unknown	RPL31
Hs_molecular_function unknown	SIRT6

Hs_molecular_function unknown	TEM1
Hs_molecular_function unknown	UBL1
Hs_molecular_function unknown	UBL5
Hs_molecular_function unknown	VMD2
Hs_molecular_function unknown	ZNF179
Hs_monooxygenase	CYP1A1
Hs_monooxygenase	CYP27B1
Hs_monooxygenase	PTGIS
Hs_monosaccharide catabolism	HK2
Hs_monosaccharide metabolism	HK2
Hs_monosaccharide metabolism	PFKP
Hs_monovalent inorganic cation transporter	ATP4A
Hs_monovalent inorganic cation transporter	ATP5A1
Hs_monovalent inorganic cation transporter	ATP5G2
Hs_morphogenesis	WNT3A
Hs_motor	DNM1
Hs_mRNA binding	DDX8
Hs_mRNA binding	LSM2
Hs_mRNA processing	DDX8
Hs_mRNA processing	HNRPA1
Hs_mRNA processing	HNRPC
Hs_mRNA processing	LSM2
Hs_mRNA splicing	DDX8
Hs_mRNA splicing	HNRPC
Hs_mRNA splicing	LSM2
Hs_muscle contraction	GALR2
Hs_muscle contraction	GNAO1
Hs_muscle contraction	KCNJ12
Hs_muscle contraction	VIPR1
Hs_myofibril	TPM2
Hs_myosin phosphatase	DUSP2
Hs_negative regulation of adenylate cyclase activi	GNAI2
Hs_negative regulation of cell adhesion	FXYD5
Hs_negative regulation of cell adhesion	SPN
Hs_negative regulation of cell proliferation	ADAMTS8
Hs_negative regulation of cell proliferation	BCL2
Hs_negative regulation of cell proliferation	EMP3
Hs_negative regulation of transcription	MBD2
Hs_negative regulation of transcription	TCF8
Hs_negative regulation of transcription, DNA-depen	TCF8
Hs_neurogenesis	FGF14
Hs_neurogenesis	GDA
Hs_neurogenesis	GNAO1
Hs_neurogenesis	HEYL
Hs_neuropeptide hormone	CORT
Hs_neuropeptide signaling pathway	RASSF1
Hs_neurotransmitter binding	CHRNA6
Hs_neurotransmitter binding	GABRA3
Hs_neurotransmitter binding	GABRQ
Hs_neurotransmitter metabolism	COMT
Hs_neurotransmitter receptor	CHRNA6
Hs_neurotransmitter receptor	GABRA3
Hs_neurotransmitter transporter	GABRQ

Hs_neutral amino acid transporter	SLC7A11
Hs_nickel superoxide dismutase	SOD2
Hs_nicotinic acetylcholine-gated receptor-channel	CHRNA6
Hs_NIK-I-kappaB/NF-kappaB cascade	NFKBIA
Hs_nitric oxide biosynthesis	ARG2
Hs_nitric oxide biosynthesis	NOS2A
Hs_nitric oxide metabolism	ARG2
Hs_nitric oxide metabolism	NOS2A
Hs_nitric oxide synthase	NOS2A
Hs_nitrogen metabolism	ARG2
Hs_nitrogen metabolism	NIT1
Hs_nitrogen metabolism	NOS2A
Hs_N-linked glycosylation	LIPC
Hs_NLS-bearing substrate-nucleus import	KPNB3
Hs_non-covalent chromatin modification	SIRT6
Hs_non-G-protein coupled 7TM receptor	FZD3
Hs_non-G-protein coupled 7TM receptor	FZD7
Hs_non-selective vesicle targeting	AP1M2
Hs_nuclear division	NEK2
Hs_nuclear division	RAD50
Hs_nuclear division	STAG3
Hs_nuclear envelope-endoplasmic reticulum network	MRVI1
Hs_nuclear membrane	KPNB3
Hs_nuclear membrane	NUP62
Hs_nuclear membrane	UBL1
Hs_nuclear pore	KPNB3
Hs_nuclear pore	NUP62
Hs_nuclear pore	UBL1
Hs_nuclease	ISG20
Hs_nuclease	RAD50
Hs_nucleobase, nucleoside, nucleotide and nucleic	ATIC
Hs_nucleobase, nucleoside, nucleotide and nucleic	DPYS
Hs_nucleobase, nucleoside, nucleotide and nucleic	FPGS
Hs_nucleobase, nucleoside, nucleotide and nucleic	GDA
Hs_nucleobase, nucleoside, nucleotide and nucleic	SLC29A2
Hs_nucleobase, nucleoside, nucleotide and nucleic	TPMT
Hs_nucleobase, nucleoside, nucleotide kinase	DLG2
Hs_nucleocytoplasmic transport	HNRPA1
Hs_nucleocytoplasmic transport	KPNB3
Hs_nucleocytoplasmic transport	NFKBIA
Hs_nucleolus	LSM2
Hs_nucleolus	MKI67
Hs_nucleolus	SLC29A2
Hs_nucleoplasm	HNRPA1
Hs_nucleoplasm	ISG20
Hs_nucleoplasm	NAP1L1
Hs_nucleoplasm	RPC32
Hs_nucleoplasm	SIRT6
Hs_nucleoside monophosphate metabolism	APRT
Hs_nucleoside phosphate metabolism	ATP5A1
Hs_nucleoside transporter	SLC29A2
Hs_nucleoside triphosphate metabolism	ATP5A1
Hs_nucleosome assembly	NAP1L1

Hs_nucleotide binding	ABCA3
Hs_nucleotide kinase	DLG2
Hs_nucleotidyltransferase	RPC32
Hs_nucleus	AIRE
Hs_nucleus	CALR
Hs_nucleus	CBL
Hs_nucleus	CCNT2
Hs_nucleus	CENPB
Hs_nucleus	DDX8
Hs_nucleus	DUSP2
Hs_nucleus	ELK3
Hs_nucleus	FOXC1
Hs_nucleus	FOXC2
Hs_nucleus	HES7
Hs_nucleus	HNRPA1
Hs_nucleus	HNRPC
Hs_nucleus	HOXA11
Hs_nucleus	HOXC13
Hs_nucleus	ID2
Hs_nucleus	ISG20
Hs_nucleus	KLF1
Hs_nucleus	KPNB3
Hs_nucleus	LGALS3
Hs_nucleus	LIG1
Hs_nucleus	LSM2
Hs_nucleus	MBD2
Hs_nucleus	MKI67
Hs_nucleus	MLH1
Hs_nucleus	MYCN
Hs_nucleus	NAP1L1
Hs_nucleus	NEK2
Hs_nucleus	NFATC2
Hs_nucleus	NR1I2
Hs_nucleus	NR1I3
Hs_nucleus	NUP62
Hs_nucleus	POU3F3
Hs_nucleus	PPIE
Hs_nucleus	RAD50
Hs_nucleus	RBBP2
Hs_nucleus	RPC32
Hs_nucleus	SIRT6
Hs_nucleus	SLC29A2
Hs_nucleus	SREBF2
Hs_nucleus	SRF
Hs_nucleus	STAG3
Hs_nucleus	STAT6
Hs_nucleus	TBX5
Hs_nucleus	TCF2
Hs_nucleus	TCF8
Hs_nucleus	TFAP2A
Hs_nucleus	UBL1
Hs_nucleus	WRB
Hs_nucleus	ZNF161

Hs_nucleus	ZNFN1A1
Hs_nutritional response pathway	GNAI2
Hs_obsolete	ABCA3
Hs_obsolete	ADAMTS8
Hs_obsolete	ANXA8
Hs_obsolete	ATP4A
Hs_obsolete	BCL2
Hs_obsolete	CBL
Hs_obsolete	CHRNA6
Hs_obsolete	CYP1A1
Hs_obsolete	CYR61
Hs_obsolete	FGF10
Hs_obsolete	FGFR1
Hs_obsolete	FOXC1
Hs_obsolete	FZD7
Hs_obsolete	GABRA3
Hs_obsolete	GJA4
Hs_obsolete	GNAI2
Hs_obsolete	HEYL
Hs_obsolete	HOXA11
Hs_obsolete	HOXC13
Hs_obsolete	KCNK10
Hs_obsolete	KLF1
Hs_obsolete	KPNB3
Hs_obsolete	LIG1
Hs_obsolete	MLH1
Hs_obsolete	MRVI1
Hs_obsolete	MYCN
Hs_obsolete	RAC1
Hs_obsolete	RHCG
Hs_obsolete	ROS1
Hs_obsolete	SLC15A2
Hs_obsolete	SLC25A11
Hs_obsolete	SLC5A5
Hs_obsolete	TBX5
Hs_obsolete	TFAP2A
Hs_obsolete	TGFB3
Hs_obsolete	TSHR
Hs_obsolete	WNT1
Hs_oligopeptide transporter	SLC15A2
Hs_O-methyltransferase	COMT
Hs_oncogenesis	ANXA8
Hs_oncogenesis	BCL2
Hs_oncogenesis	CBL
Hs_oncogenesis	CYP1A1
Hs_oncogenesis	FGFR1
Hs_oncogenesis	FZD7
Hs_oncogenesis	GNAI2
Hs_oncogenesis	MLH1
Hs_oncogenesis	MRVI1
Hs_oncogenesis	MYCN
Hs_oncogenesis	ROS1
Hs_oncogenesis	TFAP2A

Hs_oncogenesis	TSHR
Hs_oncogenesis	WNT1
Hs_one-carbon compound metabolism	CA14
Hs_one-carbon compound metabolism	CA7
Hs_one-carbon compound metabolism	FPGS
Hs_oogenesis	EBAF
Hs_organic acid biosynthesis	FASN
Hs_organic acid biosynthesis	PTGIS
Hs_organic acid metabolism	ARG2
Hs_organic acid metabolism	DCI
Hs_organic acid metabolism	FASN
Hs_organic acid metabolism	FPGS
Hs_organic acid metabolism	HPGD
Hs_organic acid metabolism	PTGIS
Hs_organic acid metabolism	PTS
Hs_organic cation transporter	RHCG
Hs_organic cation transporter	SLC25A11
Hs_organogenesis	ACCN1
Hs_organogenesis	BGLAP
Hs_organogenesis	BMPR2
Hs_organogenesis	BTK
Hs_organogenesis	FGF14
Hs_organogenesis	FGFR1
Hs_organogenesis	FOXC2
Hs_organogenesis	GDA
Hs_organogenesis	GNAO1
Hs_organogenesis	HES7
Hs_organogenesis	HEYL
Hs_organogenesis	LUM
Hs_organogenesis	MOG
Hs_organogenesis	NPR3
Hs_organogenesis	POU3F3
Hs_organogenesis	PSPN
Hs_organogenesis	PTS
Hs_organogenesis	RAC1
Hs_organogenesis	SPARC
Hs_organogenesis	TBX5
Hs_organogenesis	TFAP2A
Hs_organogenesis	TPM2
Hs_organogenesis	UNC5C
Hs_organogenesis	WNT1
Hs_organogenesis	ZNFN1A1
Hs_ossification	SPARC
Hs_outer membrane	HK2
Hs_oxidoreductase	ALDH2
Hs_oxidoreductase	BCKDHB
Hs_oxidoreductase	CYBA
Hs_oxidoreductase	CYP1A1
Hs_oxidoreductase	CYP27B1
Hs_oxidoreductase	FASN
Hs_oxidoreductase	FDFT1
Hs_oxidoreductase	HPGD
Hs_oxidoreductase	LOXL1

