Overexpression and characterization of hPHEX in the mouse

Dissertation der Fakultät für Biologie
der Ludwig-Maximilian-Universität München

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Martinsried 2005

Eingereicht am: 09. Mai 2005
bei der Fakultät für Biologie der Ludwig-Maximilian-Universität München

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2. Prüfer: Dr. A. Böttger
der mündlichen Prüfung

Tag der mündlichen Prüfung: 30. Mai 2006

Acknowledgements

The work presented in this dissertation took place under the supervision of Dr. rer. nat. Beate Lanske, Max-Planck-Institute for Biochemistry, from June 1999 to April of 2001 and from May 2001 to December 2002 in the laboratory of Privat dozent Dr. med., Dr. vet.med. Reinhold Erben, Ludwig-Maximilians University, Institute of Animal Physiology. The work was supported in part by a grant from Boehringer Mannheim GmbH (Roche).

This work is dedicated to my entire family. I want to thank my loving and supportive wife Tracy and my children Victoria, Taegan, and Katy just for being there, always ready with hugs and kisses. Mom and Dad thanks for helping me talk things through and supporting my ambitions. Thanks go to the all of my family and friends for without all of your support and patience this would not have been possible.

Abbreviations

APS	Ammonium Persulfate
BES	N,N-bis[2-Hydroxyethyl]-2-aminoethanesulfonic acid
Bis-Tris	Bis[2-Hydroxyethyl]iminotris[hydroxymethyl]methane
BSA	Bovine serum albumin
cDNA	complementary deoxyribonucleic acid
dCTP	2‘-Deoxy-cytidine-5‘-triphosphate
DEAE	Diethylaminoethyl
DMEM	Dulbecco’s Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	2‘-Deoxy-nucleoside-5‘- triphosphate
EBNA	Epstein-Barr virus nuclear antigen
EDTA	Ethylenediaminetetraacetic acid
FBS	fetal bovine serum
FGF	fibroblast growth factor
G418	geneticin
HEPES	N-[2-Hydroxyethyl]piperazine-N‘-[2-ethanesulfonicacid]
LB media	Luria-Bertani medium
kB	kilobase
MES	2-[N-Morpholino]ethanesulfonic acid
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
NaAc	sodium acetate
NH_4Ac	ammonium acetate
NP-40	IGEPAL CA-630
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PIPES	Piperazine-N,N‘-bis[2-ethanesulfonic acid]
RNA	ribonucleic acid
SDS	sodium deodecyl sulfate
ssDNA	salmon sperm deoxynucleic acid
TCA	Trichloroacetic acid
TEMED	N,N,N‘,N‘-Tetramethylethylenediamine
Tris	Tris[hydroxymethyl]aminomethane
Triton X-100	t-Octylphenoxypolyethoxyethanol
Tween 20	Polyoxyethylenesorbitan monolaurate
UV	ultraviolet

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1 Summary

Phosphate regulating **H**ormone with homologies to **E**ndopeptidases on the **X**-chromosome (PHEX, formerly identified as PEX) is the gene responsible for the hereditary disease X-linked hypophosphatemic rickets (XLH) and affects one in twenty thousand people, making it the most common form of rickets. A homologous disease has also been identified in *Mus musculus* and given the label Hyp for Hypophosphatemia. The cause of both diseases is an inactivation of the carboxy terminal end of the gene through mutation or deletion. It has been demonstrated that PHEX affects the pathway or regulatory elements for the expression of the renal sodium dependant phosphate transporter, NPT2a, and therefore phosphate resorption in the kidney. In a separate regulatory pathway PHEX affects the mineralization of osteoid, the scaffolding of hard bone.

In this thesis, I have created and analyzed transgenic mouse strains overexpressing hPHEX. The transgenic animals were classified by PCR and PHEX was pinpointed by *in situ* hybridization to be expressed in trabecular and cortical bone as expected. Phenotypical analysis of transgenic animals demonstrated that biochemical measurements were not affected by the presence of the transgene under the control of a ubiquitous promoter. The transgenic hPHEX animals were crossed with Hyp mice to establish whether a rescue or partial rescue of the mutant phenotype was possible. Phenotypical analysis of the rescue mice indicated an improvement in body weight and bone morphology, including mineralization, over the mutant hyp mice, while most biochemical parameters remained unchanged.

2 Introduction

Skeletogenesis is one of the most profound events in embryonic development, though it is one of the last to occur. Its continuing evolvement throughout the life of an organism makes bone, its precursors and associated factors a complex field of research. The skeleton has three primary functions to fulfill. First it is a mechanical device to give support and in connection with muscles facilitates voluntary movement. Secondly, it provides a storage compartment for ions, namely calcium and phosphate. Finally, it protects vital organs from external trauma.

It is the ability of bone to store large amounts of phosphate that underlies the goal of this thesis. Phosphate is a major constituent of bone mineral, which is laid down in the form of hydroxyapatite ($3Ca_3(PO_4)_2 \cdot Ca(OH)_2$) (Landis et al., 1995). With numerous pathways leading to the regulation of phosphate in the body, it is a rare situation that a physiological change in phosphate can be attributed to a single gene product. First identified in 1995 by the Hyp Consortium, a **P**hosphate regulating gene with homologies to **E**ndopeptidases on the **X** – chromosome (PEX) (Hyp consortium, 1995) (now identified as PHEX) is one such gene. A loss of function mutation in the carboxy-terminal region of this gene leads to X-linked hypophosphatemic rickets (XLH). A mouse model of this disease is found in the Hyp mouse, which carries a spontaneous deleterious mutation in the 3' region of the mouse PHEX (mPHEX) gene (Eicher, 1976). These homologous diseases are mainly characterized by hypophosphatemia, but also include increased alkaline phosphatase (ALP), bone deformity, normal serum calcium, normal PTH and inappropriately normal levels of active vitamin D_3.

The major site of expression of PHEX in human and mouse is in mature osteoblasts of calvaria and long bones, odontoblasts, with minor expression in lung, muscle, ovary and human parathyroid gland but specifically not in kidney (Beck et al., 1997; Du et al., 1996; Blydt-Hansen, 1999).

The aim of my doctoral work was to create and characterize two transgenic mouse lines. The first transgenic lineage was to overexpress the human PHEX (hPHEX) gene under the control of an osteocalcin gene promoter, in order to achieve osteoblast specific expression. The second line would overexpress hPHEX under control of a β-actin promoter for ubiquitous expression. These two lines were then analyzed for phenotypical changes. Transgenic animals expressing hPHEX were further crossed with the Hyp mice in order to rescue the phosphate wasting and osteoblast mineralization defect that characterize these mutant animals.

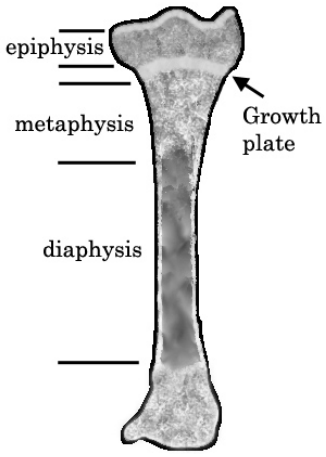
2.1 Osteogenesis

2.1.0 Physiology of bone

Bone is comprised of varied cell types, minerals and extracellular matrix. The cells of bone include chondrocytes, osteoblasts (OB), osteocytes and osteoclasts (OC). Osteoblasts and chondrocytes both differentiate from the mesenchymal cell lineage. The osteoblast is similar to the fibroblast. In fact, OBs express all gene markers of the fibroblast and only two known specific markers (Ducy et al., 2000). Osteocytes are mature OBs that have become entrapped within the matrix which they created. Osteoclasts are of hematopoietic origin and differentiate from the mononuclear/phagocytic lineage (Hattersley et al., 1991).

There are two physiologically different types of bone (flat and long) derived from intramembranous or endochondral ossification, respectively. The parts of the long bones are classified as either compact bone (cortical) or spongy bone (trabecular). Bone is defined by the amount of matrix, created by OBs, that has been mineralized. Cortical bone is generally 80-90% mineralized, whereas trabecular bone remains mostly unmineralized, with only 15-25% mineralization. Trabecular bone houses the hematopoietic bone marrow as well as blood vessels and connective tissue (Baron et al., 1999). (fig 1).

Figure 1
Long bone architecture



During embryonic development chondrocytes lay down the anlagen or templates for intermembranous bones that will later be calcified. Within the growth plate of long bones, between the epiphysis and metaphysis, it is the chondrocytes that allow for endochondral growth. Within the growth plate, chondrocytes are organized into columns of cells that differentiate through highly regulated temporal signaling. From furthest to closest to the ossification center of the bone is the reserve or resting zone, proliferating chondrocytes, transition zone, upper hypertrophic chondrocytes, lower hypertrophic chondrocytes and osteoblasts. Proliferation and differentiation of the chondrocytes is driven by indian hedgehog (Ihh) and parathyroid hormone-related peptide (PTHrP), which are expressed in the hypertrophic chondrocytes and proliferating chondrocytes, respectively (St-Jacques et al., 1999). It is between the lower hypertrophic chondrocytes and the metaphysis that initial calcification of the cartilage occurs (Wallis, 1996). After formation of the cartilage, capillaries invade allowing for migration of osteoblasts and subsequent bone formation, followed later by bone resorption activity of osteoclasts.

2.1.1 Osteoblastogenesis

The cellular maturation and differentiation from stem cell to osteoblast (OB) and osteocyte is not well understood at this time. Several specific markers as well as stimulators and inhibitors of differentiation for each of the various stages, leading to OBs have been identified. Alkaline phosphatase (ALP) and osteocalcin are generally

accepted OB markers. The transcription factor Cbfa1 induces differentiation toward OB while Msx-2 has been shown to inhibit the progression (Takeda, et al., 2001). Some of the other factors involved in OB differentiation include transforming growth factor β (TGF-β), Parathyroid hormone (PTH), Parathyroid hormone related protein (PTHrP), 1,25 dihydroxyvitamin D_3 ($1,25(OH)_2$ D_3) and bone morphogenetic proteins (BMPs) (Ducy et al., 2000).

Once the pre-osteoblast is signaled to stop proliferating and to differentiate, it changes shape from a spindle like cell to a large cuboidal OB. Mature OBs have large nuclei, an enlarged golgi complex and extensive ER. Osteoblasts form in clusters of cells (~100 to 400 cells per cluster) at each bone forming site. They are able to secrete osteoid (unmineralized extracellular matrix) in a specific direction where mineralization is desired. Within the osteoid, calcium and phosphate ions accumulate and once the osteoid components have matured, hydroxyapatite crystals bind to the type I collagen fibers through glycoproteins. While the osteoid is mineralized and bone apposition occurs, some osteoblasts become trapped within the new growth and mature into osteocytes. Osteocytes express different cell markers than the osteoblast and lining cells, for example, SB3 and CD44 (Aubin et al., 1995; Hughes et al., 1994).

2.1.2 Osteoclastogenesis

As a living organ, bone is constantly being built and resorbed at varying rates throughout the life of an organism. This process, called remodeling, is unique to bone. Bone is the

only organ whose constitutive cells work to destroy the organ itself. Compared to OBs, the function of osteoclasts (OCs) is much better understood.

Osteoclasts are the last of the skeletal cells to appear during embryogenesis. Their differentiation is under the control of numerous transcription factors and varied secreted molecules. The earliest known regulator of OC differentiation is the transcription factor Pu.1, which controls both macrophage and OC lineage differentiation. Other transcription factors involved include NF-κB, c-fos and mi. Secreted factors responsible for function and activity as well as differentiation include macrophage colony stimulating factor (M-CSF), osteoprotegrin (OPG), OPG ligand (OPGL) (Kong et al, 1999), 1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$) , RANK ligand, TNF receptor-associated factors (TRAFs) and C-SRC (Teitelbaum, 2000). OPG inhibits osteoclast differentiation and overexpression leads to an increase in bone mineral density (Simonet et al., 1997). All of the different factors which have regulatory influence are further involved in positive and negative feedback loops, as shown by the regulation of NF-κB by TRAFs, the regulation of M-CSF receptors by Pu.1 (Tondravi et al, 1997) or the stimulation of OPG secretion from osteoblastic lineages by ($1,25(OH)_2D_3$) (Hofbauer, 1998).

Osteoclasts are giant multinucleated cells that are found in contact with calcified bone surfaces. The side of the OC facing the bone surface forms a pocket enclosed by a sealing zone. The membrane of the OC within this compartment is highly folded making a ruffled border area ideal for maximizing transport capabilities. Enzymes and metalloproteinases are secreted into this pocket via vesicle transport. The pH is lowered

within the compartment through the use of a proton pump and the lysozymal enzyme cathepsin K (Baron, 1989; Li, 1999). The low pH within the compartment dissolves hydroxyapatite crystals allowing the degradation of the remaining collagen fibers. Phosphate and calcium released via this process is either released during breaks in the sealing zone, or by transport across the cell (Baron et al., 1989).

The process of remodeling trabecular bone takes 3 to 4 months to complete. The process starts with the activation of the remodeling activity and continues through the phases of resorption, reversal phase, formation and finally resting. Activation can be in response to growth, repair or humoral factors.

2.2 Phosphate regulation

Phosphate is an ion that is important for many cellular functions and involved in structural stability of lipid membranes. It is important for there to be a sufficient source of inorganic phosphate available from internal sources. The average adult human contains 600g of phosphorous with concentrations of 1.8 – 2.2 mg/dL in serum. The concentrations are not as tightly regulated as other ions such as calcium (Broadus, 1999). The concentration varies throughout the day and can vary due to age, sex, diet and hormone concentrations. Fifteen percent of inorganic phosphate is protein bound while the rest circulates as complexed monohydrogen or dihydrogen phosphate. Phosphate excretion and resorption in a normal adult human is regulated so there is no net loss or gain of phosphate. Approximately eighty-five percent of inorganic phosphorous is

reabsorbed in the renal proximal tubules. In various metabolic diseases the renal phosphate reabsorption is compromised and dietary intake can not make up for the loss.