Hs_oxidoreductase	NOS2A
Hs_oxidoreductase	PTGIS
Hs_oxidoreductase	SOD2
Hs_oxidoreductase, acting on CH-OH group of donors	FASN
Hs_oxidoreductase, acting on CH-OH group of donors	HPGD
Hs_oxidoreductase, acting on paired donors, with i	CYP27B1
Hs_oxidoreductase, acting on paired donors, with i	NOS2A
Hs_oxidoreductase, acting on superoxide radicals a	SOD2
Hs_oxidoreductase, acting on the aldehyde or oxo g	ALDH2
Hs_oxidoreductase, acting on the aldehyde or oxo g	BCKDHB
Hs_oxidoreductase, acting on the CH-NH2 group of d	LOXL1
Hs_oxidoreductase, acting on the CH-OH group of do	FASN
Hs_oxidoreductase, acting on the CH-OH group of do	HPGD
Hs_oxo-acid-lyase	CS
Hs_oxygen and reactive oxygen species metabolism	CYBA
Hs_oxygen and reactive oxygen species metabolism	SOD2
Hs_oxygen binding	CYP1A1
Hs_oxygen binding	CYP27B1
Hs_oxygen binding	PTGIS
Hs_passive proton transport, down the electrochemi	SLC15A2
Hs_passive proton transport, down the electrochemi	SLC5A5
Hs_passive proton transport, down the electrochemi	SLC8A3
Hs_pathogenesis	BACE
Hs_pathogenesis	IL9
Hs_pathogenesis	LRP1
Hs_pathogenesis	TSHR
Hs_pattern specification	EBAF
Hs_pepsin A	BACE
Hs_peptidase	PSMB4
Hs_peptidase	PSMB9
Hs_peptide binding	F2RL1
Hs_peptide binding	GALR2
Hs_peptide binding	NPR3
Hs_peptide binding	NTSR1
Hs_peptide hormone	CORT
Hs_peptide hormone	EPO
Hs_peptide hormone	INHBC
Hs_peptide hormone	INSL3
Hs_peptide receptor	F2RL1
Hs_peptide receptor	GALR2
Hs_peptide receptor	NPR3
Hs_peptide receptor	NTSR1
Hs_peptide receptor, G-protein coupled	F2RL1
Hs_peptide receptor, G-protein coupled	GALR2
Hs_peptide receptor, G-protein coupled	NPR3
Hs_peptide receptor, G-protein coupled	NTSR1
Hs_peptide transporter	SLC15A2
Hs_peptidoglycan metabolism	PGLYRP
Hs_peptidyl-dipeptidase A	ACE
Hs_peptidyl-prolyl cis-trans isomerase	PPIE
Hs_perception of biotic stimulus	MHC2TA
Hs_perception of light	CRYBB3
Hs_perception of light	CRYGD

Hs_perception of light	FOXC1
Hs_perception of light	GNAT1
Hs_perception of light	LUM
Hs_perception of light	MIP
Hs_perception of light	VMD2
Hs_perception of pest/pathogen/parasite	MHC2TA
Hs_perception of sound	CLDN14
Hs_peripheral nervous system development	ACCN1
Hs_permease	ATP4A
Hs_peroxisome	SCP2
Hs_phenol metabolism	COMT
Hs_phenylalanine metabolism	PTS
Hs_phosphatase	ALPI
Hs_phosphatase	DUSP2
Hs_phosphatase	PTPN1
Hs_phosphatase	PTPRK
Hs_phosphofructokinase	PFKP
Hs_phospholipid binding	ANXA8
Hs_phosphoric diester hydrolase	PDE1B
Hs_phosphoric monoester hydrolase	ALPI
Hs_phosphoric monoester hydrolase	DUSP2
Hs_phosphoric monoester hydrolase	PTPN1
Hs_phosphoric monoester hydrolase	PTPRK
Hs_phosphorylation	ALPI
Hs_phosphorylation	BMPR2
Hs_phosphorylation	BTK
Hs_phosphorylation	CDK5
Hs_phosphorylation	FGFR1
Hs_phosphorylation	MAK
Hs_phosphorylation	MAP2K2
Hs_phosphorylation	MAP2K3
Hs_phosphorylation	MAP2K7
Hs_phosphorylation	MAP3K8
Hs_phosphorylation	MAPK6
Hs_phosphorylation	NEK2
Hs_phosphorylation	PDGFRB
Hs_phosphorylation	ROS1
Hs_phosphorylation	RPS6KB2
Hs_phosphorylation	SNK
Hs_phosphorylation	STK23
Hs_phosphorylation	VRK1
Hs_phosphotransferase, alcohol group as acceptor	BMPR2
Hs_phosphotransferase, alcohol group as acceptor	BTK
Hs_phosphotransferase, alcohol group as acceptor	CCNT2
Hs_phosphotransferase, alcohol group as acceptor	CDK5
Hs_phosphotransferase, alcohol group as acceptor	DLG2
Hs_phosphotransferase, alcohol group as acceptor	FGFR1
Hs_phosphotransferase, alcohol group as acceptor	HK2
Hs_phosphotransferase, alcohol group as acceptor	MAK
Hs_phosphotransferase, alcohol group as acceptor	MAP2K2
Hs_phosphotransferase, alcohol group as acceptor	MAP2K3
Hs_phosphotransferase, alcohol group as acceptor	MAP2K7
Hs_phosphotransferase, alcohol group as acceptor	MAP3K8

Hs_phosphotransferase, alcohol group as acceptor	MAPK6
Hs_phosphotransferase, alcohol group as acceptor	NEK2
Hs_phosphotransferase, alcohol group as acceptor	PDGFRB
Hs_phosphotransferase, alcohol group as acceptor	PFKP
Hs_phosphotransferase, alcohol group as acceptor	ROS1
Hs_phosphotransferase, alcohol group as acceptor	RPS6KB2
Hs_phosphotransferase, alcohol group as acceptor	SNK
Hs_phosphotransferase, alcohol group as acceptor	STK23
Hs_phosphotransferase, alcohol group as acceptor	VRK1
Hs_phosphotransferase, nitrogenous group as accept	CKMT1
Hs_phosphotransferase, phosphate group as acceptor	DLG2
Hs_physiological processes	ACE
Hs_physiological processes	ANXA8
Hs_physiological processes	AQP7
Hs_physiological processes	BACE
Hs_physiological processes	CTRL
Hs_physiological processes	EPO
Hs_physiological processes	F2RL1
Hs_physiological processes	GALR2
Hs_physiological processes	GNAI2
Hs_physiological processes	IL9
Hs_physiological processes	INSL3
Hs_physiological processes	KCNJ12
Hs_physiological processes	LRP1
Hs_physiological processes	PLAT
Hs_physiological processes	SPARC
Hs_physiological processes	TSHR
Hs_physiological processes	VIPR1
Hs_pigment biosynthesis	COX10
Hs_pigment biosynthesis	OCA2
Hs_pigment metabolism	COX10
Hs_pigment metabolism	HMBS
Hs_pigment metabolism	OCA2
Hs_plasma glycoprotein	CSF1
Hs_plasma membrane	ACCN1
Hs_plasma membrane	ACE
Hs_plasma membrane	ADAM10
Hs_plasma membrane	ADAM2
Hs_plasma membrane	AP1M2
Hs_plasma membrane	AQP7
Hs_plasma membrane	ATP4A
Hs_plasma membrane	BACE
Hs_plasma membrane	BMPR2
Hs_plasma membrane	CD22
Hs_plasma membrane	CD69
Hs_plasma membrane	CHRNA6
Hs_plasma membrane	CLDN1
Hs_plasma membrane	CLDN14
Hs_plasma membrane	CLDN3
Hs_plasma membrane	CSF1
Hs_plasma membrane	DLG2
Hs_plasma membrane	DNM1
Hs_plasma membrane	EPOR

Hs_plasma membrane	F2RL1
Hs_plasma membrane	FGFR1
Hs_plasma membrane	FZD3
Hs_plasma membrane	FZD7
Hs_plasma membrane	GABRA3
Hs_plasma membrane	GABRQ
Hs_plasma membrane	GALR2
Hs_plasma membrane	GJA4
Hs_plasma membrane	GNAI2
Hs_plasma membrane	HTR6
Hs_plasma membrane	ITGA6
Hs_plasma membrane	ITGB5
Hs_plasma membrane	JAM2
Hs_plasma membrane	KCNJ12
Hs_plasma membrane	KCNJ5
Hs_plasma membrane	KCNK10
Hs_plasma membrane	KCNN4
Hs_plasma membrane	KCNQ5
Hs_plasma membrane	KPNB3
Hs_plasma membrane	LGALS3
Hs_plasma membrane	LRP1
Hs_plasma membrane	MIP
Hs_plasma membrane	MMP14
Hs_plasma membrane	MOG
Hs_plasma membrane	NTSR1
Hs_plasma membrane	PRSS21
Hs_plasma membrane	PTPRK
Hs_plasma membrane	ROS1
Hs_plasma membrane	SLC15A2
Hs_plasma membrane	SLC25A11
Hs_plasma membrane	SLC29A2
Hs_plasma membrane	SPN
Hs_plasma membrane	THY1
Hs_plasma membrane	TRHR
Hs_plasma membrane	TSHR
Hs_plasma membrane	VIPR1
Hs_plasma membrane cation-transporting ATPase	ATP4A
Hs_plastid	KPNB3
Hs_polyamine transporter	SLC7A11
Hs_porphyrin biosynthesis	COX10
Hs_porphyrin biosynthesis	HMBS
Hs_porphyrin metabolism	COX10
Hs_porphyrin metabolism	HMBS
Hs_positive regulation of cell proliferation	CSF1
Hs_positive regulation of cell proliferation	IL9
Hs_positive regulation of cell proliferation	LIF
Hs_positive regulation of cell proliferation	NAP1L1
Hs_positive regulation of cell proliferation	TSHR
Hs_positive regulation of cell proliferation	VIPR1
Hs_potassium channel	KCNAB1
Hs_potassium channel	KCNAB2
Hs_potassium channel	KCNJ12
Hs_potassium channel	KCNJ5

Hs_potassium channel	KCNK10
Hs_potassium channel	KCNN4
Hs_potassium channel	KCNQ5
Hs_potassium channel regulator	KCNAB1
Hs_potassium channel regulator	KCNAB2
Hs_potassium transport	ATP4A
Hs_potassium transport	KCNAB1
Hs_potassium transport	KCNAB2
Hs_potassium transport	KCNJ12
Hs_potassium transport	KCNJ5
Hs_potassium transport	KCNK10
Hs_potassium transport	KCNN4
Hs_potassium transport	KCNQ5
Hs_P-P-bond-hydrolysis-driven transporter	ABCA3
Hs_P-P-bond-hydrolysis-driven transporter	ATP4A
Hs_P-P-bond-hydrolysis-driven transporter	ATP5A1
Hs_P-P-bond-hydrolysis-driven transporter	ATP5G2
Hs_P-P-bond-hydrolysis-driven transporter	FXC1
Hs_P-P-bond-hydrolysis-driven transporter	OCA2
Hs_P-P-bond-hydrolysis-driven transporter	RAD50
Hs_pre-mRNA splicing factor	DDX8
Hs_pre-mRNA splicing factor	LSM2
Hs_prenylated protein tyrosine phosphatase	PTPN1
Hs_prenylated protein tyrosine phosphatase	PTPRK
Hs_prenyltransferase	COX10
Hs_prenyltransferase	FDFT1
Hs_prostaglandin biosynthesis	PTGIS
Hs_prostaglandin metabolism	HPGD
Hs_prostanoid biosynthesis	PTGIS
Hs_prostanoid metabolism	HPGD
Hs_prostanoid metabolism	PTGIS
Hs_protease inhibitor	BIRC6
Hs_protease inhibitor	C3
Hs_protease inhibitor	CSTB
Hs_protease inhibitor	SPINK1
Hs_protease inhibitor	TIMP2
Hs_protease inhibitor	TIMP4
Hs_proteasome endopeptidase	PSMB4
Hs_proteasome endopeptidase	PSMB9
Hs_protein amino acid dephosphorylation	DUSP2
Hs_protein amino acid dephosphorylation	PTPN1
Hs_protein amino acid dephosphorylation	PTPRK
Hs_protein amino acid glycosylation	LIPC
Hs_protein amino acid phosphorylation	BMPR2
Hs_protein amino acid phosphorylation	BTK
Hs_protein amino acid phosphorylation	CDK5
Hs_protein amino acid phosphorylation	FGFR1
Hs_protein amino acid phosphorylation	MAK
Hs_protein amino acid phosphorylation	MAP2K2
Hs_protein amino acid phosphorylation	MAP2K3
Hs_protein amino acid phosphorylation	MAP2K7
Hs_protein amino acid phosphorylation	MAP3K8
Hs_protein amino acid phosphorylation	MAPK6

Hs_protein amino acid phosphorylation	NEK2
Hs_protein amino acid phosphorylation	PDGFRB
Hs_protein amino acid phosphorylation	ROS1
Hs_protein amino acid phosphorylation	RPS6KB2
Hs_protein amino acid phosphorylation	SNK
Hs_protein amino acid phosphorylation	STK23
Hs_protein amino acid phosphorylation	VRK1
Hs_protein binding	BMPR2
Hs_protein binding	CALR
Hs_protein binding	CASP2
Hs_protein binding	CYR61
Hs_protein binding	ELK3
Hs_protein binding	FXYD5
Hs_protein binding	JUP
Hs_protein binding	KCNN4
Hs_protein binding	KIP2
Hs_protein binding	KLF1
Hs_protein binding	KPNB3
Hs_protein binding	LGALS3
Hs_protein binding	LRP1
Hs_protein binding	MHC2TA
Hs_protein binding	NFKBIA
Hs_protein binding	NOS2A
Hs_protein binding	NR1I2
Hs_protein binding	NR1I3
Hs_protein binding	PDE1B
Hs_protein binding	PGLYRP
Hs_protein binding	RBBP2
Hs_protein binding	SLC8A3
Hs_protein binding	SPARC
Hs_protein binding	SPTA1
Hs_protein binding	TCF8
Hs_protein binding	TFAP2A
Hs_protein binding	TMSB10
Hs_protein binding	TPM2
Hs_protein biosynthesis	EIF3S2
Hs_protein biosynthesis	GADD45G
Hs_protein biosynthesis	RPL18
Hs_protein biosynthesis	RPL31
Hs_protein biosynthesis	RPS10
Hs_protein biosynthesis	RPS3A
Hs_protein biosynthesis	RPS6KB2
Hs_protein catabolism	ACE
Hs_protein catabolism	ADAM10
Hs_protein catabolism	ADAM2
Hs_protein catabolism	ADAMTS8
Hs_protein catabolism	AGA
Hs_protein catabolism	BACE
Hs_protein catabolism	BIRC6
Hs_protein catabolism	CASP2
Hs_protein catabolism	CTRL
Hs_protein catabolism	GZMA
Hs_protein catabolism	GZMB

Hs_protein catabolism	MASP2
Hs_protein catabolism	MMP13
Hs_protein catabolism	MMP14
Hs_protein catabolism	PLAT
Hs_protein catabolism	PRSS21
Hs_protein catabolism	PSMB4
Hs_protein catabolism	PSMB9
Hs_protein catabolism	TRAF2
Hs_protein catabolism	TRAF4
Hs_protein complex assembly	CLDN14
Hs_protein complex assembly	KCNQ5
Hs_protein complex assembly	SLC7A11
Hs_protein complex assembly	TRAF2
Hs_protein folding	CCT4
Hs_protein folding	PPIE
Hs_protein kinase cascade	DUSP2
Hs_protein kinase cascade	FGFR1
Hs_protein kinase cascade	GADD45G
Hs_protein kinase cascade	NFKBIA
Hs_protein kinase CK2	BMPR2
Hs_protein kinase CK2	NEK2
Hs_protein kinase CK2	STK23
Hs_protein kinase regulator	CCNT2
Hs_protein modification	BIRC6
Hs_protein modification	BMPR2
Hs_protein modification	BTK
Hs_protein modification	CDK5
Hs_protein modification	DUSP2
Hs_protein modification	FGFR1
Hs_protein modification	LIPC
Hs_protein modification	LOXL1
Hs_protein modification	MAK
Hs_protein modification	MAP2K2
Hs_protein modification	MAP2K3
Hs_protein modification	MAP2K7
Hs_protein modification	MAP3K8
Hs_protein modification	MAPK6
Hs_protein modification	NEK2
Hs_protein modification	PDGFRB
Hs_protein modification	PLAT
Hs_protein modification	PTPN1
Hs_protein modification	PTPRK
Hs_protein modification	ROS1
Hs_protein modification	RPS6KB2
Hs_protein modification	SNK
Hs_protein modification	STK23
Hs_protein modification	VRK1
Hs_protein phosphatase	DUSP2
Hs_protein phosphatase	PTPN1
Hs_protein phosphatase	PTPRK
Hs_protein phosphatase type 2C	DUSP2
Hs_protein secretion	AP1M2
Hs_protein secretion	KIAA0905

Hs_protein serine/threonine kinase	BMPR2
Hs_protein serine/threonine kinase	CCNT2
Hs_protein serine/threonine kinase	CDK5
Hs_protein serine/threonine kinase	MAK
Hs_protein serine/threonine kinase	MAP2K2
Hs_protein serine/threonine kinase	MAP2K3
Hs_protein serine/threonine kinase	MAP2K7
Hs_protein serine/threonine kinase	MAP3K8
Hs_protein serine/threonine kinase	MAPK6
Hs_protein serine/threonine kinase	NEK2
Hs_protein serine/threonine kinase	RPS6KB2
Hs_protein serine/threonine kinase	SNK
Hs_protein serine/threonine kinase	STK23
Hs_protein serine/threonine kinase	VRK1
Hs_protein serine/threonine phosphatase	DUSP2
Hs_protein targeting	AP1M2
Hs_protein targeting	FXC1
Hs_protein targeting	KPNB3
Hs_protein targeting	NFKBIA
Hs_protein threonine/tyrosine kinase	MAP2K3
Hs_protein threonine/tyrosine kinase	MAP2K7
Hs_protein translocase	FXC1
Hs_protein transport	AP1M2
Hs_protein transport	DNM1
Hs_protein transport	FXC1
Hs_protein transport	KIAA0905
Hs_protein transport	KPNB3
Hs_protein transport	LRP1
Hs_protein transport	NFKBIA
Hs_protein transport	SLC15A2
Hs_protein transport	SNX12
Hs_protein transporter	AP1M2
Hs_protein transporter	FXC1
Hs_protein transporter	KPNB3
Hs_protein transporter	SLC15A2
Hs_protein transporter	SNX12
Hs_protein tyrosine kinase	BTK
Hs_protein tyrosine kinase	CDK5
Hs_protein tyrosine kinase	FGFR1
Hs_protein tyrosine kinase	MAP2K2
Hs_protein tyrosine kinase	MAP2K3
Hs_protein tyrosine kinase	MAP2K7
Hs_protein tyrosine phosphatase	DUSP2
Hs_protein tyrosine phosphatase	PTPN1
Hs_protein tyrosine phosphatase	PTPRK
Hs_protein tyrosine/serine/threonine phosphatase	DUSP2
Hs_protein-hormone receptor	TSHR
Hs_protein-ligand dependent protein catabolism	BIRC6
Hs_protein-ligand dependent protein catabolism	PSMB4
Hs_protein-ligand dependent protein catabolism	PSMB9
Hs_protein-lysine 6-oxidase	LOXL1
Hs_protein-mitochondrial targeting	FXC1
Hs_protein-nucleus import	KPNB3

Hs_protein-nucleus import	NFKBIA
Hs_protein-nucleus import, docking	KPNB3
Hs_proteoglycan	LUM
Hs_proteolysis and peptidolysis	ACE
Hs_proteolysis and peptidolysis	ADAM10
Hs_proteolysis and peptidolysis	ADAM2
Hs_proteolysis and peptidolysis	ADAMTS8
Hs_proteolysis and peptidolysis	BACE
Hs_proteolysis and peptidolysis	CASP2
Hs_proteolysis and peptidolysis	CTRL
Hs_proteolysis and peptidolysis	GZMA
Hs_proteolysis and peptidolysis	GZMB
Hs_proteolysis and peptidolysis	MASP2
Hs_proteolysis and peptidolysis	MMP13
Hs_proteolysis and peptidolysis	MMP14
Hs_proteolysis and peptidolysis	PLAT
Hs_proteolysis and peptidolysis	PRSS21
Hs_proteolysis and peptidolysis	PSMB9
Hs_proteolysis and peptidolysis	TRAF2
Hs_proteolysis and peptidolysis	TRAF4
Hs_proton transport	ATP4A
Hs_proton transport	ATP5A1
Hs_proton transport	ATP5G2
Hs_proton transport	SLC15A2
Hs_proton transport	SLC5A5
Hs_proton transport	SLC8A3
Hs_proton-transporting ATP synthase complex	ATP5A1
Hs_proton-transporting ATP synthase complex	ATP5G2
Hs_proton-transporting ATP synthase complex (sensu	ATP5A1
Hs_proton-transporting ATP synthase complex (sensu	ATP5G2
Hs_pteridine and derivative metabolism	PTS
Hs_P-type ATPase	ATP4A
Hs_purine nucleoside monophosphate metabolism	APRT
Hs_purine nucleoside triphosphate metabolism	ATP5A1
Hs_purine nucleotide biosynthesis	ADSL
Hs_purine nucleotide biosynthesis	APRT
Hs_purine nucleotide biosynthesis	ATIC
Hs_purine nucleotide biosynthesis	ATP5A1
Hs_purine nucleotide metabolism	ADSL
Hs_purine nucleotide metabolism	APRT
Hs_purine nucleotide metabolism	ATIC
Hs_purine nucleotide metabolism	ATP5A1
Hs_purine ribonucleoside monophosphate metabolism	APRT
Hs_purine ribonucleoside triphosphate metabolism	ATP5A1
Hs_purine ribonucleotide biosynthesis	ADSL
Hs_purine ribonucleotide biosynthesis	APRT
Hs_purine ribonucleotide biosynthesis	ATP5A1
Hs_purine ribonucleotide metabolism	ADSL
Hs_purine ribonucleotide metabolism	APRT
Hs_purine ribonucleotide metabolism	ATP5A1
Hs_RAN protein binding	KPNB3
Hs_receptor	BMPR2
Hs_receptor	CD69