2.2.1 1,25 dihydroxyvitamin D_3 (calcitriol)

Biologically active 1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$) and parathyroid hormone (PTH) are the two most influential hormones in ion homeostasis in bone. Inert vitamin D is processed in the kidney and liver into its active form. Upregulation of $1,25(OH)_2D_3$ increases the rate of intestinal absorption of phosphate and calcium ions. Increased concentrations of $1,25(OH)_2D_3$ are also associated with upregulation of OPG (inhibition of osteoclast formation) and down regulation of osteoblastic PHEX expression *in vitro* (Hofbauer et al., 1998; Ecarot and Desbarats, 1999). Long term hypophosphatemia is one cause of upregulation of $1,25(OH)_2D_3$. In the hypophosphatemic vitamin D resistant rickets mouse model (Hyp) it was shown that $1,25(OH)_2D_3$ action on osteoblasts was dependent on extracellular Pi concentrations, as measured by alkaline phosphatase (ALP) activity *in vitro* (Yamamoto et al., 1992). One of the phenotypical abnormalities of the Hyp mouse is the abnormally normal levels of $1,25(OH)_2D_3$ in the presences of hypophosphatemia.

2.2.2 Parathyroid Hormone / Parathyroid Hormone related Protein

PTH is a highly conserved protein consisting of 84 amino acids in all known mammals and is exocytosed by the parathyroid gland. PTH is able to stimulate the release of both calcium and phosphate ions from bone. In kidney, PTH has opposite actions on the two ions. Renal phosphate reabsorption is inhibited and calcium renal reabsorption is

stimulated. PTH interacts with calcium, phosphate and $1,25(OH)_2D_3$ as part of a complex regulatory mechanism to control serum calcium and phosphate ion levels. Serum calcium levels have the most profound affect on PTH production, where hypercalcemia inhibits and hypocalcemia stimulates PTH production. $1,25(OH)_2D_3$ actions are similar to those of calcium, while phosphate stimulates both secretion of PTH and parathyroid gland cell proliferation (Slatopolsky et al., 1996).

Parathyroid hormone related protein (PTHrP) was the second member of the PTH family described as a tumor product and identified as the causative factor in humoral hypercalcemia in malignancy (HHM) (Wysolmerski, 1994). PTHrP is a polyhormone with multiple splice variants, creating three isoforms of 139, 141, and 173 amino acids. PTH and the amino terminal PTHrP share a common receptor, the PTH/PTHrP receptor. The PTH/PTHrP receptor is found in varied tissues, but most abundantly in kidney and bone (Lee et al., 96). PTH and PTHrP also have other separate and unique receptors, though some of their biological functions have yet to be elucidated. PTHrP is expressed in multiple tissues, with varying actions. Expression found in bone inhibits bone resorption and in chondrocytes it is demonstrated to promote proliferation while inhibiting terminal differentiation and apoptosis (Lee et al., 96). PTHrP expressed in chondrocytes is controlled by the developmental patterning gene Indian Hedgehog, which is expressed by prehypertrophic chondrocytes (Vortkamp et al., 1996). Increased levels of PTH (associated with hyperparathyroidism), through indirectly increased osteoclast activity, lead to the release of calcium and phosphate ions from bones. In distal proximal tubules of the kidney phosphate is primarily reabsorbed through a sodium dependant cotransporter (Npt2). Increased levels of PTH act to degrade Npt2, therefore leading to

fewer membrane bound sodium dependant phosphate transporters and renal phosphate wasting (Pfister et al., 1998).

2.3 Phosphate regulating hormone with homologies to Endopeptidases on the X – chromosome (PHEX)

2.3.1 X-Linked Hypophosphatemic Rickets

X-linked hypophosphatemic rickets (XLH) is an X-linked dominant disorder that has little or no gene dosage effects and affects close to 1:20000 people (Whyte et al., 1996). The XLH disorder is characterized by hypophosphatemia, a short stature (including lower extremity deformation), tooth abscesses, enthesopathy, increased alkaline phosphatase (ALP), normal serum calcium, normal PTH and inappropriately normal levels of $1,25(OH)_2D_3$. Hypophosphatemia, which is the only consistent characteristic of this disorder, is caused by renal phosphate wasting in the proximal tubule. This wasting has been attributed to the reduction in both RNA and protein levels of the high affinity, low-capacity Na^+ dependent phosphate transporter type II (NPT2) by 50% (Tenenhouse et al., 1994). Fluorescent in situ hybridization studies of NPT2 located its chromosomal position at 5q35, excluding it as a causative gene for XLH.

2.3.2 Hyp, mouse model for XLH

In 1966, six mice were observed to have a shortened trunk and hind limbs. These mice were bred further and eventually backcrossed into the C57BL6/6J background. These mice, identified by the symbol Hyp, were described in 1976 as being the mouse homologue to XLH (Eicher et al., 1976. Hyp mice present the same phenotypical evidence as XLH, most importantly: hypophosphatemia, shortened hind limbs and inappropriately normal levels of $1,25(OH)_2D_3$. To examine osteoblast function, normal periosteum and osteoblasts from Hyp and normal mice were transplanted into the gluteal muscle of normal and Hyp mice. As expected, the normal matrix implanted into Hyp mice did not mineralize normally. The Hyp cells transplanted into normal mice did not show a complete mineralization as the normal cells transplanted into normal mice did. This inability to achieve normal mineralization indicates that Hyp mice also have a primary osteoblast defect (Ecarot-Charrier et al., 1988).

In order to see if the causative factor was circulating or not, Hyp mice were used in a parabiosis experiment. Hyp mice were joined to normal littermates allowing sharing of circulating factors. In both short and long term analysis of phosphate levels the normal littermate develops hypophosphatemia (Meyer et al., 1989). Transplantation studies of the Hyp kidneys into normal mice and of normal kidneys into Hyp mice identified no primary renal defect, but rather an intrinsic defect of the environment within the Hyp mouse (Nesbitt et al., 1992).

2.3.3 Identification and characterization of PHEX

The phosphate regulating gene with endopeptidase activity on the X chromosome (PHEX) was identified in 1995 through positional cloning by the efforts of the Hyp Consortium, in an effort to find the gene responsible for XLH. The gene is comprised of 22 small exons spread over 220Kb of genomic DNA. It encodes a 749 amino acid protein which is a type II integral membrane glycoprotein of the family of neutral endopeptidases. Other members of this family include neprilysin (NEP), endothelin converting enzyme 1 and 2 (ECE-1 and ECE-2) and the KELL antigen (KELL). PHEX has a short N-terminal region, a single transmembrane domain and a long carboxy terminal region containing the catalytic site (Hyp Consortium, 1995). Conserved features include ten cysteine residues, a pentapeptide zinc binding motif (HEXXH), a second zinc binding motif (ENXADXGG), and N-glycosylation sites (Oefner, 2000). It has been proposed that PHEX is an inactivating endopeptidase. At this time the definitive substrate(s) for PHEX have not been identified, though fibroblast growth factor 23 (FGF-23) does seem a likely candidate (Bowe, 2001).

In one study, 83% of XLH patients had missense or deletion mutations in the 3' region of the PHEX gene (Rowe, 1997). The Hyp mouse was also found to have a 3' deletion in the PHEX gene between exons 15 and 16. Expression of PHEX is localized to flat and long bone, teeth, lung, muscle, ovary and human parathyroid gland (Beck et al., 1997; Du et al., 1996; Blydt-Hansen et al., 1999). Bone and teeth show a much higher level of expression than other tissues and there is no expression in the kidney. This is interesting as many endopeptidases have been shown to have high expression at the point of activity.

2.3.4 Endopeptidase activity

The first evidence of endopeptidase activity came from cleavage experiments on PTH. Recombinant hPHEX transiently expressed in COS-7 cells was incubated with both human PTH (1-38) and (1-34). Cleavage products of PTH were seen by reverse phase high pressure liquid chromatography (HPLC) for both species and were not detected when incubated with vector transfected COS-7 cells (Lipman et al., 1998) Further examination of PHEX endopeptidase activity used a secreted form of PHEX (secPHEX) that involved deletion or replacement of amino acids in the signal peptide / membrane anchor domain (SA domain). The secreted form was purified from cultured LLC-PK cells and assayed for activity with multiple human substrates to include: PTH1-34, PTH1-84, [Leu]enkephalin, PTHrP1-34, PTHrP107-139, big-endothelin-1, endothelin-1 and others. The only substrate showing cleavage was PTHrP107-139 (Boileau et al., 2001). This study indicates that the extracellular catalytic domain of PHEX requires interaction with other membrane proteins for true physiological activity. More recently it was shown that PHEX can cleave wild-type FGF-23, but not the mutant FGF-23 (FGF-23(R179Q)) found to be the cause of autosomal dominant hereditary rickets (ADHR) (ADHR Consortium, 2000). A high level of FGF-23 expression has been found in tumors from patients with tumor-induced osteomalacia (TIO). Expression of FGF-23 in osteoblasts has thus far proved to be negative, and the biological point of expression has been shown only in brain and thymus (Yamashita, 2000). TIO is also known as oncogenic hypophosphatemic osteomalacia (OHO) and displays the same phenotypical characteristics as for XLH patients (Kumar, 2000). The secreted FGF-23 protein from

tumors found in patients with this disorder is a circulating factor with a molecular mass of 32kDa (White et al., 2001).

ZAAL-pNA and [Leu]enkephalin, the substrates for ECE1 and NEP respectively, have been demonstrated to be cleaved by PHEX. The catalytic domain of NEP was crystallized and using sequence homology compared to other endopeptidases. The C terminal domain retains 39% similarity which is enough to suggest common tertiary folding patterns. Ten cysteine residues involved in disulfide bridges, 3 zinc binding residues and 6 ligand binding residues are also conserved between the two proteins (Oefner et al., 2000).

2.4 Goal of this project

In this doctoral work we attempted to create transgenic mouse lineages expressing hPHEX through the use of pronuclear injection using two different transgene constructs. The first transgene contained the hPHEX cDNA under the control of the osteocalcin promoter (OG2) for specific expression of hPHEX in osteoblasts. The second transgene used the β-actin promoter for ubiquitous expression of the hPHEX cDNA. Once expression of hPHEX in the two lineages was established, the lineages were analyzed for phenotypical differences. Analysis included, but was not limited to, body weight, serum and urine phosphate, calcium, and potassium levels, serum PTH, ALP and creatinine, bone length and densitometry, immunohistochemical staining and *in situ* hybridization. Mice showing expression of hPHEX were then bred with Hyp mice. The offspring were subjected to the same phenotypical analysis to determine if a rescue of the Hyp phenotype was possible from osteoblast specific or ubiquitous overexpression of hPHEX. Due to negative expression in the first OG2 transgenic mice, only the transgenic mice with ubiquitous expression of hPHEX and subsequent rescue mice were analyzed during this doctoral work.

3 Materials and methods

3.1 Materials

3.1.1 Solutions and buffers

1x TBE 100 mM Tris-HCl, pH 8.3 43 mM Boric acid 2 mM EDTA autoclave	**5x DNA-Loading buffer** 40 % (w/v) Sucrose 0.25 % (w/v) Xylene cyanol 0.25 % (w/v) Bromphenol blue in 5x TBE
Protein staining Brilliant Blue R250-concentrate diluted in H_2O(Sigma)	**Destain Solution** 40 % (v/v) Methanol 10 % (v/v) Acetic acid, in H_2O
Cellophane prep solution 25% Ethanol 5% Glycerol	**5x SDS-Loading buffer** 65 mM Tris-HCl, pH 6.8 10% (v/v) Glycerol 2.3% (w/v) SDS 0.01% (w/v) Bromphenol blue (5% (v/v) β-Mercaptoethanol
10x SDS-PAGE-Running buffer 250 mM Tris base 1.9 M Glycine 1% (w/v) SDS	**1X TNE** 10 mM Tris-HCl pH 7. 500 mM NaCl 1 mM EDTA
1x TNT 10 mM Tris-HCl, pH 8.0 150 mM NaCl 0.05% (v/v) Tween 20	**Ponceau staining** 0.1% (w/v) Ponceau S (Sigma) 5% (w/v) Acetic acid in H_2O
2x BBS 50 mM BES 280 mM NaCl 1.5 mM Na_2HPO_4 sterile filtered	**20x SSC pH 7.0** 3 M NaCl 0.3 M Sodium citrate dihydrate
1x PBS 137 mM NaCl 2.7 mM KCl 8.1 mM Na_2HPO_4 1.5 mM KH_2PO_4 autoclaved	**TBST** 0.04% (v/v) Tween 20, in TBS

10x BTPE 100 mM PIPES 300 mM Bis-Tris autoclaved 1X solution is pH 6.5	**PET** 500 ml PBS (1x) 0.005% (w/v) EDTA (Sigma) 0.05% (w/v) Trypsin, (PBS autoclaved, EDTA and Trypsin sterile filtered)
Gelatin solution 10 g powdered gelatin 0.05% Thymol-Kristall antimicrobial bi-distilled water to 1000ml stored at 4°C for 2 weeks	**Glyoxal mix** 10% (v/v) 6M Glyoxal, deionized (Sigma) 10% (v/v) 10x BTPE 75% (v/v) DMSO 5% (v/v) 80% Glycerol (sterile) stored at -80°C, thawed only once
Chromealium -Gelatine solution 1000 ml Gelatin solution 40 ml Chromealium solution stored at 4°C for 1 week	**Chromealium Solution** 4 g Chromealium (Kalium-Chrom(III)-Sulfat) bi-distilled water to 100ml stored in dark at room temperature
MMA I 60 ml Methylmethacrylate 35 ml Butylmethacrylate 5 ml Methylbenzoate 1.2 ml Polyethyleneglycol 400	**APES solution** 4 ml APES (Sigma) 200 ml Acetone
MMA III 60 ml Methylmethacrylate 35 ml Butylmethacrylate 5 ml Methylbenzoate 1.2 ml Polyethyleneglycol 400 0.8 g Benzoylperoxoid	**MMA II** 60 ml Methylmethacrylate 35 ml Butylmethacrylate 5 ml Methylbenzoate 1.2 ml Polyethyleneglycol 400 0.4 g Benzoylperoxoid

***In situ* Hybridization solution** 50% Formamide 10 mM Tris-HCl pH 7.6 200 µg/ml tRNA 1X Denhardts solution 10% Dextran Sulfate 600 mM NaCl 0.25 % SDS to 100 % volume	**MMA polymerization mixture I** 60 ml Methylmethacrylate 35 ml Butylmethacrylate 5 ml Methylbenzoate 1.2 ml Polyethyleneglycol 400 0.8 g Benzoylperoxoid 400 µl N,N-Dimethyl-p-toluidin
	Denhardts Solution (100X) 5 g Ficoll 400 5 g Polyvinylpyrolidone 5 g BSA Bi-distilled water to 250 ml then filtered at 0.45 µm then stored in 50 ml aliquots at -20°C

Ready to use solutions, buffers and materials

Roti-Phenol	Carl Roth GmbH
Roti-Phenol/Chloroform	Carl Roth GmbH
NTB-2 Nuclear Track Emulsion	Integra Biosciences;
Trizol	Invitrogen
Nitroplus supported Nitrocellulose	Sud-Laborbedarf

3.1.2 Cell lines and Bacterial strains

Mammalian Cell lines

293-EBNA	Human Embryonic Kidney including nuclear Epstein-Barr antigen Invitrogen, Karlsruhe, Germany Catalog no. R620-07
COS-7	African Green Monkey Kidney DSMZ - Deutsche Sammlung von Mikro-organismen und Zellkulturen GmbH Braunschweig, Germany

E. Coli

DH5α deoR	$T1^R$: F- ϕ80lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17(r_k- , m_k+) phoA supE44

	thi-1gyrA96 relA1 tonA
TOP10:	F- mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZDM15 ΔlacX74 deoR recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (Str^R) endA1 nupG
BL21	(DE3) pLysS: F- ompT $hsdS_B$ (r_B-m_B-) gal dcm (DE3) pLysS (Cam^R)

3.1.3 Bacterial and Eukaryotic cell culture

All media are autoclaved after preparation or the components are sterile filtered.