Hs_receptor	CHRNA6
Hs_receptor	CLDN3
Hs_receptor	EPOR
Hs_receptor	EVI2A
Hs_receptor	F2RL1
Hs_receptor	FGFR1
Hs_receptor	FZD3
Hs_receptor	FZD7
Hs_receptor	GABRA3
Hs_receptor	GABRQ
Hs_receptor	GALR2
Hs_receptor	HTR6
Hs_receptor	ITGA6
Hs_receptor	ITGB5
Hs_receptor	LRP1
Hs_receptor	NPR3
Hs_receptor	NR1I2
Hs_receptor	NR1I3
Hs_receptor	NTSR1
Hs_receptor	PDGFRB
Hs_receptor	PGLYRP
Hs_receptor	PTPRK
Hs_receptor	ROS1
Hs_receptor	SPN
Hs_receptor	SRP9
Hs_receptor	TRAF4
Hs_receptor	TRHR
Hs_receptor	TSHR
Hs_receptor	UNC5C
Hs_receptor	VIPR1
Hs_receptor binding	ADAM2
Hs_receptor binding	ADAMTS8
Hs_receptor binding	C3
Hs_receptor binding	CORT
Hs_receptor binding	CSF1
Hs_receptor binding	EBAF
Hs_receptor binding	EPO
Hs_receptor binding	F2RL1
Hs_receptor binding	FGF10
Hs_receptor binding	FGF14
Hs_receptor binding	IFNG
Hs_receptor binding	IL16
Hs_receptor binding	IL9
Hs_receptor binding	INHBC
Hs_receptor binding	INSL3
Hs_receptor binding	LIF
Hs_receptor binding	PGLYRP
Hs_receptor binding	PSPN
Hs_receptor binding	TGFB3
Hs_receptor mediated endocytosis	DNM1
Hs_receptor signaling protein	GNAO1
Hs_receptor signaling protein	KPNB3
Hs_receptor signaling protein	TIAM2

Hs_regulation of adenylate cyclase activity	GNAI2
Hs_regulation of blood pressure	ACE
Hs_regulation of CDK activity	CCNT2
Hs_regulation of cell adhesion	FXYD5
Hs_regulation of cell adhesion	SPN
Hs_regulation of cell cycle	BCL2
Hs_regulation of cell cycle	CCT4
Hs_regulation of cell cycle	FGF10
Hs_regulation of cell cycle	HK2
Hs_regulation of cell cycle	MKI67
Hs_regulation of cell cycle	RPS6KB2
Hs_regulation of cell cycle	TGFB3
Hs_regulation of cell cycle	TSC1
Hs_regulation of cell growth	CYR61
Hs_regulation of cell growth	IFNG
Hs_regulation of cell proliferation	ADAMTS8
Hs_regulation of cell proliferation	BCL2
Hs_regulation of cell proliferation	CSF1
Hs_regulation of cell proliferation	EMP3
Hs_regulation of cell proliferation	IL9
Hs_regulation of cell proliferation	LIF
Hs_regulation of cell proliferation	NAP1L1
Hs_regulation of cell proliferation	TSHR
Hs_regulation of cell proliferation	VIPR1
Hs_regulation of DNA recombination	RAD50
Hs_regulation of heart	KCNJ12
Hs_regulation of mitosis	NEK2
Hs_regulation of neurotransmitter levels	COMT
Hs_regulation of protein-nucleus import	NFKBIA
Hs_regulation of transcription from Pol II promote	ELK3
Hs_regulation of transcription from Pol II promote	MYCN
Hs_regulation of transcription from Pol II promote	SREBF2
Hs_regulation of transcription from Pol II promote	SRF
Hs_regulation of transcription from Pol II promote	STAT6
Hs_regulation of transcription from Pol II promote	TCF8
Hs_regulation of transcription from Pol II promote	TFAP2A
Hs_regulation of transcription from Pol II promote	ZNF161
Hs_regulation of transcription from Pol III promot	RPC32
Hs_regulation of transcription, DNA-dependent	AIRE
Hs_regulation of transcription, DNA-dependent	CALR
Hs_regulation of transcription, DNA-dependent	CCNT2
Hs_regulation of transcription, DNA-dependent	FOXC1
Hs_regulation of transcription, DNA-dependent	FOXC2
Hs_regulation of transcription, DNA-dependent	HEYL
Hs_regulation of transcription, DNA-dependent	HOXA11
Hs_regulation of transcription, DNA-dependent	HOXC13
Hs_regulation of transcription, DNA-dependent	KLF1
Hs_regulation of transcription, DNA-dependent	MHC2TA
Hs_regulation of transcription, DNA-dependent	NFATC2
Hs_regulation of transcription, DNA-dependent	NR1I2
Hs_regulation of transcription, DNA-dependent	NR1I3
Hs_regulation of transcription, DNA-dependent	POU3F3
Hs_regulation of transcription, DNA-dependent	RBBP2

Hs_regulation of transcription, DNA-dependent	SIRT6
Hs_regulation of transcription, DNA-dependent	TBX5
Hs_regulation of transcription, DNA-dependent	TCF2
Hs_regulation of transcription, DNA-dependent	ZNFN1A1
Hs_regulation of translation	EIF3S2
Hs_regulation of translation	SRP9
Hs_regulation of translational initiation	EIF3S2
Hs_reproduction	ADAM2
Hs_reproduction	EBAF
Hs_reproduction	INSL3
Hs_reproduction	MAK
Hs_reproduction	TMSB10
Hs_reproduction	WNT1
Hs_response to bacteria	NFKBIA
Hs_response to biotic stimulus	AIRE
Hs_response to biotic stimulus	BCL2
Hs_response to biotic stimulus	BECN1
Hs_response to biotic stimulus	C3
Hs_response to biotic stimulus	CD22
Hs_response to biotic stimulus	CD69
Hs_response to biotic stimulus	CD74
Hs_response to biotic stimulus	CSF1
Hs_response to biotic stimulus	GZMA
Hs_response to biotic stimulus	IFNG
Hs_response to biotic stimulus	IL16
Hs_response to biotic stimulus	IL9
Hs_response to biotic stimulus	KCNN4
Hs_response to biotic stimulus	LIF
Hs_response to biotic stimulus	LTF
Hs_response to biotic stimulus	MASP2
Hs_response to biotic stimulus	MHC2TA
Hs_response to biotic stimulus	NFKBIA
Hs_response to biotic stimulus	PGLYRP
Hs_response to biotic stimulus	PRG2
Hs_response to biotic stimulus	PSMB9
Hs_response to biotic stimulus	PSME2
Hs_response to biotic stimulus	RAC1
Hs_response to biotic stimulus	RAD50
Hs_response to biotic stimulus	SOD2
Hs_response to biotic stimulus	SPN
Hs_response to biotic stimulus	TCF8
Hs_response to biotic stimulus	VIPR1
Hs_response to biotic stimulus	ZNF161
Hs_response to chemical substance	DPYS
Hs_response to chemical substance	MMP13
Hs_response to chemical substance	NR1I2
Hs_response to chemical substance	PRG2
Hs_response to DNA damage	RAD50
Hs_response to external stimulus	AIRE
Hs_response to external stimulus	BCL2
Hs_response to external stimulus	BECN1
Hs_response to external stimulus	C3
Hs_response to external stimulus	CD22

Hs_response to external stimulus	CD69
Hs_response to external stimulus	CD74
Hs_response to external stimulus	CLDN14
Hs_response to external stimulus	CRYBB3
Hs_response to external stimulus	CRYGD
Hs_response to external stimulus	CSF1
Hs_response to external stimulus	DPYS
Hs_response to external stimulus	FOXC1
Hs_response to external stimulus	GNAT1
Hs_response to external stimulus	GZMA
Hs_response to external stimulus	IFNG
Hs_response to external stimulus	IL16
Hs_response to external stimulus	IL9
Hs_response to external stimulus	KCNN4
Hs_response to external stimulus	LIF
Hs_response to external stimulus	LTF
Hs_response to external stimulus	LUM
Hs_response to external stimulus	MASP2
Hs_response to external stimulus	MHC2TA
Hs_response to external stimulus	MIP
Hs_response to external stimulus	MMP13
Hs_response to external stimulus	NFKBIA
Hs_response to external stimulus	NR1I2
Hs_response to external stimulus	PGLYRP
Hs_response to external stimulus	PRG2
Hs_response to external stimulus	PSMB9
Hs_response to external stimulus	PSME2
Hs_response to external stimulus	RAC1
Hs_response to external stimulus	RAD50
Hs_response to external stimulus	SOD2
Hs_response to external stimulus	SPN
Hs_response to external stimulus	TCF8
Hs_response to external stimulus	VIPR1
Hs_response to external stimulus	VMD2
Hs_response to external stimulus	ZNF161
Hs_response to light	CRYBB3
Hs_response to light	CRYGD
Hs_response to light	FOXC1
Hs_response to light	GNAT1
Hs_response to light	LUM
Hs_response to light	MIP
Hs_response to light	VMD2
Hs_response to oxidative stress	SOD2
Hs_response to pathogenic bacteria	NFKBIA
Hs_response to radiation	CRYBB3
Hs_response to radiation	CRYGD
Hs_response to radiation	FOXC1
Hs_response to radiation	GNAT1
Hs_response to radiation	LUM
Hs_response to radiation	MIP
Hs_response to radiation	VMD2
Hs_response to stress	BCL2
Hs_response to stress	BECN1