LB-Medium 1% (w/v) Tryptone peptone (Difco) 0.5% (w/v) Yeast extract (Difco) 0.5% (w/v) NaCl 0.01% (v/v) 10 M NaOH, autoclaved Plates are made with the additon of 1.0 % (w/v) Agar (Difco) before autoclaving. Antiboditics are added and plates are poured at 50°C	**SOC-Transfection media** 2 % (w/v) Tryptone peptone (Difco) 0.5 % (w/v) Yeast extract (Difco) 10 mM NaCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 2.5 mM KCl autoclaved 20 mM. Glucose (sterile filtered)
RPMI 1640 media (pH 7,0-7,2) 500 ml RPMI 1640 C 10%(v/v) FBS (Invitrogen) 2 mM L-Glutamin (Glutamax™II, Invitrogen) 100 µg/ml Streptomycin 100 U/ml Penicillin (Invitrogen) 0.5 µg/ml Fungizone (Invitrogen)	**Selective Antibiotics** **Bacterial** 50 µg/ml Ampicillin(Sigma) **Eukaryotic** 350 µg/ml G418(Invitrogen) or 500 µg/ml Zeocine(Invitrogen) dependent on cell line
DMEM/F12 media (pH 7,0-7,2) 500 ml DMEM/F12 (Invitrogen) 10%(v/v) FBS (Invitrogen) 2 mM L-Glutamin (Glutamax™II, Invitrogen) 100 µg/ml Streptomycin (Invitrogen) 100 U/ml Penicillin (Invitrogen) 350 µg/ml G418 (for selection of transfected cells only) 0.5 µg/ml Fungizone (Invitrogen)	**Serumfree (conditioned) Media** 500 ml DMEM/F12 (Invitrogen) 2 mM L-Glutamin (Glutamax™II, Invitrogen) 100 µg/ml Streptomycin (Invitrogen) 100 U/ml Penicillin (Invitrogen) 0.5 µg/ml Fungizone (Invitrogen)

Transfection Media		**Freezing Media**	
90% (v/v)	DMEM/Eagle (Serva)	50% (v/v)	DMEM/F12 or RPMI 1640
10% (v/v)	FBS (Invitrogen)	40% (v/v)	FBS (Invitrogen)
2 mM	L-Glutamin (Glutamax™I, Invitrogen)	10% (v/v)	DMSO
100 µg/ml	Streptomycin (Invitrogen)		
100 U/ml	Penicillin (Invitrogen)		
0.5 µg/ml	Fungizone (Invitrogen)		

3.1.4 Enzymes

Restriction endonucleases	New England Biolabs; Frankfurt a. Main, Germany Roche Diagnostics; Mannheim, Germany
T4 DNA ligase	New England Biolabs; Frankfurt a. Main, Germany
AmpliTaq DNA polymerase	Roche Diagnostics; Mannheim, Germany
Superscript II Reverse Transcriptase	Roche Diagnostics; Mannheim, Germany
Alkaline Phosphatase	Roche Diagnostics; Mannheim, Germany
Sequenase 7-deaza dGTP DNA Sequencing kit	USB; Cleveland, Ohio, U.S.A
RNase A	Sigma; Diesenhofen, Germany
DNA Polymerase I Large (Klenow) Fragment	New England Biolabs; Frankfurt a. Main, Germany
Proteinase K	Sigma; Diesenhofen, Germany
T3 RNA Polymerase SP6 RNA Polymerase T7 RNA Polymerase	Promega; Mannheim, Germany
T4 polynucleotide kinase	New England Biolabs; Frankfurt a. Main, Germany

3.1.5 Oligonucleotides

All oligonucleotides were synthesized by Metabion GmbH. (www.metabion.com)

Martinsried, Germany

2.1.6 Primers

All oligonucleotides were produced by Metabion, Martinsried.

pCDNA AS Intron	5' – TTC CAC CAC TGC TCC CAT TCA TC – 3'
PHEX2	5' – GTT ATG CTC ATG TGA GGT GC – 3'
Hyp ex3sense	5' – TCT TGT CAA ACA GTG TTC TGG – 3'
Hyp ex3antisense	5' – CCA GGG AGA CAT TTG AGG AG – 3'
Hyp 19sense	5' – GCT TGG GCT AGT TTG CTA TCT C – 3'
Hyp 19antisense	5' – TGA GTT GGT GCT ATA CAC GGA G – 3'
RHP4	5' – GCT GGC ACT GTG CAA CTG TCT – 3'
OC Sense	5' – TCT CTC TGC TCA CTC TGC TG– 3'
OC antisense	5' – AGA GCA GCC AAA GCC GAG C– 3'

3.1.7 Vectors

pCDNA4/HisMax	Invitrogen; Karlsruhe, Germany
pCDNA	Invitrogen; Karlsruhe, Germany
pBluescript	Stratagene; Heidelberg, Germany
pBI	Clontech; Palo Alto, CA U.S.A.

3.1.8 Isotopes

α-^{32}P-dCTP	Amersham Pharmacia Biotech; Freiberg, Germany
α-^{32}P-dATP	Amersham Pharmacia Biotech; Freiberg, Germany
γ-^{32}P-dATP	Amersham Pharmacia Biotech; Freiberg, Germany

3.1.9 Antibodies

Antibodies were purchased from Santa Cruz Biotechnology, CA, U.S.A.

3.1.10 Molecular weight and protein standards

All DNA and RNA markers were obtained from New England Biolabs, Frankfurt a. Main, Germany (figure 2).

3.1.11 Kits

Geneclean II	Dianova; Hamburg, Germany
DNA Maxiprep	Qiagen; Hilden, Germany
Rat Intact PTH ELISA	Immutopics Inc; San Clemente, CA U.S.A.
Osteocalcin	Immundiagnostik; Bensheim, Germany

3.2 Methods

3.2.1 Sub cloning

3.2.1.1 DNA plasmid cultures

Bacteria containing the plasmid DNA of interest were suspended in 0.5 ml of LB and spread on a LB agar plate containing antibiotics specific to the plasmid being prepared. In this work, all plasmids were selected for with ampicillin. Using a flame sterilized glass rod, 250 µl and 100 µl of solution were spread on LB-agar plates (100 µl ampicillin/ml) and incubated overnight at 37°C (minimum of 16 hours) to produce single isolated colonies.

3.2.1.2 DNA Miniprep

Two milliliters of LB media with 100 µl ampicillin/ml was inoculated with a single bacterial clone from a LB-agar plate and grown overnight at 37°C with constant agitation. 1.5 ml of the culture was centrifuged at 14,000 rpm for one minute in an eppindorf tube at room temperature. The supernatant was removed and the pellet resuspended in 100 ml of ice cold solution I (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0). 200 µl of solution II (0.2 N NaOH, 1% SDS) was added and mixed by inversion 5 – 10 times then incubated on ice five minutes. 150 µl of ice cold solution III (5 M KAc pH 4.8) was added, mixed, then incubated on ice for five minutes. The solution was centrifuged five minutes at 14000 rpm and the supernatant transferred to a fresh tube. An equal volume of phenol/chloroform (Roth) was added and vortexed, then centrifuged for five minutes at 14000 rpm at room temperature. The aqueous upper layer was transferred to a clean tube and mixed with an equal volume of phenol/chloroform/isoamyl alcohol (Roth) by shaking. The solution was centrifuged for five minutes at 14000 rpm at room temperature and the aqueous layer

transferred to a clean tube. DNA was then precipitated with one tenth w/v of NaAc pH 5.6 and two volumes of cold EtOH. DNA pellet was then washed with 1 ml of 70% EtOH, then air dried for ten minutes and resuspended in 30 µl TE pH 8.0. One microliter of RNase A was added and incubated for 15 minutes at 37°C.

3.2.1.3 DNA Maxiprep

DNA maxipreps were prepared using Qiagen Maxiprep kits. The protocol is briefly described here.

150 ml of LB media was inoculated with antibiotics. Cultures were then inoculated from a single clone and incubated overnight at 37°C (minimum of 16 hours) with constant agitation. 100 ml of culture was then centrifuged for ten minutes at 4000 rpm. Supernatant was discarded and the bacterial pellet resuspended in 10 ml of Buffer P1 (with RNase A added). Ten milliliters of Buffer P2 were added and mixed by inversion five to ten times. The solution was then transferred to a 40 milliliter centrifuge tube and ten milliliters of chilled Buffer P3 added. The solution was incubated 20 minutes on ice then centrifuged 30 minutes in a GSA rotor at 4˚C at 14000 rpm. The supernatant was collected in a fresh centrifuge tube and centrifuged a second time for 20 minutes at 4°C at 14000 rpm to produce a cleared lysate. Ten milliliters of buffer QBT were applied to a supplied Qiagen-tip 500 column and allowed to flow through by gravity. The cleared lysate was then applied to the wet column and allowed to flow through by gravity. The column was then washed twice with 30 ml of buffer QC. The DNA was eluted from the column with 15 ml of buffer QF. The DNA was then precipitated from the elutate in 30 milliliter glass corex tubes with 10.5 milliliters of isopropanol by centrifugation for 30 minutes at 14000 rpm and

4°C. Pelleted DNA was washed with 5 ml of 70% EtOH and centrifuged 10 minutes at 14000 rpm and 4°C. Precipitated DNA was air dried ten minutes and resuspended in 300 µl of TE pH 8.0. OD^{260} and OD^{280} were read to determine concentration and quality.

3.2.1.4 Restriction endonuclease digest

3.2.1.4.1 Single digest

Two microliters of DNA (1 ug/µl) were mixed with 3 µl of buffer, 3 µl 10X BSA (if required by enzyme), 2 µl of restriction endonuclease 5 U/µl –20 U/µl), and bidistilled water to final volume of 30 µl. Digestions were incubated two hours at the temperature recommended by the manufacturer.

3.2.1.4.2 Double digest

Two microliters of DNA (1 ug/µl) were mixed with 4 µl of buffer (compatible with both enzymes), 4 µl 10X BSA (if required by one of the enzymes), 2 µl of each restriction endonuclease and bidistilled water to final volume of 40 µl. Digestions were incubated two hours at the temperature recommended by the manufacturer.

3.2.1.4.3 Southern DNA digest

Ten microliters of genomic DNA (tail lysis prep see Sec 3.2.1.0) were mixed with 4 µl of buffer, 4 µl 10X BSA(if required by enzyme), 3 µl of restriction endonuclease 20 U/µl –100 U/µl), and bi-distilled water to final volume of 40 µl. The digestion was then mixed by passing through a pipet tip five times. Digestions were incubated four hours at the temperature recommended by the manufacturer.

3.2.1.5 DNA fragment purification

Purifications were done using the Geneclean II Kit (Dianova). An agarose gel was made dependant on the DNA size. Fragments sized one kilobase or smaller were purified on a 1.2% agarose gel. Those fragments larger than one kilobase were purified on 0.8% agarose gels. Agarose was mixed with 100 milliliters TAE and boiled. When cooled to 50˚C, 2 µl ethidium bromide (10 mg/ml, Roth) or vista green nucleic acid stain (Sigma, RPN5786) were added to the solution then poured into a gel chamber with comb and allowed to solidify. The DNA samples were loaded in slots next to an appropriate sized DNA ladder and run at 5 V/cm of gel until separation of bands was achieved as determined by UV transillumination. Using a sterile scalpel blade, the band of interest was cut from the gel and placed in a pre-tared eppindorf tube and weighed. Three volumes of Ultrasalt (NaI) were added and the gel slice incubated at 50˚C for five minutes. Glass silica was added 1 µl per µg DNA expected plus 1 µl (5 µg DNA would require 6 µl silica) and incubated for five minutes at room temperature. DNA bound to the silica particles was centrifuged at 14000 rpm for 5 sec and the supernatant discarded. The pellet was washed twice with 1 ml of Ultra Wash and allowed to air dry for 10 minutes. The DNA was eluted by stirring the pellet with 10 µl of TE pH 8.0, centrifuging at 14000 rpm for 5 seconds and removing the supernatant. The supernatant was centrifuged a second time to remove all silica and the clean DNA supernatant placed in a fresh tube. Purified DNA was quantified by $UV^{260/280}$ spectrophotometery then stored at -20°C.

3.2.1.6 Quantification

Quantification of DNA and RNA was determined using cuvettes with 1cm path length in a Beckman DU 530 UV/VIS Spectrophotometer. Two microliters of sample were

diluted in 198 µl of bi-distilled water and UV260 and UV 280 measured against a blank of bi-distilled water.

µg DNA = (Absorbance)*(50)*(dilution factor)*(mL solution)

µg RNA = (Absorbance)*(40)*(dilution factor)*(mL solution)

3.2.1.7 Phosphatase treatment

Two microliters of DNA (1 µg/µl) were mixed with 1 µl dephosphorylation buffer, 1 µl alkaline phosphatase and 6 µl bidistilled water then incubated for 60 minutes at 37°C. The enzyme was then inactivated by addition of $1/10^{th}$ volume of 200 mM EGTA and heating at 65°C for 10 minutes.

3.2.1.8 Ligation

Ligation reactions were set up with molar ratios of 1 to 3 and/or 1 to 5 vector to insert. The formula for the amount of insert used is calculated as follows:

ng of insert = [(ng of vector)(kb size of vector)(molar ratio of insert to vector)] / kb size of vector

Generally 50 nanograms of vector were used per ligation reaction. One microliter of T4 Ligase buffer was mixed with 1 µl of T4 ligase, 50ng vector DNA, corresponding molar ratio of insert DNA and bidistilled water to a final volume of 10 µl. The reaction mixture was incubated overnight at 16°C. Any ligation reaction not used for immediate transformation was frozen at -20°C.

3.2.1.9 Transformation

Transformations were performed using heat shock (Stratagene). DH5α cells, stored at -80°C, were thawed on ice for 30 minutes. Five microliters of ligation reaction were added to the cells and incubated on ice for an additional 10 minutes. Cells were incubated at 42°C for 2 minutes. One milliliter of LB media was added to the cells and incubated for 1 hour at 37°C. Cells were then pelleted by centrifugation at 5000 rpm, the supernatant removed and the bacterial pellet resuspended in 200 µl fresh LB media. 100 µl and 50 µl of cells were spread with flame sterilized glass rods on LB plates containing 100 µg/milliliters ampicillin and grown overnight at 37°C.

3.2.2 Cell culture

Cos-7 (African green monkey kidney) cells were grown to confluence in DMEM with 10% FBS (heat inactivated 56°C, 30 minutes), 10 U/ml penicillin streptomycin, and 125 µg/ml Fungizone (Invitrogen) at 5% CO_2 and 100% humidity.

EBNA 293 human embryonic kidney cells were grown to confluence in DMEM with 10% FBS (heat inactivated 56°C, 30 minutes), 10 U/ml penicillin streptomycin and 125 µg/ml Fungizone (Invitrogen) at 5% CO_2 and 100% humidity.

3.2.2.1 Passaging

Cells were passaged once they had achieved 90 – 100% confluence. Media was removed by aspiration and cells washed with 10 milliliters of sterile PBS. 0.5 milliliter of 0.05% Typsin/0.53 mM EDTA in PBS was added and the cells were incubated 5 minutes at 37°C until cells dissociated from the culture dish surface.

Cells were diluted and stained with 0.5% trypan-blue in PBS and counted with a hemacytometer using the following formula:

cells/ml = (average number of cells per square)(dilution factor)($10^{4)}$

where each square is 1 X 1 mm and the depth is 0.1 mm. 5 X 10^4 cells/milliliter were then transferred to a fresh culture dish and pre-warmed media added to 10 milliliters.

3.2.2.2 Freezing

Media was removed from 75% confluent cells by aspiration and washed with 10 milliliters of sterile PBS. 0.5 milliliter of 1X trypsin/EDTA (0.05% trypsin, 0.53 mM EDTA) in PBS was added and the cells were incubated 5 minutes at 37°C until cells dissociated from the culture dish surface. Cells were then collected in 5ml media and centrifuged 5 minutes at 300X g in a swing bucket rotor. Media was removed and cells were resuspended in 1 milliliter of freezing medium (60% media, 10% DMSO, 30% FBS). Cells were counted on a hemacytometer as previously described (3.2.2.1), and the cells further diluted to a concentration of 10^7 cells/milliliter and frozen in 1 milliliter aliquots. Cells were then frozen at -80°C overnight and then placed in liquid nitrogen for long term storage.

3.2.2.3 Thawing

Frozen cells were removed from liquid nitrogen and placed directly in a 37°C water bath until thawed (~ 1 minute). Five milliliters of media were added to the cell suspension then centrifuged 5 minutes at 300X g and the supernatant removed by aspiration. The cell pellet was resuspended in 10 milliliters of media and placed in a 10 cm culture dish to grow to confluency.

3.2.2.4 Transfection

Transfections were performed using the Qiagen Effectene transfection kit. Transfections were performed according to the manufacture's protocol for 100 mm culture dishes. Cells for transfection were split the day prior to transfection at a ratio of 1 to 8. Sixteen microliters of enhancer were added to 2 μg of DNA diluted in TE and the volume brought to 300 μl with bi-distilled water then mixed by vortexing. Samples were incubated for 5 minutes at room temperature then centrifuged briefly. Sixty microliters of effectene reagent was mixed in by vortexing 10 seconds and samples were incubated 10 minutes at room temperature. One milliliter of growth medium was added and the transfection mix immediately added drop-wise to the newly passaged cells. Growth medium was removed the next day, the cells washed once with PBS and fresh medium added. Expression assays were then carried out after the appropriate incubation period.

2.2.3 *In vitro* transcription and translation

The $T_NT^{®}$ Quick Coupled Transcription/Translation (Promega) system was used to determine if the constructs were capable of producing RNA and protein. The manufacturer's protocols were followed and are briefly described here.

3.2.3.1 Transcription and translation reactions

Two microliters of [^{35}S]methionine (1000 Ci/mmol at 10m Ci/ml) were added to 40 μl $T_NT^{®}$ Quick Master Mix and 7 μl bi-distilled water followed by one microliter of circular plasmid DNA (1 μg/μl). The reaction mixture was mixed with a pipet tip and incubated for 90 minutes at 30°C.

3.2.3.2 Western gel analysis

One to five microliters of sample were removed from the reaction mixture and added to SDS loading buffer. The sample was loaded on a 4-12% bis-acrylamide pre-cast gel (Novex) and run for one hour in 1X running buffer at 200V. The proteins in the gel were then stained with Brilliant Blue R250 for 10 minutes and the background stain removed with consecutive washes in destain solution. The gel was then soaked in cellophane prep solution for 24 hours. The moist gel was placed between sheets of cellophane and air bubbles removed by rolling with a glass rod. The sandwiched gel was placed in a drying frame, the cellophane stretched tight, while excluding air bubbles, and left to dry.

2.2.4 Animals

C57BL/6J mice were obtained from The Jackson Laboratory, Bar Harbor Maine, U.S.A. Hyp mice were a kind gift of Dr. Jueppner. All animal were raised in accordance with German standard animal laboratory practices. Rat/mouse breeding chow (Altromin #1314, Lage Germany) and water were available ad libitum to all animals except during overnight urine collection where food was restricted.

3.2.5 PCR

3.2.5.1 Amplification

Polymerase chain reactions were carried out using a Perkin Elmer (PE) Applied Biosystems GeneAmp PCR System 9700 Thermocycler machine for all reactions except sequencing. Sequencing reactions were carried out in a TRIO-Thermoblock (BIOmetra, Göttingen). The annealing temperature was dependent on primer pairs and salt concentrations and determined using a PE Applied Biosystems GeneAmp

PCR System 9700 Thermocycler Tm utility program.

3.2.5.2 Genotyping of PHEX transgenic animals

The human PHEX transgene overexpressed in the mice was constructed to contain a SV40 poly A sequence from the vector pCDNA 1 (Invitrogen). This sequence is of artificial origins is not found in known genomic DNA sequences and therefore specific to the transgene created. PCR using one primer from this region and one primer from within the coding sequence of PHEX was used for the genotyping of transgenic animals.

100 ng of genomic tail DNA was combined with 5 µl 10XPCR buffer, 3 µl 25 mM $MgCl_2$, 2 µl 2.5 mM dNTPs, 1 µl 10 µM PHEX2 primer, 1 µl 10 µM pCDNA AS Intron primer, 0.5 U Taq polymerase and bi-distilled water to 50 µl. PCR was carried out as follows:

5 minutes 94°C
30 cycles of
45 seconds 94°C,
45 seconds 64°C,
45 seconds 72°C
and
7 minutes 72°C

3.2.5.3 Genotyping of Hyp mice

Hyp mice carry a spontaneous mutation that causes a deletion (between exons 15 and 16) of the 3' portion of the PHEX gene and can therefore be identified by multiplex PCR using primer sets from exon 3 and exon 19.

100 ng of genomic tail DNA was combined with 5 µl 10XPCR buffer, 3 µl 25 mM

$MgCl_2$, 2 µl 2.5 mM dNTPs, 1 µl 10 µM hyp ex3sense primer, 1 µl 10 µM hyp ex3antisense primer, 1 µl 10 µM hyp ex19sense primer, 1 µl 10 µM hyp ex19antisense primer, 0.5 U Taq polymerase and bi-distilled water to 50 µl. PCR was carried out as follows:

5 minutes 94°C
30 cycles of
45 seconds 94°C,
45 seconds 58°C,
45 seconds 72°C
and
7 minutes 72°C

3.2.5.4 Sequencing

Sequencing was performed using ABI Prism BigDye Terminator Cycle Sequencing. 500 ng of DNA was mixed with 4 µl Big Dye, 5 pmol primer and filled to 10 µl with bidistilled water. Reaction was overlaid with mineral oil and PCR carried out as follows:

1 minute 96°C
30 cycles of
30 seconds 96°C,
15 seconds 50°C,
finished with
4 minutes 60°C

Analysis was performed by M. Boicu (Department of Molecular Structural Biology, MPI for Biochemistry, Martinsried) using a 373A DNA Sequencer (Applied Biosystems). Computational analysis of DNA sequences were performed using DNA strider (Dr. Christian Marek) and pDRAW32 (AcaClone Software).

3.2.5.5 Reverse transcription PCR (RT-PCR)

One microliter of total RNA was mixed with one microliter oligo dT and 9 µl bi-distilled water then incubated 10minutes at 70°C followed by a quick chill on ice.

One microliter dNTPs (10 mM), 2 µl DTT (100 mM) and 4 µl 5X first strand synthesis buffer were added to the RNA solution, mixed and incubated 2 minutes at 42°C. One microliter Superscript II was added and incubated 60 minutes at 42°C followed by 15 minutes inactivation at 70°C. Four microliters of the RT reaction mixture was removed and used for PCR as described in section 3.2.5.2.

3.2.6 Southern Blotting and hybridization

3.2.6.1 Genomic DNA tail preparation

One centimeter of mouse tail was placed in an eppendorf tube containing 500 ul tail lysis buffer, 100 µg/milliliter proteinase K (Sigma) and incubated overnight at 55°C with constant shaking. Solution was shaken strongly by hand for 3 minutes then centrifuged for 10 minutes at 14000rpm to precipitate hair. Supernatant was poured into a clean eppendorf tube with 0.5 ml isopropanol and inverted several times to precipitate DNA. DNA was removed with a clean pipette tip and placed into 500 µl of bi-distilled water. DNA was shaken by hand for 5 minutes or until DNA went into solution. 500 µl of phenol/chloroform/isoamylalcohol was added and mixed by inverting. Mixture was centrifuged 5 minutes at 14000rpm at room temperature and the aqueous (upper) phase transferred to a clean eppi. A second phenol/chloroform/isoamylalcohol extraction was performed with the aqueous phase again being transferred to a clean eppendorf. The DNA was precipitated from the aqueous phase with 1/10 volume sodium acetate (pH 5.2) and 2 volumes ice cold ethanol, then centrifuged for 10 minutes at 4°C and 14000rpm. Supernatant was removed and the pellet washed by inversion in 1 milliliter of 70% ethanol. Pellets were then centrifuged 5 minutes at 4°C and 14000rpm, the supernatant removed and

the pellet air dried for 5 minutes at 50°C. The DNA was resuspended in 40 µl of TE pH8.0 and stored at 4°C.

3.2.6.2 Agarose gel electrophoresis

A 0.8% agarose gel was prepared by dissolving 3.2g of agarose in 400ml of boiling 1X TAE. After cooling to 50°C with stirring, 12 µl of EtBr (10 mg/ml) was added and the agarose poured into a gel chamber, the appropriate gel comb set, then allowed to solidify. 1X TAE was added to the running chamber and the gel comb removed. 10 µl of restriction enzyme digested genomic DNA was mixed with 2 µl of 6X DNA loading buffer and loaded into the wells with 500 ng of 1kb DNA ladder (NEB) loaded as a standard. Agarose gels were run at room temperature and 2.5 V/cm of gel for a minimum of 8 hours. DNA was visualized on a UV transilluminator next to a metric ruler and a digitized image kept for records.

3.2.6.3 Transfer to solid support

Agarose gel was soaked for 1 hour in denaturing buffer(0.6 M NaCl, 2 M NaOH), washed briefly with bidistilled water then soaked in neutralizing buffer (0.6 M NaCl, 1 M Tris-HCl pH8.0) for 1 hour. A gel sandwich was then created by overlapping 2 sheets filter paper (Whatman 3mm) into 20X SSC buffer. Gel sized layers were then placed on top of the first overlapping pieces of filter paper in the following order: 2 sheets filter paper, 0.8% agarose gel, supported nitrocellulose (presoaked in 20X SSC), 2 sheets filter paper, flat fold paper towels. Each layer was added to the stack and air bubbles removed from between layers by rolling with a glass rod. A light weight was placed on top of the stack and allowed to transfer via capillary action overnight (figure 3).

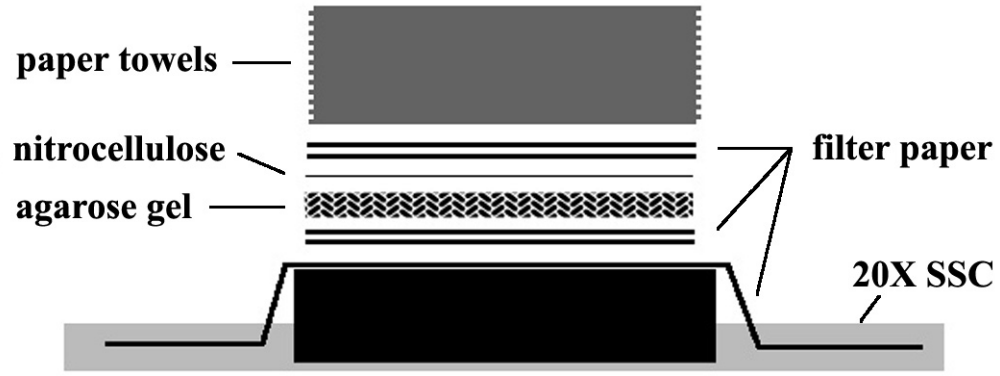


Figure 3. Building a Southern blot

3.2.6.4 Fixation on solid support

Fixation of DNA onto supported nitrocellulose was done with a UV Stratalinker 2400 (Stratagene). Each blot, still moist with SSC from the transfer to solid support, was stratalinked at 1200 J/cm^2.

3.2.6.5 Radiolabeling of probes

Circular DNA vectors containing the probes were digested with appropriate restriction enzymes to release the double stranded DNA. Digestions were run on a 0.8 – 1.2% agarose gel and the correct size DNA was isolated and quantified (see section 3.2.7.2/3).