Hs_response to stress	C3
Hs_response to stress	CD22
Hs_response to stress	CSF1
Hs_response to stress	EPO
Hs_response to stress	GADD45G
Hs_response to stress	IL9
Hs_response to stress	LTF
Hs_response to stress	MAP2K7
Hs_response to stress	MASP2
Hs_response to stress	MHC2TA
Hs_response to stress	NFKBIA
Hs_response to stress	PRG2
Hs_response to stress	RAC1
Hs_response to stress	RAD50
Hs_response to stress	SOD2
Hs_response to stress	SPN
Hs_response to stress	ZNF161
Hs_response to toxin	DPYS
Hs_response to wounding	BECN1
Hs_response to wounding	C3
Hs_response to wounding	IL9
Hs_response to wounding	PRG2
Hs_response to wounding	RAC1
Hs_response to wounding	SPN
Hs_response to wounding	ZNF161
Hs_response to xenobiotic stimulus	MMP13
Hs_response to xenobiotic stimulus	NR1I2
Hs_response to xenobiotic stimulus	PRG2
Hs_retinoid binding	CRABP1
Hs_Rho protein signal transduction	TSC1
Hs_Rho small monomeric GTPase	RAC1
Hs_rhodopsin-like receptor	F2RL1
Hs_rhodopsin-like receptor	GALR2
Hs_rhodopsin-like receptor	HTR6
Hs_rhodopsin-like receptor	NTSR1
Hs_rhodopsin-like receptor	TRHR
Hs_ribonucleoprotein complex	DDX8
Hs_ribonucleoprotein complex	GADD45G
Hs_ribonucleoprotein complex	HNRPA1
Hs_ribonucleoprotein complex	HNRPC
Hs_ribonucleoprotein complex	LSM2
Hs_ribonucleoprotein complex	RPL18
Hs_ribonucleoprotein complex	RPL31
Hs_ribonucleoprotein complex	RPS10
Hs_ribonucleoprotein complex	RPS3A
Hs_ribonucleoside monophosphate metabolism	APRT
Hs_ribonucleotide biosynthesis	ADSL
Hs_ribonucleotide biosynthesis	APRT
Hs_ribonucleotide biosynthesis	ATP5A1
Hs_ribosome	GADD45G
Hs_ribosome	RPL18
Hs_ribosome	RPL31
Hs_ribosome	RPS10

Hs_ribosome	RPS3A
Hs_RNA binding	DDX8
Hs_RNA binding	ENTPD2
Hs_RNA binding	HNRPA1
Hs_RNA binding	HNRPC
Hs_RNA binding	LSM2
Hs_RNA binding	PPIE
Hs_RNA binding	RPL18
Hs_RNA binding	RPL31
Hs_RNA binding	RPS10
Hs_RNA binding	RPS3A
Hs_RNA binding	SRP9
Hs_RNA dependent adenosinetriphosphatase	DDX8
Hs_RNA dependent adenosinetriphosphatase	ENTPD2
Hs_RNA helicase	DDX8
Hs_RNA helicase	ENTPD2
Hs_RNA localization	HNRPA1
Hs_RNA polymerase II transcription factor	ELK3
Hs_RNA polymerase II transcription factor	HOXC13
Hs_RNA polymerase II transcription factor	MHC2TA
Hs_RNA polymerase II transcription factor	NR1I2
Hs_RNA polymerase II transcription factor	NR1I3
Hs_RNA polymerase II transcription factor	SREBF2
Hs_RNA polymerase II transcription factor	SRF
Hs_RNA polymerase II transcription factor	TBX5
Hs_RNA polymerase II transcription factor	TFAP2A
Hs_RNA polymerase II transcription factor	ZNF161
Hs_RNA polymerase II transcription factor, enhance	TFAP2A
Hs_RNA splicing	DDX8
Hs_RNA splicing	HNRPC
Hs_RNA splicing	LSM2
Hs_RNA-nucleus export	HNRPA1
Hs_S phase of mitotic cell cycle	LIG1
Hs_S phase of mitotic cell cycle	MLH1
Hs_S phase of mitotic cell cycle	NAP1L1
Hs_S-acyltransferase	FASN
Hs_S-adenosylmethionine-dependent methyltransferas	COMT
Hs_S-adenosylmethionine-dependent methyltransferas	FASN
Hs_S-adenosylmethionine-dependent methyltransferas	TPMT
Hs_sarcomere	TPM2
Hs_second-messenger-mediated signaling	CORT
Hs_second-messenger-mediated signaling	GALR2
Hs_second-messenger-mediated signaling	GNAI2
Hs_second-messenger-mediated signaling	HTR6
Hs_second-messenger-mediated signaling	TSHR
Hs_second-messenger-mediated signaling	VIPR1
Hs_secretin-like receptor	VIPR1
Hs_secretory pathway	AP1M2
Hs_secretory pathway	KIAA0905
Hs_serine protease inhibitor	SPINK1
Hs_serine-type endopeptidase	CTRL
Hs_serine-type endopeptidase	GZMA
Hs_serine-type endopeptidase	GZMB

Hs_serine-type endopeptidase	LTF
Hs_serine-type endopeptidase	MASP2
Hs_serine-type endopeptidase	PLAT
Hs_serine-type endopeptidase	PRSS21
Hs_sexual reproduction	ADAM2
Hs_sexual reproduction	EBAF
Hs_sexual reproduction	INSL3
Hs_sexual reproduction	MAK
Hs_sexual reproduction	TMSB10
Hs_sexual reproduction	WNT1
Hs_signal recognition particle	SRP9
Hs_signal transducer	ADAM2
Hs_signal transducer	ADAMTS8
Hs_signal transducer	BMPR2
Hs_signal transducer	C3
Hs_signal transducer	CBL
Hs_signal transducer	CD69
Hs_signal transducer	CHRNA6
Hs_signal transducer	CLDN3
Hs_signal transducer	CORT
Hs_signal transducer	CSF1
Hs_signal transducer	EBAF
Hs_signal transducer	EPO
Hs_signal transducer	EPOR
Hs_signal transducer	EVI2A
Hs_signal transducer	F2RL1
Hs_signal transducer	FGF10
Hs_signal transducer	FGF14
Hs_signal transducer	FGFR1
Hs_signal transducer	FZD3
Hs_signal transducer	FZD7
Hs_signal transducer	GABRA3
Hs_signal transducer	GABRQ
Hs_signal transducer	GALR2
Hs_signal transducer	GNAI2
Hs_signal transducer	GNAO1
Hs_signal transducer	GNAT1
Hs_signal transducer	HTR6
Hs_signal transducer	IFNG
Hs_signal transducer	IL16
Hs_signal transducer	IL9
Hs_signal transducer	INHBC
Hs_signal transducer	INSL3
Hs_signal transducer	ITGA6
Hs_signal transducer	ITGB5
Hs_signal transducer	KPNB3
Hs_signal transducer	LIF
Hs_signal transducer	LRP1
Hs_signal transducer	NPR3
Hs_signal transducer	NR1I2
Hs_signal transducer	NR1I3
Hs_signal transducer	NTSR1
Hs_signal transducer	PDGFRB

Hs_signal transducer	PGLYRP
Hs_signal transducer	PSPN
Hs_signal transducer	PTPRK
Hs_signal transducer	ROS1
Hs_signal transducer	SPN
Hs_signal transducer	SRP9
Hs_signal transducer	STAT6
Hs_signal transducer	TGFB3
Hs_signal transducer	TIAM2
Hs_signal transducer	TRAF2
Hs_signal transducer	TRAF4
Hs_signal transducer	TRHR
Hs_signal transducer	TSHR
Hs_signal transducer	UNC5C
Hs_signal transducer	VIPR1
Hs_signal transducer	WNT1
Hs_signal transducer	WNT3A
Hs_signal transducer	WNT4
Hs_signal transduction	C3
Hs_signal transduction	CABP2
Hs_signal transduction	CHRNA6
Hs_signal transduction	CRABP1
Hs_signal transduction	ELK3
Hs_signal transduction	EPO
Hs_signal transduction	EPOR
Hs_signal transduction	FGF10
Hs_signal transduction	FGF14
Hs_signal transduction	FZD3
Hs_signal transduction	GABRQ
Hs_signal transduction	GNAI2
Hs_signal transduction	GNAO1
Hs_signal transduction	GNAT1
Hs_signal transduction	KCNK10
Hs_signal transduction	MAP2K3
Hs_signal transduction	MAP2K7
Hs_signal transduction	MAPK6
Hs_signal transduction	NR1I2
Hs_signal transduction	NR1I3
Hs_signal transduction	PDE1B
Hs_signal transduction	PDGFRB
Hs_signal transduction	PTPN1
Hs_signal transduction	ROS1
Hs_signal transduction	RPS6KB2
Hs_signal transduction	SPN
Hs_signal transduction	SRF
Hs_signal transduction	TFAP2A
Hs_signal transduction	TGFB3
Hs_signal transduction	TRAF2
Hs_signal transduction	UNC5C
Hs_signaling (initiator) caspase	CASP2
Hs_skeletal development	BGLAP
Hs_skeletal development	BMPR2
Hs_skeletal development	FGFR1

Hs_skeletal development	LUM
Hs_skeletal development	NPR3
Hs_skeletal development	SPARC
Hs_small GTPase mediated signal transduction	RAC1
Hs_small GTPase mediated signal transduction	TSC1
Hs_small GTPase regulatory/interacting protein	KPNB3
Hs_small GTPase regulatory/interacting protein	TIAM2
Hs_small molecule transport	ABCA3
Hs_small molecule transport	ATP4A
Hs_small molecule transport	CHRNA6
Hs_small molecule transport	GABRA3
Hs_small molecule transport	GJA4
Hs_small molecule transport	KCNK10
Hs_small molecule transport	RHCG
Hs_small molecule transport	SLC15A2
Hs_small molecule transport	SLC25A11
Hs_small molecule transport	SLC5A5
Hs_small monomeric GTPase	RAC1
Hs_small nucleolar ribonucleoprotein complex	LSM2
Hs_small protein conjugating enzyme	BIRC6
Hs_small protein conjugating enzyme	UBL1
Hs_small ribosomal subunit	RPS10
Hs_small ribosomal subunit	RPS3A
Hs_S-methyltransferase	TPMT
Hs_sodium channel	ACCN1
Hs_sodium transport	ACCN1
Hs_sodium transport	SLC5A5
Hs_sodium transport	SLC8A3
Hs_sodium/potassium-exchanging ATPase complex	ROS1
Hs_soluble fraction	ACE
Hs_soluble fraction	COMT
Hs_soluble fraction	CORT
Hs_soluble fraction	INSL3
Hs_soluble fraction	JUP
Hs_soluble fraction	PTPN1
Hs_soluble fraction	WNT1
Hs_solute:cation antiporter	SLC8A3
Hs_solute:cation symporter	SLC15A2
Hs_solute:solute antiporter	SLC8A3
Hs_specific RNA polymerase II transcription factor	NR1I2
Hs_specific RNA polymerase II transcription factor	NR1I3
Hs_specific RNA polymerase II transcription factor	ZNF161
Hs_spectrin	SPTA1
Hs_spermatid development	TMSB10
Hs_spermatogenesis	INSL3
Hs_spermatogenesis	MAK
Hs_spermatogenesis	TMSB10
Hs_spermatogenesis	WNT1
Hs_spliceosome complex	DDX8
Hs_steroid biosynthesis	FDFT1
Hs_steroid biosynthesis	SCP2
Hs_steroid hormone receptor	NR1I2
Hs_steroid hormone receptor	NR1I3