Radiolabeling of probes was carried out with the Random primed radiolabeling kit (Roche, 1004760). 100 ng of double stranded DNA was mixed with bi-distilled water to a volume of 9 µl, boiled for 5 minutes then placed on ice. DNA was centrifuged briefly and mixed with 1µl each of dATP, dGTP, dTTP and 2 µl of hexanucleotide primer mix. Five microliters (50 µCi) dCTP was added, then mixed with 1 µl Klenow

enzyme. The reaction mixture was briefly vortexed, centrifuged, then incubated 1 hour at 37°C. The reaction was stopped with the addition of 2 µl of 0.2 M EDTA pH8.0. The mixture was then brought to 100 µl with 78 µl TE pH8.0.

A G-50 Sephadex column was prepared by placing two circles of 3M Whatman filter in a 1 milliliter insulin syringe followed by pre-soaked G-50 Sephadex(Pharmacia Biotech). The column was centrifuged 2 minutes at 2000 X G at 25°C. Sephadex was added without introducing air bubbles and centrifuged until a dry volume of 1 ml was achieved.

The 100 µl of reaction mixture was added to the sephadex column and centrifuged 5 minutes at 2000X G and 25°C. The radio-labeled probe (in the flowthrough) was collected into a fresh eppendorf and the column disposed of. The probe's activity was measured in a Packard Tricarb 5430 counter using 2 µl of probe in 10 ml of scintillation fluid (Quickszint 212, Zinsser Analytic, Frankfurt). Probes with activity of more than 400k counts per µl were considered acceptable.

3.2.6.6 Hybridization, washing and signal detection

Blots on nitrocellulose were rolled and placed in hybridization tubes with 20 ml hybridization juice and incubated at 42°C for 2 hours.

500 µl of Herring Sperm genomic DNA (SSDNA, Sigma) was denatured at 100°C for 10 minutes then placed on ice in a 50 ml Falcon tube. Radio-labeled probe (1 million counts per milliliter hybridization juice) was added to the ssDNA on ice. Fifty microliters of 10N NaOH was added and mixed by swirling at room temperature. 300 µl 2 M Tris-HCl (pH 8.0) was added and mixed by swirling, then 475 µl of 1 M HCl

was added drop-wise while swirling to prevent precipitating out of solution. Denatured probe was then added to 20 ml hybridization solution, mixed and used to replace the pre-hybridization solution. Blots were incubated overnight at 42°C for a minimum of 16 hours.

Hybridization solution was removed the following day and washed with agitation 30 minutes at 37°C in 2X SSC, 0.1% SDS, followed by 30 minutes of agitation in 0.5X SSC, 0.1% SDS at 50°C.

Blots were placed on Polytrap 295 PE paper covered with plastic wrap, a Goligo autoradiography Marker (Stratagene) applied and exposed to BioMax MS or X-Omat film (Kodak) for 1 to 3 days at -80°C. Films were then processed in an Agfa Curix HT 330U film developer. The Goligo markers were matched from the films to the blots and the lanes marked. Size of bands was determined by comparison to the metric ruler in the transillumination image of the gel.

3.2.7 Northern Blotting and hybridization

3.2.7.1 RNA extraction

RNA extraction from tissues and cell cultures was carried out using Trizol (Invitrogen) following the manufactures recommended protocol, briefly described here.

Tissues were taken from animals, frozen in liquid nitrogen and stored at -80°C. 200 mg of tissue was placed in 2 ml Trizol and homogenized using a Polytron

homogenizer (Brinkmann). Cells grown in 6 cm diameter dishes in monolayer were lysed directly in the dish using 2 ml Trizol and a cell scraper. Cell lysate was homogenized by passing the lysate through a 1 ml pipet tip. Homogenized sample was incubated for 5 minutes at room temperature. 0.4 ml chloroform was added and mixed by vigorous shaking. Samples were incubated for 2 minutes at room temperature then centrifuged at 12000 X g for 15 minutes at 4°C. The colorless upper aqueous layer was removed to a clean tube. RNA was precipitated from the aqueous layer by the addition of 1 milliliter of isopropyl alcohol and incubation for 10 minutes at room temperature. Sample was centrifuged at 12000 X g for 10 minutes at 4°C. Pellet was then washed with 2 ml of 70% ethanol and centrifuged at 7500 X g for 5 minutes at 4°C. Pellet was air dried for 10 minutes and dissolved in 200 µl DEPC treated bi-distilled water. RNA was quantified as described in 3.2.1.6 and stored at -80°C.

3.2.7.2 Glyoxal Northern gel

Ten µg of RNA was concentrated using a speedvac to a volume of 6 µl, mixed with 20 µl of Glyoxal mix and incubated for 1 hour at 55°C then placed on ice. A 1.2% BPTE agarose gel was made by boiling 1.2 g RNase free agarose in 100 ml of 1X BPTE, cooling and pouring into a gel form at 50°C, adding a comb, then allowing to solidify. The gel was place in an electrophoresis chamber covered with 1X BPTE buffer, comb removed and the samples loaded into wells. The gel was then electrophoresised at 100V (5 V/cm gel) at 4°C for 3.5 hours (method adapted from Chomczynskiand Sacchi, 1986). The gel was visualized on a UV transilluminator and position of 28S and 18S bands noted.

3.2.7.3 Transfer to solid support

A gel sandwich was then created by overlapping 2 sheets filter paper (Whatman 3mm) into 10X SSC buffer. Gel sized layers were then placed on top of the first overlapping pieces of filter paper in the following order: 2 sheets filter paper, 1.2% agarose gel, nylon membrane (presoaked in bi-distilled water then 10X SSC), 2 sheets filter paper, flat fold paper towels. Each layer was added to the stack and air bubbles removed from between layers by rolling with a glass rod. A light weight was placed on top of the stack and the RNA transferred via capillary action overnight.

3.2.7.4 Fixation onto solid support

Fixation of DNA onto supported nitrocellulose was done with a UV stratalinker (Stratagene). The nylon membrane was soaked for 5 minutes in 0.5 M NaOAc containing Methyl Blue to visualize the bands. 28S and 18S bands were marked with pencil and the membrane was stratalinked at 1200 J/cm^2 in a Stratalinker 2400.

3.2.7.5 Radiolabeling of probes

Circular DNA vectors containing the probes were digested with appropriate restriction enzymes to release the double stranded DNA. Digestions were run on a 0.8 – 1.2% agarose gel and the correct size DNA was isolated and quantified (see section 3.2.7.2/3).

Radiolabeling of probes was carried out with the Random primed radiolabeling kit (Roche, 1004760). 100 ng of double stranded DNA was mixed with bi-distilled water to a volume of 9 µl, boiled for 5 minutes then placed on ice. DNA was centrifuged briefly and mixed with 1 µl each of dATP, dGTP, dTTP, 2 µl of hexanucleotide

primer, 5 µl (50 µCi) dCTP and 1 µl Klenow enzyme. The reaction mixture was briefly vortexed then incubated for 1 hour at 37°C. The reaction was stopped with the addition of 2 µl of 0.2 M EDTA pH8.0. The mixture was then brought to 100 µl with 78 µl TE pH8.0.

A G-50 Sephadex column was created by placing two circles of 3M Whatman filter in a 1 ml insulin syringe followed by pre-soaked G-50 Sephadex (Pharmacia Biotech). The column was centrifuged 2 minutes at 2000X G and 25°C. Sephadex was added without introducing air bubbles and centrifuged, then more sephadex added, until a dry volume of 1 ml was achieved.

The 100 µl of reaction mixture was added to the sephadex column and centrifuged 5 minutes at 25°C and 2000X G. The radiolabeled probe was collected into a fresh eppendorf and the column disposed of. The probes activity was measured in a Packard Tricarb 5430 counter using 2 µl of probe in 10 ml of scintillation fluid (Quickzint 212 Zinsser Analytic) Probes with activity of more than $4X10^5$ counts per microliter were considered acceptable.

3.2.7.6 Hybridization, washing and signal detection

Membranes were pre-hybridized for 2 hours in church buffer at 60°C in glass roller tubes. 500 µl of ssDNA was denatured 10 minutes at 100°C then placed on ice in a 50 ml Falcon tube. Radiolabeled probe (1 million counts per milliliter hybridization juice) was added to the ssDNA on ice. Fifty microliters of 10N NaOH was added and mixed by swirling. 300 µl 2 M Tris-HCl pH 8.0 was added and mixed by swirling, then 475 µl of 1 M HCl was added drop-wise while swirling to prevent precipitating

out of solution. Denatured probe was then added to 20 ml of pre-warmed church buffer. Hybridization buffer with radiolabeled probe then replaced the pre-hybridization buffer and the membrane incubated overnight (minimum of 16 hours) at 60°C. Tubes were rolled throughout hybridization to insure even distribution of probe over the membrane.

The following day the membrane was removed from the hybridization tube and washed for 20 minutes in Church wash Solution at 50°C. Blots were then measured for background activity with a hand-held Geiger counter and washed an additional ten minutes when required due to high background counts. Blots were then placed on Polytrap 295 SE paper covered with plastic wrap, a goligo autoradiography marker (Stratagene) added, then exposed for 1 to 5 days to BioMax MS film (Kodak) at -80°C. Films were processed in an Agfa Curix HT 330U developer, the Goligo autoradiography markers matched and the lanes marked.

3.2.8 *In Situ* hybridization

3.2.8.1 Preparation of probe

The plasmid containing the probe was linearized with the appropriate restriction endonuclease. Ten micrograms DNA, 4 µl 10X RE buffer, 4 µl 10X BSA (if needed) and 40-200 units RE were mixed and the volume brought to 40 µl with nuclease free water. The digestion was incubated 2-3 hours at the manufacturers recommended temperature for the RE. The digestion volume was brought to 100 µl with bi-distilled water then 100 µl phenol/chloroform added and mixed by shaking. The mixture was centrifuged 2 minutes at14000 rpm and the upper aqueous phase transferred to a fresh eppendorf. DNA was then precipitated by adding 10 µl 3 M NaAc pH 5.2, 250 µl

cold 100% EtOH and incubating 30 minutes at -20°C. The precipitate was centrifuged for 10 minutes at 4°C and 14000 rpm, the supernatant removed and the pellet washed once with 1 ml of cold 70% EtOH. The pellet was air dried, resuspended in 10 µl nuclease free water, the concentration determined (see section 3.2.1.6) and stored with a final concentration of 1 µg/µl at -80°C.

3.2.8.2 Radiolabeling

Gemini transcription kits (Promega) were used for radiolabeling of probes as recommended by the manufacturer and briefly described here.
A reaction mixture was created by mixing 2 µl 5X transcription buffer, 1 µl 100 mM DTT, 0.5 µl rATP, 0.5 µl rCTP, 0.5 µl rGTP, 3 µl (25 µCi/µl) ^{35}S-UTP, 0.5 µl RNasin, 1 µl 1 µg/µl linerized plasmid, and 1 µl plasmid dependant polymerase. The reaction mixture was vortexed, centrifuged briefly, then incubated for 1 hour at 37°C followed by a brief centrifugation. An additional 1 µl of polymerase was added and incubated again for1 hour more followed by a brief centrifugation. 0.5 µl RNasin and 0.5 µl DNase were added then vortexed, centrifuged and incubated 15 minutes at 37°C. The reaction mixture was then centrifuged and stored on ice.

Unincorporated nucleotides were removed by running through a Nuctrap column (Stratagene #400702). Seven microliters 0.5 M EDTA, 14 µl 5X NTE (100 mM Tris(pH 7.5), 500 mM NaCl, 50 mM EDTA), 38 µl H_2O were added to the reaction mixture. Seventy microliters of NTE were added to the column and pushed through. The reaction mixture was then applied to the column and pushed through into a fresh eppendorf. Seventy microliters NTE was applied a second time to the column and pushed through into the same eppendorf. Total elutant collected was approximately

100 µl. One microliter of labeled probe was measured in a beta counter (Packard Tricarb 5430). Probes of 1 X 10^6 counts /µl were aliquoted into 50 µl samples and frozen for later use.

3.2.8.3 Hybridization, washing and visualization

100 µl of complete hybridization solution was used per slide. Complete hybridization solution was prepared as follows:

Five microliters radiolabeled probe (1 X 10^6 counts /µl) were denatured for 3 minutes at 85°C then placed on ice. The probe and 5 µl 1M DTT were added to 100 µl 85°C pre-heated *In situ* hybridization solution.

Slides to be hybridized were placed on trays in a moisture chamber with hybridization solution in the bottom section. 100 µl of complete hybridization solution was dropped onto each section and covered with a square of parafilm M. The chamber was closed and incubated overnight at 50°C

Hybridized slides were washed in baths the following day (without allowing them to dry out at any time) as follows:

20 minutes 50°C 5X SSC, remove parafilm M, 30 minutes 50°C 2X SSC/50% formamide, 10mins 37°C 1X TNE, 30 minutes 37°C 1X TNE /10 µg/ml RNase A, 10 minutes 37°C 1X TNE, 20 minutes 50°C 2X SSC, 20 minutes 50°C 0.2X SSC, 20 minutes 50°C 0.2X SSC, 5 minutes room temperature 70%EtOH, 5 minutes room temperature 95% EtOH and air dry. Slides were exposed overnight to BioMax MR film, the film developed and development time for the emulsion decided.

3.2.8.4 Emulsion and development

Slides were dipped in Kodak autoradiography emulsion NTB2 as follows (all handling of emulsion was carried out in absolute dark):

Approximately 30 ml of NTB2 emulsion was melted in a falcon tube using a water bath at 45°C. Twenty milliliters of 2%glycerol was preheated to 45°C and a water bath, with magnetic stirring, preheated to 50°C. The emulsion was added slowly to the glycerol and mixed without creating air bubbles then poured into a slide dipping vessel in the 50°C water bath. A blank slide was first dipped to verify no air bubbles were in the emulsion. Slides with hybridized sections were then dipped three times into the emulsion and placed vertically in a drying rack with the frosted writing surface facing down. Slides were allowed to dry for 1 hour then placed in light tight containers with desiccant and sealed. Slides were stored at 4°C until developed.

Slides were developed by incubating 2 minutes in Kodak D-76 developer (6.8 mg/ml), dipping briefly in water, incubating 4 minutes in Kodak fixer (18 mg/ml), dipping briefly in water and allowing to dry. Emulsion was removed from the back side of the slides and sections were coverslipped.