Hs_steroid metabolism	FDFT1
Hs_steroid metabolism	NR1I2
Hs_steroid metabolism	SCP2
Hs_steroid metabolism	SREBF2
Hs_sterol biosynthesis	FDFT1
Hs_sterol carrier	SCP2
Hs_sterol metabolism	FDFT1
Hs_sterol metabolism	SREBF2
Hs_striated muscle thin filament	TPM2
Hs_structural constituent of cytoskeleton	SPTA1
Hs_structural constituent of eye lens	CRYBB3
Hs_structural constituent of eye lens	CRYGD
Hs_structural constituent of eye lens	MIP
Hs_structural constituent of muscle	TPM2
Hs_structural constituent of ribosome	GADD45G
Hs_structural constituent of ribosome	RPL18
Hs_structural constituent of ribosome	RPL31
Hs_structural constituent of ribosome	RPS10
Hs_structural constituent of ribosome	RPS3A
Hs_structural molecule	BGLAP
Hs_structural molecule	CLDN1
Hs_structural molecule	CLDN14
Hs_structural molecule	CLDN3
Hs_structural molecule	JUP
Hs_structural molecule	PRPH
Hs_structural molecule	TUBA8
Hs_sugar binding	CD22
Hs_sugar binding	CD69
Hs_sugar binding	LGALS3
Hs_sugar binding	PRG2
Hs_sugar binding	TEM1
Hs_superoxide metabolism	CYBA
Hs_superoxide metabolism	SOD2
Hs_symport	SLC15A2
Hs_symport	SLC5A5
Hs_symporter	SLC15A2
Hs_symporter	SLC5A5
Hs_synaptic transmission	ACCN1
Hs_synaptic transmission	CHRNA6
Hs_synaptic transmission	COMT
Hs_synaptic transmission	CORT
Hs_synaptic transmission	DNM1
Hs_synaptic transmission	GABRA3
Hs_synaptic transmission	GABRQ
Hs_synaptic transmission	GALR2
Hs_synaptic transmission	HTR6
Hs_synaptic transmission	KCNQ5
Hs_synaptic transmission	MOG
Hs_synaptic transmission	NTSR1
Hs_synaptic transmission	VIPR1
Hs_synaptonemal complex	STAG3
Hs_taxis	IL16
Hs_taxis	SPN

Hs_TCA cycle enzyme complex (sensu Eukarya)	BCKDHB
Hs_telomere maintenance	RAD50
Hs_tetrahydrobiopterin metabolism	PTS
Hs_TGFbeta receptor signaling pathway	EBAF
Hs_thiolester hydrolase	FASN
Hs_threonine endopeptidase	PSMB4
Hs_threonine endopeptidase	PSMB9
Hs_thrombin receptor	F2RL1
Hs_thylakoid	KPNB3
Hs_tight junction	CLDN1
Hs_tight junction	CLDN14
Hs_tight junction	CLDN3
Hs_toxin	PRG2
Hs_transcription co-activator	KLF1
Hs_transcription co-activator	MHC2TA
Hs_transcription co-activator	NR1I2
Hs_transcription co-activator	NR1I3
Hs_transcription co-activator	TCF8
Hs_transcription co-activator	TFAP2A
Hs_transcription cofactor	CALR
Hs_transcription cofactor	ELK3
Hs_transcription cofactor	KLF1
Hs_transcription cofactor	MHC2TA
Hs_transcription cofactor	NR1I2
Hs_transcription cofactor	NR1I3
Hs_transcription cofactor	TCF8
Hs_transcription cofactor	TFAP2A
Hs_transcription co-repressor	CALR
Hs_transcription co-repressor	TCF8
Hs_transcription factor	AIRE
Hs_transcription factor	CBL
Hs_transcription factor	ELK3
Hs_transcription factor	FOXC1
Hs_transcription factor	FOXC2
Hs_transcription factor	HEYL
Hs_transcription factor	HOXA11
Hs_transcription factor	HOXC13
Hs_transcription factor	KLF1
Hs_transcription factor	MBD2
Hs_transcription factor	MHC2TA
Hs_transcription factor	MYCN
Hs_transcription factor	NFATC2
Hs_transcription factor	NR1I2
Hs_transcription factor	NR1I3
Hs_transcription factor	POU3F3
Hs_transcription factor	RBBP2
Hs_transcription factor	SREBF2
Hs_transcription factor	SRF
Hs_transcription factor	STAT6
Hs_transcription factor	TBX5
Hs_transcription factor	TCF2
Hs_transcription factor	TCF8
Hs_transcription factor	TFAP2A

Hs_transcription factor	ZNF161
Hs_transcription factor binding	CALR
Hs_transcription factor binding	ELK3
Hs_transcription factor binding	KLF1
Hs_transcription factor binding	MHC2TA
Hs_transcription factor binding	NFKBIA
Hs_transcription factor binding	NR1I2
Hs_transcription factor binding	NR1I3
Hs_transcription factor binding	TCF8
Hs_transcription factor binding	TFAP2A
Hs_transcription factor complex	RPC32
Hs_transcription factor, cytoplasmic sequestering	NFKBIA
Hs_transcription from Pol II promoter	CCNT2
Hs_transcription from Pol II promoter	ELK3
Hs_transcription from Pol II promoter	FOXC2
Hs_transcription from Pol II promoter	KLF1
Hs_transcription from Pol II promoter	MYCN
Hs_transcription from Pol II promoter	RBBP2
Hs_transcription from Pol II promoter	SREBF2
Hs_transcription from Pol II promoter	SRF
Hs_transcription from Pol II promoter	STAT6
Hs_transcription from Pol II promoter	TCF8
Hs_transcription from Pol II promoter	TFAP2A
Hs_transcription from Pol II promoter	ZNF161
Hs_transcription from Pol III promoter	RPC32
Hs_transcription, DNA-dependent	AIRE
Hs_transcription, DNA-dependent	CALR
Hs_transcription, DNA-dependent	CCNT2
Hs_transcription, DNA-dependent	ELK3
Hs_transcription, DNA-dependent	FOXC1
Hs_transcription, DNA-dependent	FOXC2
Hs_transcription, DNA-dependent	HEYL
Hs_transcription, DNA-dependent	HOXA11
Hs_transcription, DNA-dependent	HOXC13
Hs_transcription, DNA-dependent	KLF1
Hs_transcription, DNA-dependent	MHC2TA
Hs_transcription, DNA-dependent	MYCN
Hs_transcription, DNA-dependent	NFATC2
Hs_transcription, DNA-dependent	NR1I2
Hs_transcription, DNA-dependent	NR1I3
Hs_transcription, DNA-dependent	POU3F3
Hs_transcription, DNA-dependent	RBBP2
Hs_transcription, DNA-dependent	RPC32
Hs_transcription, DNA-dependent	SIRT6
Hs_transcription, DNA-dependent	SREBF2
Hs_transcription, DNA-dependent	SRF
Hs_transcription, DNA-dependent	STAT6
Hs_transcription, DNA-dependent	TBX5
Hs_transcription, DNA-dependent	TCF2
Hs_transcription, DNA-dependent	TCF8
Hs_transcription, DNA-dependent	TFAP2A
Hs_transcription, DNA-dependent	ZNF161
Hs_transcription, DNA-dependent	ZNFN1A1

Hs_transcriptional activator	FOXC1
Hs_transcriptional gene silencing	SIRT6
Hs_transcriptional repressor	MBD2
Hs_transferase	APRT
Hs_transferase	ATIC
Hs_transferase	AUP1
Hs_transferase	B4GALT2
Hs_transferase	BMPR2
Hs_transferase	BTK
Hs_transferase	CCNT2
Hs_transferase	CDK5
Hs_transferase	CKMT1
Hs_transferase	COMT
Hs_transferase	COX10
Hs_transferase	DLG2
Hs_transferase	FASN
Hs_transferase	FDFT1
Hs_transferase	FGFR1
Hs_transferase	HK2
Hs_transferase	MAK
Hs_transferase	MAP2K2
Hs_transferase	MAP2K3
Hs_transferase	MAP2K7
Hs_transferase	MAP3K8
Hs_transferase	MAPK6
Hs_transferase	NEK2
Hs_transferase	PDGFRB
Hs_transferase	PFKP
Hs_transferase	ROS1
Hs_transferase	RPC32
Hs_transferase	RPS6KB2
Hs_transferase	SIRT6
Hs_transferase	SNK
Hs_transferase	STK23
Hs_transferase	TKT
Hs_transferase	TPMT
Hs_transferase	VRK1
Hs_transferase, transferring acyl groups	AUP1
Hs_transferase, transferring acyl groups	FASN
Hs_transferase, transferring alkyl or aryl (other	COX10
Hs_transferase, transferring alkyl or aryl (other	FDFT1
Hs_transferase, transferring alkyl or aryl groups,	COX10
Hs_transferase, transferring alkyl or aryl groups,	FDFT1
Hs_transferase, transferring glycosyl groups	APRT
Hs_transferase, transferring glycosyl groups	B4GALT2
Hs_transferase, transferring groups other than ami	AUP1
Hs_transferase, transferring groups other than ami	FASN
Hs_transferase, transferring hexosyl groups	B4GALT2
Hs_transferase, transferring one-carbon groups	ATIC
Hs_transferase, transferring one-carbon groups	COMT
Hs_transferase, transferring one-carbon groups	FASN
Hs_transferase, transferring one-carbon groups	TPMT
Hs_transferase, transferring pentosyl groups	APRT

Hs_transferase, transferring phosphorus-containing	BMPR2
Hs_transferase, transferring phosphorus-containing	BTK
Hs_transferase, transferring phosphorus-containing	CCNT2
Hs_transferase, transferring phosphorus-containing	CDK5
Hs_transferase, transferring phosphorus-containing	CKMT1
Hs_transferase, transferring phosphorus-containing	DLG2
Hs_transferase, transferring phosphorus-containing	FGFR1
Hs_transferase, transferring phosphorus-containing	HK2
Hs_transferase, transferring phosphorus-containing	MAK
Hs_transferase, transferring phosphorus-containing	MAP2K2
Hs_transferase, transferring phosphorus-containing	MAP2K3
Hs_transferase, transferring phosphorus-containing	MAP2K7
Hs_transferase, transferring phosphorus-containing	MAP3K8
Hs_transferase, transferring phosphorus-containing	MAPK6
Hs_transferase, transferring phosphorus-containing	NEK2
Hs_transferase, transferring phosphorus-containing	PDGFRB
Hs_transferase, transferring phosphorus-containing	PFKP
Hs_transferase, transferring phosphorus-containing	ROS1
Hs_transferase, transferring phosphorus-containing	RPC32
Hs_transferase, transferring phosphorus-containing	RPS6KB2
Hs_transferase, transferring phosphorus-containing	SNK
Hs_transferase, transferring phosphorus-containing	STK23
Hs_transferase, transferring phosphorus-containing	VRK1
Hs_transforming growth factor-beta receptor	BMPR2
Hs_transforming growth factor-beta receptor ligand	EBAF
Hs_transforming growth factor-beta receptor ligand	INHBC
Hs_transforming growth factor-beta receptor ligand	TGFB3
Hs_transition metal transport	LTF
Hs_translation initiation factor	EIF3S2
Hs_translation regulator	EIF3S2
Hs_translation regulator	ELK3
Hs_translation regulator	KLF1
Hs_translation regulator	MHC2TA
Hs_translation regulator	NFATC2
Hs_translation regulator	NR1I3
Hs_translation regulator	SREBF2
Hs_translation regulator	SRF
Hs_translation regulator	TCF2
Hs_translation regulator	TCF8
Hs_translation regulator	ZNF161
Hs_translation regulator	ZNFN1A1
Hs_translational elongation	SRP9
Hs_translational initiation	EIF3S2
Hs_transmembrane receptor	CD69
Hs_transmembrane receptor	CLDN3
Hs_transmembrane receptor	EVI2A
Hs_transmembrane receptor	GABRQ
Hs_transmembrane receptor	SPN
Hs_transmembrane receptor protein kinase	BMPR2
Hs_transmembrane receptor protein kinase	FGFR1
Hs_transmembrane receptor protein kinase	PDGFRB
Hs_transmembrane receptor protein kinase	ROS1
Hs_transmembrane receptor protein phosphatase	PTPRK

Hs_transmembrane receptor protein serine/threonine	BMPR2
Hs_transmembrane receptor protein serine/threonine	EBAF
Hs_transmembrane receptor protein tyrosine kinase	FGFR1
Hs_transmembrane receptor protein tyrosine kinase	PDGFRB
Hs_transmembrane receptor protein tyrosine kinase	ROS1
Hs_transmembrane receptor protein tyrosine kinase	FGFR1
Hs_transmembrane receptor protein tyrosine kinase	PDGFRB
Hs_transmembrane receptor protein tyrosine kinase	ROS1
Hs_transmembrane receptor protein tyrosine phospha	PTPRK
Hs_transmission of nerve impulse	ACCN1
Hs_transmission of nerve impulse	CHRNA6
Hs_transmission of nerve impulse	COMT
Hs_transmission of nerve impulse	CORT
Hs_transmission of nerve impulse	DNM1
Hs_transmission of nerve impulse	GABRA3
Hs_transmission of nerve impulse	GABRQ
Hs_transmission of nerve impulse	GALR2
Hs_transmission of nerve impulse	HTR6
Hs_transmission of nerve impulse	KCNQ5
Hs_transmission of nerve impulse	MOG
Hs_transmission of nerve impulse	NTSR1
Hs_transmission of nerve impulse	VIPR1
Hs_transport	ABCA3
Hs_transport	ACCN1
Hs_transport	AP1M2
Hs_transport	AQP7
Hs_transport	ATP4A
Hs_transport	ATP5A1
Hs_transport	ATP5G2
Hs_transport	CHRNA6
Hs_transport	CRABP1
Hs_transport	DNM1
Hs_transport	FXC1
Hs_transport	FXYD5
Hs_transport	GABRA3
Hs_transport	GABRQ
Hs_transport	HNRPA1
Hs_transport	KCNAB1
Hs_transport	KCNAB2
Hs_transport	KCNJ12
Hs_transport	KCNJ5
Hs_transport	KCNK10
Hs_transport	KCNN4
Hs_transport	KCNQ5
Hs_transport	KIAA0905
Hs_transport	KPNB3
Hs_transport	LRP1
Hs_transport	LTF
Hs_transport	MIP
Hs_transport	NFKBIA
Hs_transport	NUP62
Hs_transport	OCA2
Hs_transport	RAD50

Hs_transport	RHCG
Hs_transport	SCP2
Hs_transport	SLC15A2
Hs_transport	SLC25A11
Hs_transport	SLC29A2
Hs_transport	SLC5A5
Hs_transport	SLC7A11
Hs_transport	SLC8A3
Hs_transport	SNX12
Hs_transporter	ABCA3
Hs_transporter	AQP7
Hs_transporter	ATP5A1
Hs_transporter	ATP5G2
Hs_transporter	CRABP1
Hs_transporter	MIP
Hs_transporter	OCA2
Hs_transporter	RHCG
Hs_transporter	SLC15A2
Hs_transporter	SLC25A11
Hs_transporter	SLC5A5
Hs_triacylglycerol lipase	LIPC
Hs_tricarboxylic acid cycle	CS
Hs_trypsin	CTRL
Hs_trypsin	GZMA
Hs_trypsin	GZMB
Hs_trypsin	MASP2
Hs_trypsin	PLAT
Hs_trypsin	PRSS21
Hs_tumor suppressor	MRVI1
Hs_tumor suppressor	TSC1
Hs_ubiquitin conjugating enzyme	UBL1
Hs_ubiquitin cycle	BIRC6
Hs_ubiquitin-dependent protein catabolism	BIRC6
Hs_ubiquitin-dependent protein catabolism	PSMB4
Hs_ubiquitin-dependent protein catabolism	PSMB9
Hs_uptake permease	ATP4A
Hs_urea cycle intermediate metabolism	ARG2
Hs_vacuole	AGA
Hs_vesicle coat	AP1M2
Hs_vesicle coat	KIAA0905
Hs_vesicle-mediated transport	AP1M2
Hs_vesicle-mediated transport	DNM1
Hs_vesicle-mediated transport	KIAA0905
Hs_vesicle-mediated transport	LRP1
Hs_vision	CRYBB3
Hs_vision	CRYGD
Hs_vision	FOXC1
Hs_vision	GNAT1
Hs_vision	LUM
Hs_vision	MIP
Hs_vision	VMD2
Hs_vitamin metabolism	CYP27B1
Hs_voltage-gated ion channel	KCNAB1

Hs_voltage-gated ion channel	KCNAB2
Hs_voltage-gated ion channel	KCNJ12
Hs_voltage-gated ion channel	KCNJ5
Hs_voltage-gated ion channel	KCNK10
Hs_voltage-gated ion channel	KCNQ5
Hs_voltage-gated potassium channel	KCNAB1
Hs_voltage-gated potassium channel	KCNAB2
Hs_voltage-gated potassium channel	KCNJ12
Hs_voltage-gated potassium channel	KCNJ5
Hs_voltage-gated potassium channel	KCNQ5
Hs_voltage-gated potassium channel complex	KCNJ5
Hs_voltage-gated potassium channel complex	KCNN4
Hs_voltage-gated potassium channel complex	KCNQ5
Hs_water transport	AQP7
Hs_Wnt receptor signaling pathway	FZD3
Hs_Wnt receptor signaling pathway	FZD7
Hs_Wnt receptor signaling pathway	WNT1
Hs_Wnt receptor signaling pathway	WNT3A
Hs_Wnt receptor signaling pathway	WNT4
Hs_xenobiotic metabolism	MMP13
Hs_xenobiotic metabolism	NR1I2
Hs_xenobiotic metabolism	PRG2
Hs_zinc binding	ACE
Hs_zinc binding	ADAM10
Hs_zinc binding	ADAMTS8
Hs_zinc binding	CA14
Hs_zinc binding	CA7
Hs_zinc binding	FASN
Hs_zinc binding	GDA
Hs_zinc binding	MMP13
Hs_zinc binding	MMP14
Hs_zinc binding	TCF8
Hs_zinc binding	TRAF2
Hs_zinc binding	TRAF4
Hs_zinc binding	ZNF179

GENERAL DISCUSSION

Examination of structural changes in bone physiology, relating to osteoporosis and aging, illustrates a reciprocal relationship between bone volume and bone marrow adiposity, which is believed to be an active process(256). The reason for the increase in bone marrow adiposity associated with bone loss is currently unknown, however, one theory holds that bone marrow adipocytes may be a consequence of the inappropriate differentiation of pluripotent mesenchymal cells, diverted from other lineages such as osteoblasts or hematopoietic support cells(175, 204). This balance between bone volume and bone marrow adiposity, has led to speculation whether limiting the number of adipocytes within the bone marrow, will result in an increase in osteoblast numbers and enhanced bone formation(279). This hypothesis remains unclear, however, certain agents used for the treatment of osteoporosis are also able to inhibit adipocyte differentiation. Both high density lipoprotein (HDL), and Genistein have been shown to affect osteoblast biology(71, 136, 163). *In vitro*, HDL has been shown to stimulate proliferation of osteoblast-like ROS cells, and *in vivo*, Genistein has been shown to inhibit bone resorption by upregulating osteoprotegrin expression(372). Both agents are also able to downregulate PPARγ by MAPK signaling, thereby inhibiting the terminal differentiation of adipocytic cells(71, 136, 163). Therefore, agents that may enhance bone formation, may also limit bone marrow adiposity by limiting the adipogenic effect of PPARγ.

Previously, PTHrP was demonstrated to be anabolic with respect to bone formation when administered intermittently. This may be attributed to enhanced proliferation and differentiation of osteoprogenitor cells, or decreased apoptosis of

differentiated osteoblasts and osteocytes(185, 303). PTHrP heterozygous null mice express lower levels of PTHrP and develop a premature form of osteoporosis, that is characterized by a deficiency in bone formation and increased fat within the bone marrow(9). This would suggest that PTHrP affects the differentiation process of mesenchymal cells. Our initial series of studies focused on whether PTHrP could influence adipogenesis and osteogenesis(125, 279).

Our studies demonstrate that when 3T3-L1 pre-adipocytic cells overexpress PTHrP, they proliferate at an accelerated rate and resist acquiring a mature adipocyte phenotype. The mechanism by which PTHrP is able to affect 3T3-L1 pre-adipocytes is believed to occur through the PTH1-R, which activates MAPK, which then phosphorylates and downregulates the adipocyte determining factor PPARγ. PTHrP stimulation of MAPK may occur through activation of the cAMP-PKA and the DAG-PKC signaling pathways, as both pathways are linked to the PTH-1R(3, 39). In osteoblast-like cells and rat duodenal cells (enterocytes), PTH and PTHrP stimulation of both pathways appears to contribute to the activation of MAPK(119). Other reports have suggested that activation of MAPK by cAMP-PKA signaling is subject to cross talk with DAG-PKC signaling, as has been demonstrated for pituitary adenylate cyclase activating peptide (PACAP) in neuronal PC12 cells(35). However, in 3T3-L1 pre-adipocytes, MAPK activation by PTHrP appears to be dependent on PKA activation alone, as specific inhibitors for PKC signaling do not appear to affect MAPK activity.

cAMP-PKA mediated activation of MAPK requires Rap1, a member of the Ras-like family of small GTPases that is activated within the cAMP→PKA→Rap1→B-Raf→MEK→MAPK signaling cascade(374, 375). PKA signaling appears to be a pre-

requisite for MAPK activation by Rap1 in PC12 neuronal cells and may also apply to 3T3-L1 cells. To date, no studies have been performed on Rap1 expression in adipocytic cells in spite of the dual requirements of cAMP and MAPK activity in the early differentiation process of committed pre-adipocytes. As PKA mediated activation of MAPK requires Rap1, confirmation of the expression of this protein would give further credence to the described mechanism.

PTHrPs ability to stimulate MAPK may have other consequences, asides from its ability to inhibit the differentiation of pre-adipocytes. MAPK has also been implicated in the differentiation and commitment of pluripotent mesenchymal cells. The commitment and differentiation of primary bone marrow stromal cells towards the osteoblastic lineage was shown to require MAPK signaling, whereas suppression of MAPK leads to general adipogenesis(232). If PTHrP is able to stimulate MAPK in primary bone marrow stromal cells, this may favor osteogenesis and inhibit adipogenesis of these cells.