3.2.9 Phenotypical analysis of transgenic animals

3.2.9.1 Metabolic cages for urine collection

Mice were placed in stainless steel metabolic cages from 17:00 to 10:00 the next day, with free access to water but no food. Overnight urine was collected in 15 ml falcon tubes. Urine was frozen for later analysis (sect. 3.2.9.3).

3.2.9.2 Necropsy and sample collection

Animals were anesthetized by peritoneal injection of ketamine/xylazine (70/7 mg/kg). One heparinized capillary was filled with ~100 µl blood from the retro-orbital venous plexus and analyzed in an AVL 9180 (AVL Medizintechnik GmbH) electrolyte analyzer for serum Na, K and Ca levels. Remaining blood was taken from the vena cava, incubated for 1 hour at 30°C then further processed (see sect. 3.2.9.3). Samples of liver, kidney, spleen, heart and lung were removed and placed in 4% paraformaldehyde for fixation and sectioning. The left femur was removed and cleaned for peripheral quantitative computed tomography (pCQT) bone densitometry analysis. The left tibia was removed, cleaned and snap frozen in liquid nitrogen then stored at -80°C for RNA preparation. The right femur was removed and cleaned for MMA embedding and sectioning. The first lumbar vertebra was removed and cleaned for MMA embedding and sectioning. The remaining spinal column was removed and placed in 70% EtOH for bone densitometry analysis.

3.2.9.3 Serum and urine analysis

Two dilutions of urine were prepared for analysis. Dilution A consisted of 125 µl of urine diluted with 125 µl of 2N HCl for analysis by flame photometry in an EFOX 5053 (Eppendorf, Hamburg, Germany). Dilution B consisted of 30 µl of Dilution A mixed with 300 µl bi-distilled water for analysis in Hitachi 766 autoanalyzer (Boehringer Mannheim, Mannheim, Germany) for ion content analysis. Serum was diluted 1:2 for analysis in the same equipment as the urine samples.

Urine samples were analyzed for sodium, potassium, calcium, phosphate and

creatinine. Serum samples were analyzed for sodium, potassium, calcium, alkaline phosphatase, GOT, HAST, CK, t-billirubin, albumin, creatinine, phosphate, PTH, and osteocalcin. $1,25(OH)_2$ vitamin D_3 was measured by Immudiagnostik AG (Belisheim, Germany).

3.2.9.4 PTH ELISA

Serum levels of intact PTH (1-84) were measured using the Rat intact PTH ELISA kit (Immutopics). The manufacturer's recommended protocol was used and is briefly described here. In duplicate twenty five microliters of sample were loaded into a streptavadin coated microtiter plate with the provided rat intact PTH standards and controls. 100 µl of working antibody solution (1:1 mixture of rat biotinylated antibody with rat HRP conjugated antibody) were added to each well, the plate sealed and covered with aluminum foil. The plate was incubated for three hours at room temperature with constant shaking. The plate was unsealed, the contents of each well aspirated, then washed five times with the provided working wash solution with the final wash being aspirated. 150 µl of ELISA HRP substrate were aded to each well, the plate seasled and covered with aluminum foil and incubated 30 minutes at room temperature with constant shaking. The plate was unsealed and the 595nm absorbance was read from each well within 5 minutes using the 0 pg/ml standard wells as a blank. 100 µl of ELISA stop solution was added to the wells and the 450 nm absorbance was read within ten minutes using 150 µl of substrate and 100 µl of stop solution as a reagent blank. For PTH values that fell within 0 to 800 pg/ml the 450 nm absorbance was used to determine concentration. The corrected absorbance was calculated by subtracting the 0 pg/ml standard levels from all other OD values. The corrected absorbance was plotted on a log-log graph against the concentration and unknown values determined by linear regression analysis.

3.2.9.5 Osteocalcin measurement

Serum osteocalcin was analyzed using a mouse osteocalcin IRMA kit (Immundiagnostik, Bensheim Germany). The manufactures protocol was followed and is briefly described here. Standards provided were dissolved in one milliliter bi-distilled water. Ten microliters of the controls and samples were diluted with 100 µl zero standard. Twenty five microliters of samples, controls and standards were aliquoted into eppendorf tubes and 200 µl I^{125} labeled osteocalcin antibody added and vortexed. One bead was added to each reaction mixture and allowed to incubate for 24 hours. Wash solution was diluted from stock and allowed to equilibrate overnight at room temperature. After 24 hour incubation the supernatant was removed and washed three times with 2 ml wash solution. Beads were then measured using a gamma counter. Sample values were calculated from the standard curve generated.

3.2.10 Histology

3.2.10.1 Paraffin embedding

Tissues harvested from animals were placed in embedding cassettes and fixed for 24 hours in 4% paraformaldehyde at 4°C, washed overnight in 0.1 M phosphate buffer, then placed in 70% ethanol. Tissues were processed in a Hypercenter 2 (Shandon) and embedded using a Tissue-TEKII tissue embedding center (Ames).

3.2.10.2 Methylacrylate embedding

3.2.10.2.1 Fixation and preparation

After fixation for 24 hours in 4% paraformaldehyde, the tissues were washed for 24 hours in 0.1 M phosphate buffer with 10% sucrose added at 4°C with stirring and one change of buffer. Tissues were then placed in a container that allowed for a magnetic

stirrer and were kept at 4°C while in solutions. The specimens were processed 2 days in 70% EtOH, 2 days in 95% EtOH, 2 days in 100% isopropanol (changed once), 2 days in xylene (solution changed once), 2 days in MMA I, 2 days in MMA II, 2 days in MMA III (Erben, 1997).

3.2.10.2.2 Embedding

Immunohistochemical

The tissues were embedded in round glass vials 2.5 cm in diameter and 6 cm in height with MMA polymerization mixture II precast in the bottom. The embedding vials were kept cold at all times during the embedding process. Vials were filled with MMA polymerization mixture I, tissues were placed against the hardened bottom, vials sealed and placed in a climate chamber at -20°C to polymerize. Once polymerized, the glass vials were broken and the blocks trimmed for sectioning.

3.2.10.3 Slide preparation and Sectioning

Slides used for sections were first cleaned in acetone for 10 minutes at room temperature, rinsed with water, incubated 5 minutes in acetone followed by 5 minutes incubation in (3-Aminopropyl)triethoxysilane (APES) solution. Slides were then washed twice in bi-distilled water then air dried at 50°C. Slides were stored at room temperature. Sections were cut with a Microm HM 355 rotation microtome. Paraffin sections were cut with a thickness of 8 μm and methylacrylate sections were prepared with a thickness of 4 μm.

Deplasticization

Slides were placed in 3 successive solutions of methoxyethylacetate, 20 minutes per

solution. Methoxyethylacetate was changed after processing 40 slides. Slides were washed twice for 5 minutes in acetone then washed twice for 5 minutes in bi-distilled water.

Deparaffinization

Slides were placed in 2 successive washes of xylol for 15 minutes, 5 minutes in 100% EtOH, 5 minutes in 70% EtOH, 5 minutes in 40% EtOH and washed for 5 minutes in bi-distilled water.

3.2.10.4 Staining and visualization of sections

Hematoxylin and Eosin

Slides were incubated for one minute in filtered Harris Hematoxylin then rinsed in flowing water until the incubation chamber had been cleared of stain. Sections were then incubated one minute in 1.0% Eosin Y. Sections were dehydrated five minutes in ascending alcohol solutions of 70%, 80%, 95%, and twice in 100% ethanol. Slides were cleared with two series of five minutes incubation in xylene.

Modified Van Kossa

Slides were rehydrated then transferred to a solution of 1% silver nitrate for 15-60 minutes under a strong light. Blackening of the mineralized bone can be seen macroscopically. Sections were gently washed in three successive bi-distilled water baths then treated with thiosulfate for 5 minutes and again rinsed well with bi-distilled water. Section were counterstained for 3 minutes with methyl green, rinsed in three successive washed of n-butanol then either immersed in 2 successive xylol baths of five minutes each or further processed using the McNeal protocol. Sections were

coverslipped with Roti-histokit and glass coverslip.

McNeal

Sections were immersed in bi-distilled water instead of xylol after counterstaining in the Von Kossa staining protocol. Slides were immersed for 20 to 60 minutes in freshly prepared 5% tetrachrome then rinsed twice in bi-distilled water, briefly rinsed twice in 2-propanol, then soaked 5 minutes in xylol and finally coverslipped with Roti-histokit and glass coverslip.

Visualization

Sections were visualized using a Zeiss axioskop microscope (Carl Zeiss Mikroscopie, Berlin Germany) and images captured using a Spot Diagnostic inc. model 1.3.0 digital image capture devise (Visitron Systems, Puchheim Germany) and Adobe Photoshop software.

3.2.11 Peripheral quantitative computed tomography

Left femurs were excised during necropsy, fixed and stored in 70% ethanol for a minimum of two weeks. Four slices from each femur were analyzed by peripheral quantitative computed tomography (pQCT) (Statech Medizintechnik, Pforzheim Germany). Three slices were within the distal femoral metaphysis and the fourth was at mid-diaphysis. (Figure 4)

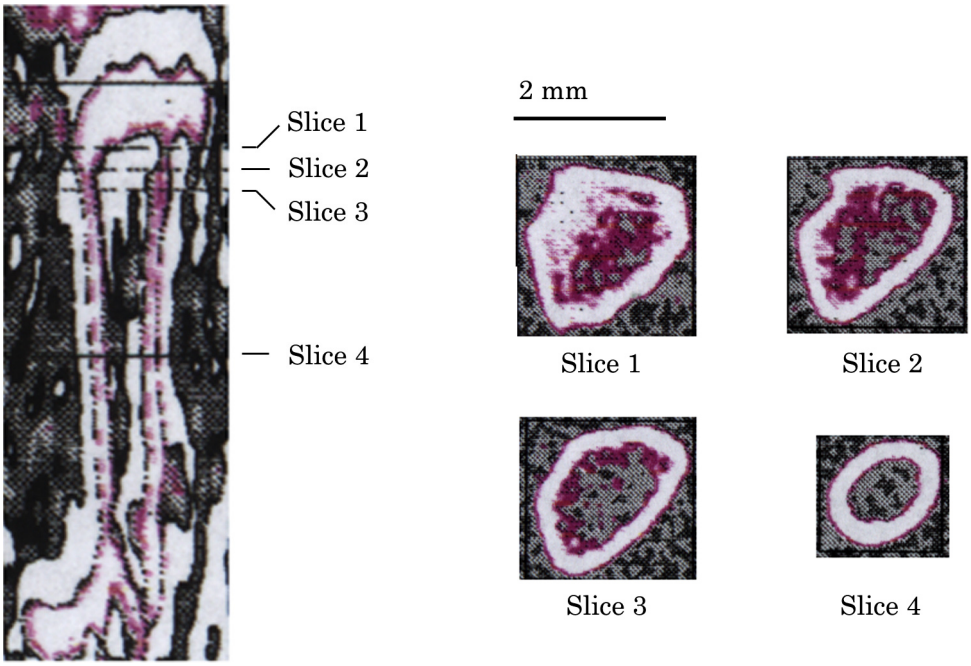


Figure 4

Definition of slices measured for densitometry.
3 distal femur measurements and one mid-diaphysis

4 Results

4.1 Assay for competent transgenic constructs

4.1.1 Blueprints of transgenes

Osteocalcin-hPHEX (figure 5)

The 2.4kb hPHEX coding sequence was ligated in a three way ligation with pCDNA I (Invitrogen) and the Osteocalcin (OPG2) promoter (gift from Dr. Karsenty).

pCDNA I	Xho I / Bam HI	4.1 Kb
OPG2 Promoter	Bam HI / Hind III (blunt)	1.3 Kb
hPHEX cDNA	Sma I / Xho I	2.4 Kb

ß-actin hPHEX (figure 5)

Multiple cloning steps were used to create a final plasmid containing the following fragments:

β-actin promoter	Hind III(blunted) / SalI(blunted)	4.0 Kb
hPHEX cDNA	Sma I / Xho I	2.4 Kb
pCDNA I	Xho I / Bam HI	4.0 Kb
pBluescript	Bam HI / EcoRV	30 bp

Both constructs contain an SV40 polyadenylation site from the plasmid pCDNA I (Invitrogen). This commercial plasmid contains an intronic sequence of 70bp. The use of this intronic sequence permits RNA transcribed from the transgene to be distinguished from endogenous mPHEX, which has high nucleotide similarity.

The OG2-hPHEX transgene was digested with Kpn I to verify the expected DNA sizes of 4.66Kb and 3.23Kb. The β-actin-hPHEX transgene was digested with Kpn I

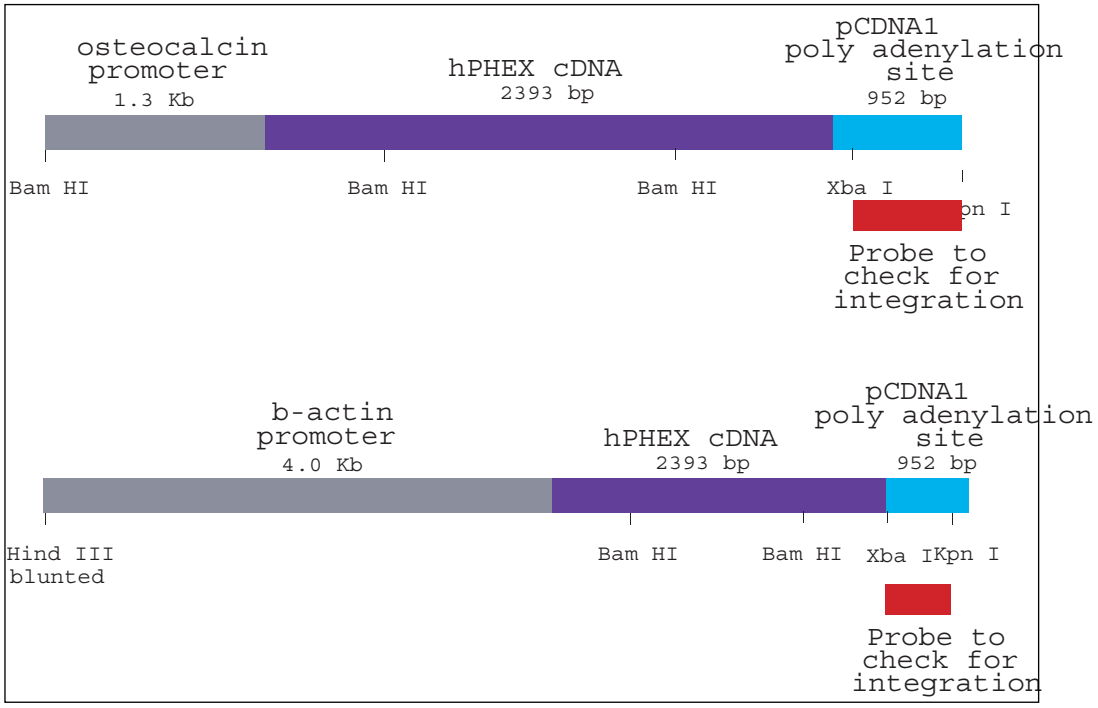


Figure 5
Transgene constructs for osteocalcin – hPHEX and β-actin – hPHEX showing hybridization probe for southern blots.

to verify fragment sizes of 7.4 and 3.0. Both transgenes were sequenced to verify correct orientation of all fragments and subcloning junctions' integrity.

4.1.2 Assay for *in vitro* expression

Once the transgene constructs were verified, they were subjected to TnT Quick Coupled transcription and translation (Promega) reactions. One microliter of reaction sample was used for western analysis. A primary band was seen at 125 kDa in both constructs indicating a

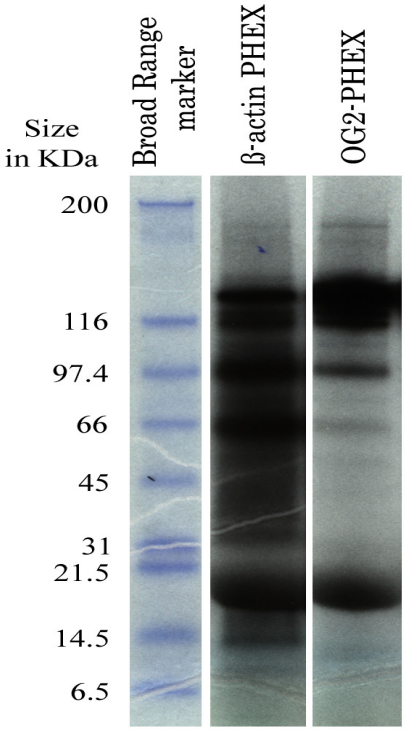


Figure 6
TnT Quick Transcription Translation of OG2-hPHEX and β-actin-hPHEX constructs

primary transcription start site.

Also seen were multiple smaller bands indicating alternate transcription start sites, or possible translational proteolytic cleavage products. An artifact band for globin appeared in both samples in the lower kDa range that has been previously report with this system (figure 6).

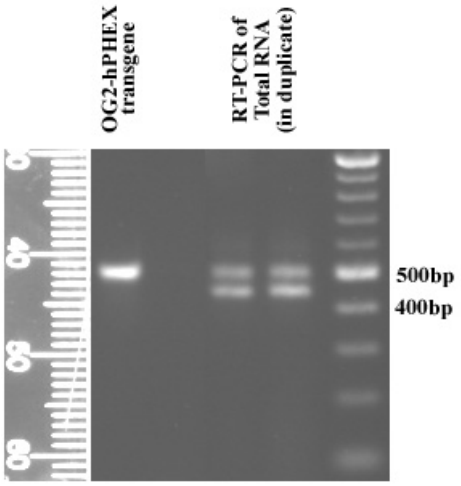


Figure 7
PCR of OG2-hPHEX transgene and reverse transcribed RNA from transiently transfected COS-7 cells.

4.1.3 Assay for *in vivo* expression

The OG2-PHEX transgene was transiently transfected into COS-7 cells, incubated 48 hours at 37°C and total RNA prepared. Total RNA and the plasmid transgene were treated to RT-PCR using the primers pCDNA – AS Intron and PHEX2 for 30 cycles. Genomic DNA produced a band of 521bp and the mRNA, due to an intron within the PCR amplified region, was 451bp. The plasmid DNA produced a single band of 521bp, and the mRNA from the transient transfection produced bands of 521bp and 451bp (figure 7).

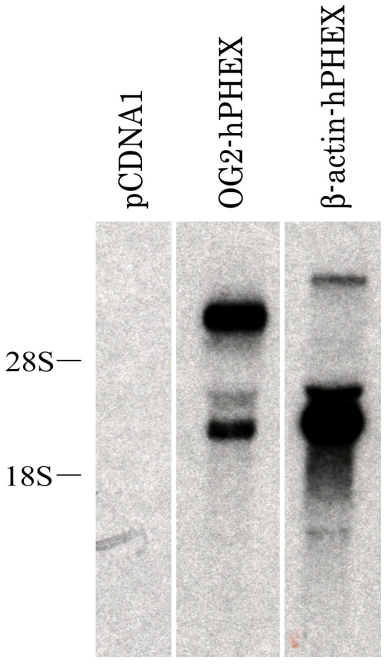


Figure 8
Northern of transient transfected COS-7 cells

One microgram each of the OG2-hPHEX and β-actin-hPHEX transgene constructs were transiently transfected

into COS-7 cells while an equal amount of pCDNA1 was transfected as a negative control. The cells were allowed to express for 48 hours then the monolayer cells were harvested and total RNA prepared using Trizol. Six micrograms of total RNA were used in a glyoxal Northern. The RNA was incubated overnight in Church buffer at 55°C using the radiolabeled 1.57Kb BamHI fragment of hPHEX as a probe. The blot was exposed to BioMax film for 24 hours at -80°C, allowed to thaw to room temperature and developed. The pCDNA1 RNA showed no hybridization while the OG2-hPHEX produced bands of 6.0 and 3.4 Kb . The β-actin-hPHEX hybridized with the probe at 8.0, 4.0 and 3.5 Kb (figure 8).

4.1.4 *In situ* Hybridization visualization

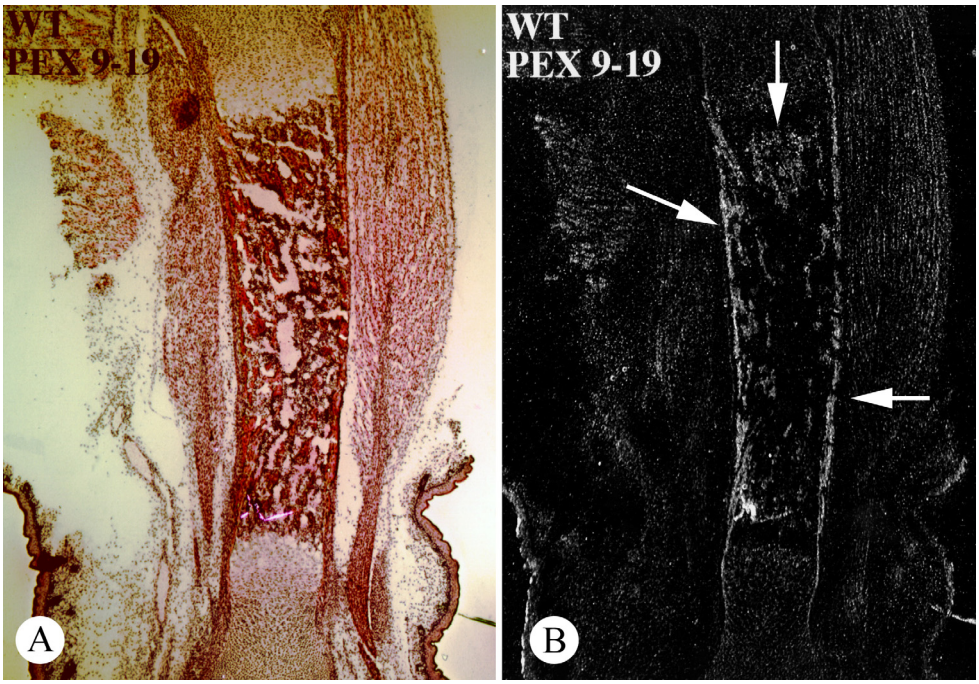


Figure 9 In situ hybridization of mouse tibia.
2.5X A) light field and B) dark field microscopy.

In situ hybridization was carried out using a probe consisting of exons 9 to 19 of the mPHEX gene. Positive expression is most clearly seen in the dark field microscopy

image (figure 9, arrows). Expression is localized to trabecular and cortical bone. These results clearly indicate that hPHEX if overexpressed specifically in bone or overexpressed ubiquitously could be identified through in situ hybridization. In situ hybridization of hPHEX transgenic mice did not show any appreciable difference to wild-type sections (data not shown).

4.2 Confirmation of germline transmission (First Pronuclear Injection)

RCC Ltd, (Füllinsdorf, Switzerland) was contracted to perform the pronuclear injections of the OG2-hPHEX transgene and return 3 transgenic animals without clause for expression of the transgene. Two lines of mice were established by Rcc Ltd. with a third being too sick for further breeding. The lines designated 47 and 79 were subjected to Southern blot analysis to ensure the germ-line transmission of the transgene to offspring. Lineage 47 displayed a major band at 3.0Kb and a single band at 8.2Kb. Lineage 79 displayed a major band also at 3.0Kb and minor bands at 4.6Kb and 4.9Kb (figure 10).

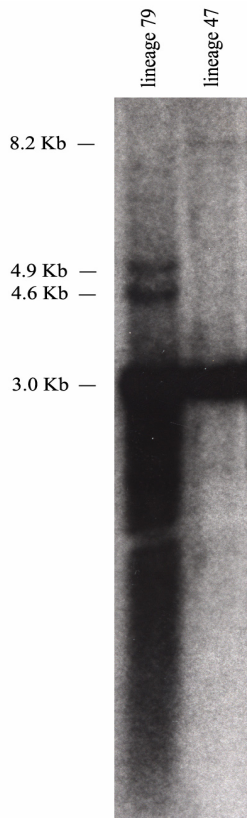


Figure 10
Southern of lineage 47 and 79.

Mice from both lineages were sacrifice and tissues removed for total RNA extraction. Total RNA was used for RT-PCR (64°C, 30 cycles) to determine expression of the hPHEX transgene, using primers pCDNA – AS Intron and PHEX2. As control, osteocalcin primers were used in a PCR of the same reverse transcription reaction. Bands of 521bp, indicative of genomic DNA, were seen for the transgenic animals from both lineages but not for the WT littermates, no bands of 451bp,

indicative of RNA were seen for any of the animals. (figure 11)

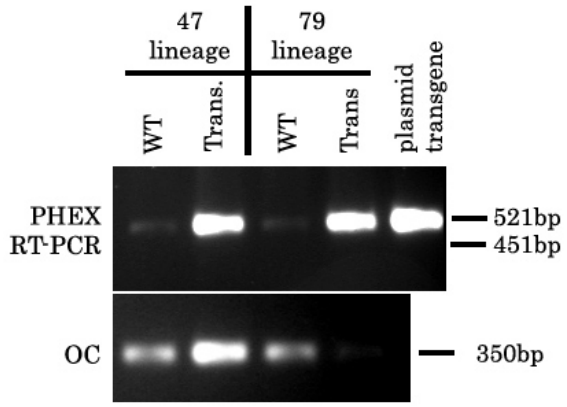


Figure 11
RT-PCR of lineage 47 and 79 mice for OG2-hPHEX transgene with control PCR from same RT reaction for osteocalcin

Northern blot analysis was carried out on different mice from both lineages. Calvaria and femur were extracted from mice, snap frozen in liquid nitrogen and used for total RNA preparations. Five micrograms of total RNA was used for each tissue type from each animal. The RNA was hybridized overnight in Church buffer at 55°C using the radiolabeled 1.57Kb BamHI fragment of hPHEX as a probe. No hybridization bands were seen for any of the samples.

From the analysis of the Southern, Northern and RT-PCR from the two lineages of mice provided by RCC Ltd., we concluded that there was no expression of the hPHEX mRNA or protein. Without expression of the mRNA there was no possibility of examining our hypothesis.

4.3 Confirmation of germline transmission (Second pronuclear injections)

The OG2-hPHEX transgene and the β-actin-hPHEX transgene were sent to Dr. Harold Jueppner (Harvard Medical School, Boston) for a second attempt at pro-

nuclear injection, in order to create transgenic mice that expressed the hPHEX protein. Two lines of mice with the β-actin-hPHEX transgene and a single line of OG2-hPHEX transgenic mice were created.

To determine whether the transgene was transmitted to offspring, a PCR using primers pCDNA – AS Intron and PHEX2 was performed (64°C, 30 cycles). The mice from all lineages show a single band at 521bp if they are positive for the transgene and no band if the transgene has not been transmitted (i.e. WT mice) (figure 12).

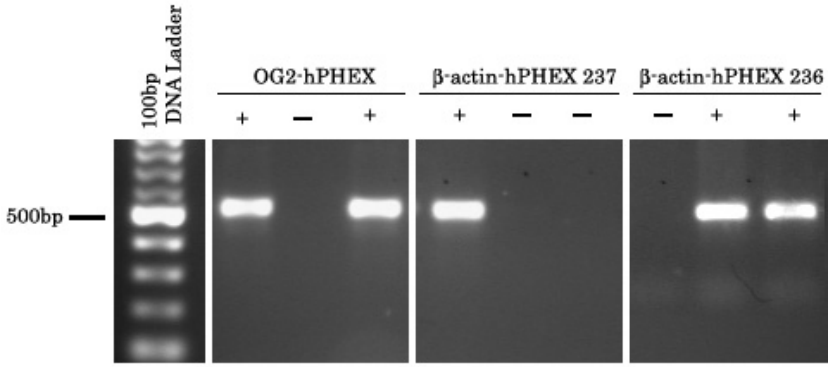


Figure 12
PCR for hPHEX transmission to offspring using PEX2 and pCDNA AS Intron

In order to see the copy number and number of transgene integration sites, a southern blot analysis was run using the Xba I / Kpn I probe on all transgenic lineages returned. OG2-hPHEX produced a transgenic mouse with four integration sites. One integration site at 2.8Kb contained a markedly greater number of construct copies than the other three sites at 3.3Kb, 4.0Kb and 6.4Kb. The first β-actin-hPHEX transgenic lineage, designated β-actin 236, displayed a doublet on southern blot analysis at 2.0Kb and 5.6Kb. The second β-actin-hPHEX lineage, designated β-actin 237, showed a single band at 6.4Kb. The β-actin 237 lineage probably has a greater copy number at the integration site compared to the two integration sites of the β-actin 236 lineage (figure 13).