Therefore, PTHrPs ability to promote bone formation may occur by stimulation of MAPK, which then directs earlier progenitor cells to undergo osteogenesis. PTHrP may also inhibit the terminal differentiation of adipocytic cells within the bone marrow which is a physiological change associated with bone loss.

Based on the inhibitory effect of PTHrP on adipogenesis of 3T3-L1 cells and the reciprocal relationship between marrow adiposity and bone volume found in heterozygous null PTHrP mice(9), we investigated whether PTHrP might affect adipogenesis and osteogenesis of an earlier pluripotent mesenchymal cell. We designed an *in vitro* model based on the clonal C3H 10T½ cell line, which has the ability to differentiate into adipocytes and osteoblasts, following treatment with low doses and high

doses of BMP2 respectively. The use of BMP2 has the added advantage of inducing adipogenesis of C3H 10T½ cells without limiting their osteogenic potential(376), thereby allowing us to assess the role of PTHrP in both processes simultaneously. Our findings suggest that PTHrP is able to inhibit BMP2 induced adipogenesis and concurrently enhance osteogenesis of C3H 10T½ by a PKC dependent mechanism. These findings would differ from earlier studies demonstrating that PTH and PTHrP enhance osteoblast differentiation of committed osteoprogenitors through a cAMP-PKA pathway, whereas DAG-PKC signaling enhances proliferation of these cells. This difference may be due to the relatively undefined nature of C3H 10T½ mesenchymal cells as compared to that of a committed osteoprogenitor cells. In the current scenario, PKC may be required at an earlier stage of differentiation to enhance osteoblast differentiation, as compared to the effect of PKA on the differentiation of committed osteoprogenitors.

Osteoporosis is a pathological consequence of the aging process in bone that is associated with hormone deprivation. However, the process of bone loss begins prior to any changes in systemic hormone levels and may be the result of the aged cells capacity to respond to hormonal stimuli. This has been shown to occur with respect to vitamin D, growth hormone, PDGF and IGF(73, 92, 310).

As the commitment process of pluripotent mesenchymal cells or osteoblast differentiation is dependent on exposure to varying concentrations of BMP2, we hypothesized that PTHrP might be amplifying BMP2 signaling. BMP2 signaling initially requires the formation of a ligand-type II receptor complex which can then effectively present the ligand to the type I receptor which is responsible for transducing the intracellular signal. We found that addition of PTHrP and TPA enhanced BMP-IA

receptor expression and increased cell sensitivity to extracellular levels of BMP2. Overexpression of BMP-IA alone, was sufficient to enhance BMP2 induced osteogenesis. This would suggest that the availability of BMP-IA may serve as a means of regulating BMP2 signaling.

In aging bone, it is possible that with time, pluripotent mesenchymal cells within the bone marrow may be less responsive to BMP2 signaling, due to either reduced BMP-IA receptor expression or BMP2 signaling effectors. Reduced BMP2 signaling in these cells would lead to increased adipogenesis and reduced osteogenesis within the bone marrow, as observed in osteoporosis. Introduction of PTHrP may offset the reduction in responsiveness of these cells to BMP2, by upregulation of BMP-IA receptor expression, thereby facilitating osteoblast commitment and inhibiting adipocyte commitment of marrow stromal cells. This mechanism may contribute to the increases in bone formation, observed with intermittent doses of PTHrP.

Although there is substantial evidence to suggest that PTHrP affects osteoblast and adipocyte differentiation by acting on the PTH1-R, an intracrine function for PTHrP may also be relevant. Screening of a yeast 2-hybrid osteosarcoma library with PTHrP as bait identified the repressor Rev ErbA as a potential nuclear partner for PTHrP. The nuclear orphan receptor Rev ErbA has been postulated to serve a function in adipocyte biology, such that expression of Rev ErbA mRNA increases during the progression of adipocyte differentiation(52). Furthermore, Rev ErbA has been shown to recognize similar DNA sequences as monomeric PPARγ ((A/T)A(A/T)NT(A/G)GGTCA), and homodimers of Rev ErbA are able to inhibit the transactivation elicited by PPARα on the DR-2 motif with a 5'-extended binding site(162). In spite of these findings, no

antagonism by Rev ErbA has been observed of PPARγ function, from naturally occurring PPARγ responsive DR-1 motifs. Whether Rev ErbA contributes to the inhibitory effect of nuclear PTHrP on adipogenesis remains unclear. It will therefore be of interest to determine whether overexpression of PTHrP with Rev ErbA can affect the transcriptional activity of PPARγ from DR-1 elements. This might provide further evidence for the interaction of PTHrP with Rev ErbA *in vitro*.

Although Rev ErbA does not contribute to the inhibitory effects of PTHrP on adipogenesis, we determined that overexpression of Rev ErbA enhances osteoblast differentiation of C3H 10T½ cells when induced with adipogenic doses of BMP2 and this ability is further enhanced with overexpression of nuclear PTHrP. Two possible mechanisms could be attributed to the function of nuclear PTHrP in Rev ErbA function. The first is that Rev ErbA acts as a suppressor of the osteoblast phenotype by inhibiting expression of genes required for osteoblast differentiation and that PTHrP inhibits this function. The second involves PTHrP enhancing the suppression of non-osteoblastic lineage repressive genes by Rev ErbA. Further studies will be required to examine these possibilities.

To date, Rev ErbA expression has not been delineated in osteoblasts or pre-osteoblasts. Examination of the Rev ErbA deficient mice, demonstrated no obvious defects in skeletal muscle or adipose tissue, where its expression is found to be highest. No examinations were performed on the bones of these animals. Accordingly, it will be important to examine these animals for skeletal defects, to ensure that the interaction of Rev ErbA with PTHrP to enhance osteogenesis also occurs *in vivo*.

If PTHrP expression should decrease over time, this may be relevant to age related osteoporosis. Since both the secreted form and nuclear form of PTHrP may affect adipocyte and osteoblast development, it is conceivable that lower levels of PTHrP over time, would favor adipocyte differentiation over osteoblast differentiation in the bone marrow. Therefore, decreases in PTHrP expression in aged cells might contribute to the development of the osteopenic state.

Genomic scanning by itself is a powerful tool that enables researchers to identify potential genes that are regulated "directly" (1^{st} order) by specific transcription factors. Our scanning methodology was refined to include a filter for identifying genes with similar response elements in complementary positions in the mouse and human genome and genes with similar conditions were scored as positives. In our studies, genomic scanning was effective in identifying 379 genes that harbor consensus CBFA1 responsive elements in the mouse and human genome at similar distances.

We also used this data to validate findings from previous publications. In certain cases, reports of CBFA1 responsive genes such as MMP13 and BGLAP (i.e. Osteocalcin) demonstrate a conserved regulatory element amongst species however the reported distance separating the corresponding responsive elements differs from our findings. In examining the reported distance by Jimenez et al., they identified corresponding elements in the mouse and human genome for MMP13 and BGLAP which differed by 3 nucleotides and 19 nucleotides respectively, relative to the mRNA transcription initiation site(186). We identified their reported response elements in our scan; however, we found the distance to be 18 nucleotides and 64 nucleotides for MMP13 and BGLAP respectively. This difference was attributed to their choice of mRNA initiation site, which

differed from the start sites reported by NCBI. In the case of the CBFA1 responsive element in the mouse BGLAP promoter, we found their reported distance to be identical to that of NCBI. However, their reported distance of -149 differs from NCBIs value of -194 for the CBFA1 response element in the human proximal promoter of BGLAP. In the case of MMP 13, their reference point for the CBFA1 responsive element in the human MMP13 proximal promoter is identical to that of the NCBI reported value. However, their mouse reference point for the mRNA initiation site places the responsive element at position -125 which differs from the NCBI reported value of -150. Therefore, the differences noted can be attributed solely to their choice of mRNA initiation site.

We also employed the scanning methodology to verify reports of CBFA1 responsive genes based on studies involving the proximal promoter of only one species. Osteoprotegrin had been previously reported to be a transcriptional target for CBFA1 based on studies of the human version of its proximal promoter region. Maximal transcriptional activity is found to be encoded in the first 1.5 kb of the human promoter sequence which harbors two CBFA1 responsive elements(345). However, using the genomic scanning method, complementary sequences cannot be identified within this region of the mouse proximal promoter. We were however, able to identify a complementary CBFA1 sequence at position -1604 in the mouse promoter which is equivalent to a promoter element at -1614 in the human promoter. Nevertheless, this element did not appear to contribute to CBFA1 transcriptional activation of the human osteoprotegrin promoter in β-galactosidase reporter assays(345). These differences may reflect regulatory differences amongst species for osteoprotegrin, however, the lack of

corresponding responsive elements in one genome raises questions as to the importance of the human regulatory elements as conservation argues favorably for functionality.

With genomic scanning, target genes can be identified for any transcription factor and should be useful in examining other regulatory genes associated with mesenchymal cell differentiation, such as Sox9(34), PPARγ(353) and MyoD(42). Furthermore, it can be applied to searches of combinations of transcription factors and co-factors, thus raising the elucidation of specific signaling networks. In the future, the scanning methodology may be enhanced by scanning intronic sequences and by searching for enhancer elements to identify less obvious targets of transcription factors. We also hope to combine the scanning methodology with micro-array analysis to validate potential transcriptional target genes.

In summary, we examined the effects of PTHrP on adipocyte differentiation and identified an autocrine/paracrine and also an intracrine mechanism by which PTHrP is able to inhibit adipogenesis. The autocrine/paracrine mechanism involves activation of PKA which subsequently leads to activation of MAPK which then phosphorylates and downregulates the adipocyte determining factor PPARγ. The nuclear mechanism may involve the nuclear orphan receptor Rev ErbA. We also demonstrated that PTHrP is able to enhance osteoblast commitment and differentiation of C3H 10T½ cells by upregulation of the BMP IA receptor which sensitizes a cell to BMP signaling and by a nuclear mechanism involving Rev ErbA. Finally, we have presented the framework for a novel methodology that involves genomic scanning for transcription responsive elements which should be highly useful for the identification of target genes of specific transcription factors.

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Original Contributions to Science

1. The identification of an autocrine/paracrine role for PTHrP in inhibiting adipogenesis by downregulating PPARγ activity.
2. The description of functional PTH/PTHrP receptors in pre-adipocytic cells.
3. Characterization of an autocrine/paracrine role for PTHrP in enhancing osteogenesis by a PKC dependent mechanism.
4. Establish that BMP2 receptor mediated signaling can be regulated by expression levels of BMP-IA.
5. Demonstrate an intracrine action of PTHrP that involves inhibition of adipocyte differentiation.
6. Describe a function for the nuclear receptor Rev ErbA in promoting osteoblast differentiation.
7. Provide evidence for an intracrine role for PTHrP in enhancing osteoblast differentiation in conjunction with Rev ErbA.
8. Identification of 379 genes with consensus CBFA1 response elements in their proximal promoters that are conserved amongst the human and mouse genome.