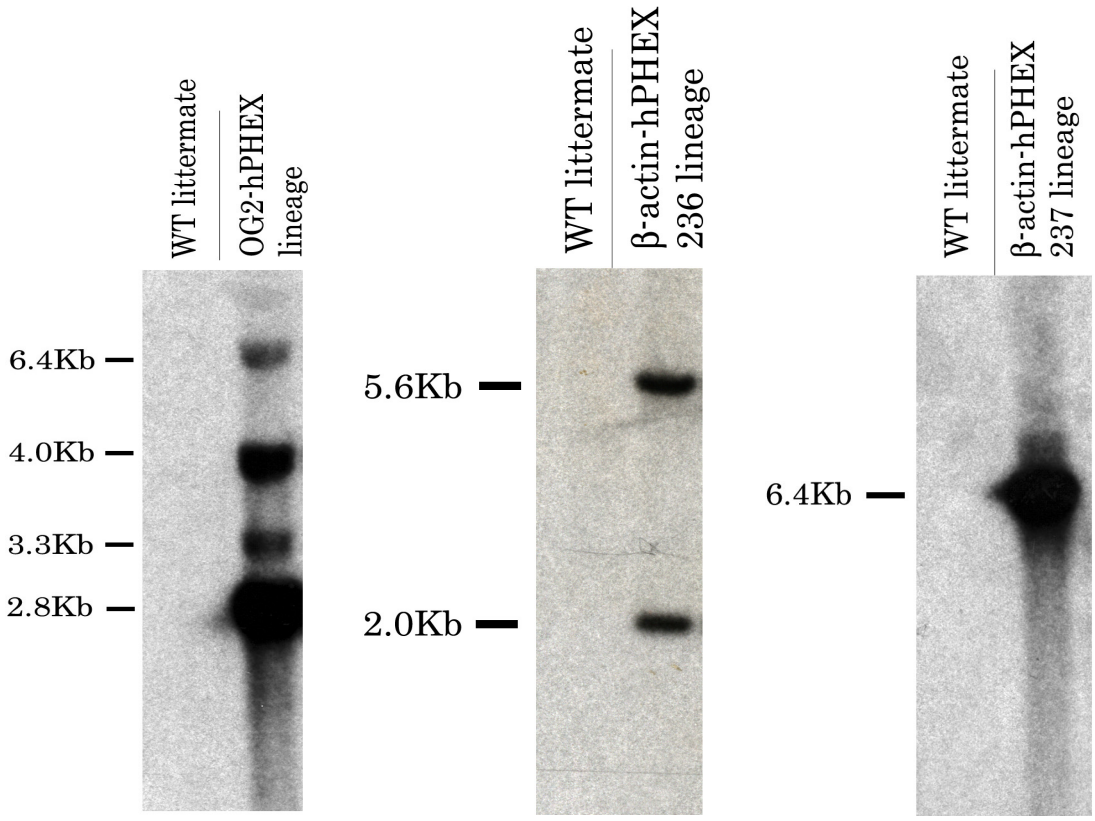


Figure 13
Southern blot analysis of OG2-hPHEX and β-actin-hPHEX transgenic lineages with corresponding Wild-type littermates.

4.3.1 Distribution of tissue expression

RT-PCR was used to determine expression of the transgene in different tissues. Total RNA from liver and skin from β-actin-hPHEX lineages and calvaria and femur from OG2-hPHEX was produced using Trizol extraction. PCR of the reverse transcribed DNA was carried out at 64°C for 30 cycles using primers PHEX2 and pCDNA AS Intron. Wild-type littermates were used as negative controls. The negative controls showed no bands at all, while the OG2-hPHEX lineage displayed a band at 521bp corresponding to genomic DNA. β-actin-hPHEX 237 and β-actin-hPHEX 236 liver

and skin samples displayed bands at 521 and 451 corresponding to DNA and mRNA bands. The skin sample of β-actin-hPHEX 236 and both samples of the β-actin-hPHEX 237 lineage displayed a lighter band at 451bp than the β-actin-hPHEX 236 liver (figure 14).

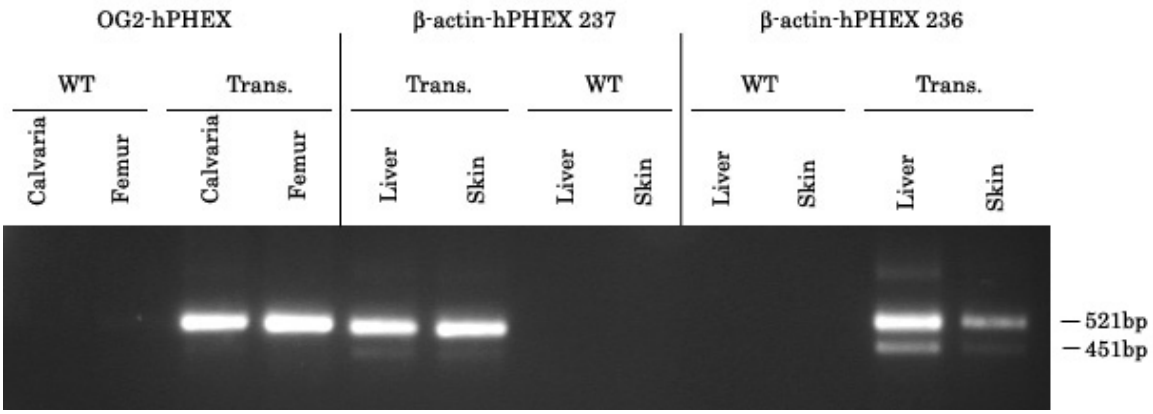
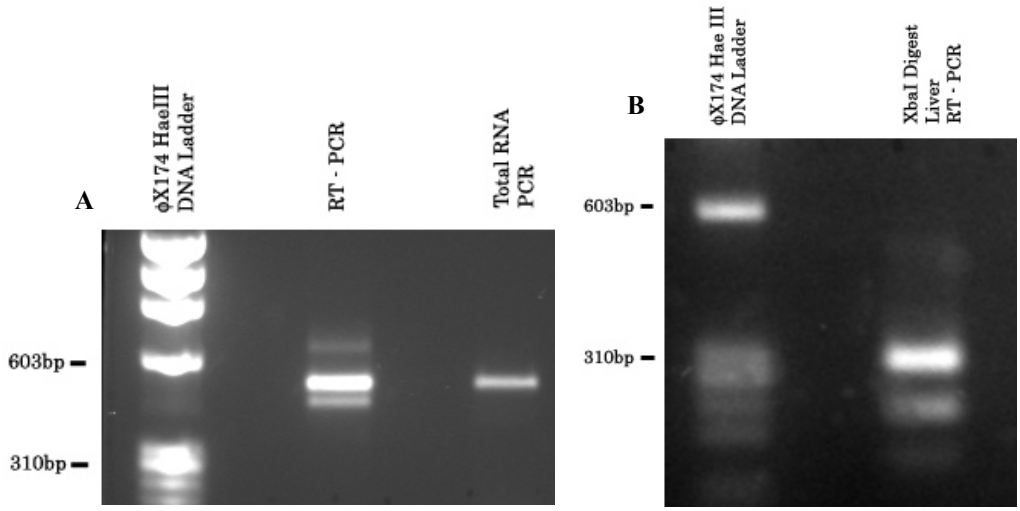


Figure 14 RT-PCR of transgenic lineages. Genomic DNA 521bp, mRNA 451bp

In order to determine if the 451bp band was due to the presence of the hPHEX transgene the PCR was repeated for the β-actin-hPHEX 236 liver sample. PCR using the same primers was run with material from the reverse transcription reaction side by side with the material from the total RNA preparation. The RT-PCR product was digested with XbaI. The genomic DNA product was cut into bands of 301bp and 220bp. The mRNA product was digested to bands of 301bp and 150bp. A large band was seen for the cleaved 301bp band present in both species, while the 220 and 150bp bands are present but weaker (figure 15).

Figure 15
β-actin-hPHEX 236 liver.

a) PCR of RT and total RNA. b) XbaI digestion of RT-PCR.



Northern Blot Analysis

Northern blot analysis was used on total RNA from liver, kidney, and bone with exposure times up to four weeks on Kodak BioMax MS film. No distinguishable bands were seen when using the full length hPHEX cDNA as a radiolabeled probe.

4.4 Assay of biochemical parameters

The disease XLH and its mouse homologue, Hyp, present in the same manner. Abnormalities in the biochemical state of many measurable parameters in the affected individual are readily apparent. We measured the values for the following parameters: body weight, serum values for calcium, potassium, sodium, GOT, alkaline phosphatase, creatine kinase, albumin, HAST, creatinine, phosphorus, osteocalcin, PTH, urine values for sodium, potassium, calcium, creatinine, and phosphorus. Mean values for all measurements follow in the appendices. Significance was determined through unpaired t-test for comparison of two groups and ANOVA for groups of three test subjects (Minitab statistical software)

4.4.1 Serum phosphate levels remain unchanged in β-actin - hPHEX mice

236 β-actin hPHEX

Female animals from the β-actin 236 lineage were sacrificed at 12 weeks of age and samples taken as described in methods. A p value of $> .05$ shows there is no

Figure 16
Comparison of β-actin 236 transgenic and wild-type body weight

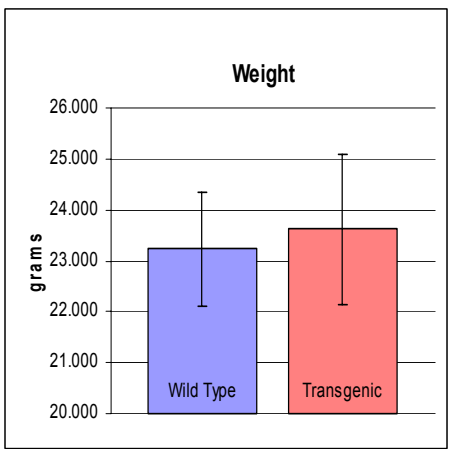
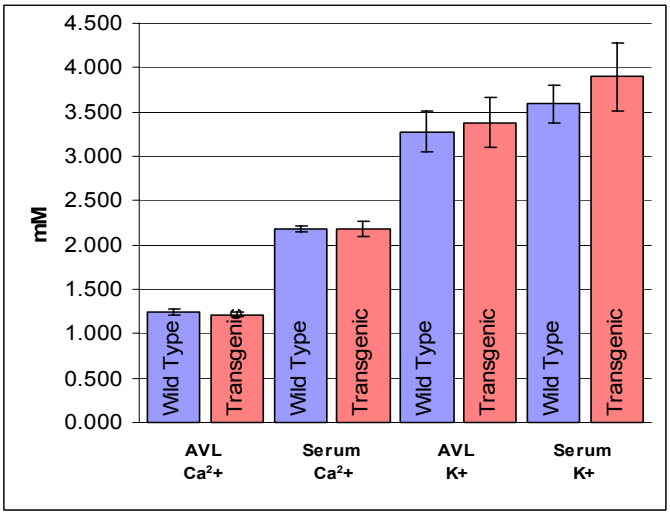
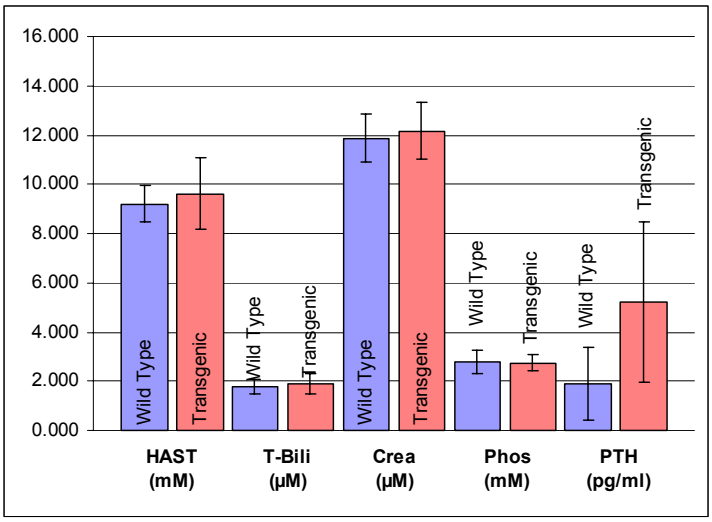


Figure 17
Comparison of β-actin 236 transgenic and wild-type serum values.



significant difference between body weight in the transgenic β-actin 236 and wild-type mice (figure 16). Further comparison of the ion concentrations from AVL and normal serum tests demonstrate unchanged values between groups (figure 17).

Figure 18
Comparison of β-actin 236 transgenic and wild-type serum values



A primary attribute of the Hyp mice is the depressed levels of serum phosphate. As indicated by the results in figure 18, the phosphate levels remain unaffected in the β-actin 236 transgenic mice in relationship to wild-type mice. There is a large difference seen in the PTH levels between groups but the large standard of deviation and a p value of more than 0.05 indicate the results are not significant. In the analysis of all biochemical parameters examined for the β-actin 236 female transgenic mice there were no significant differences compared to wild-type mice (tables 1 – 3).

Qui et. al. were able to establish there is no gender dependent effect of the PHEX gene. In order to confirm this and confirm no gender effects of the β-actin hPHEX transgene, male and female mice of the 237 lineage were analyzed for the same

biochemical parameters. Body weight of both genders remained constant between transgenic and wild-type animals (figure 19). Blood drawn by retro-orbital puncture at the time of animal sacrifice as well cardiac serum samples displayed no significant differences in calcium or potassium values (figure 20).

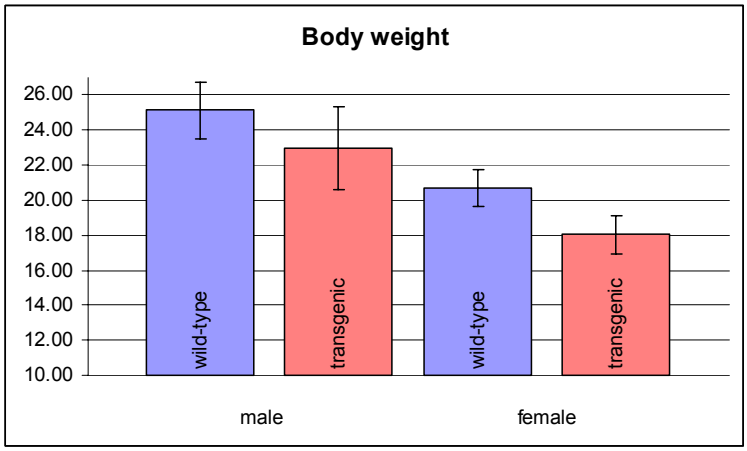


Figure 19
Body weight of male and female 237 b-actin hPHEX mice compared to wild-ttype littermates/

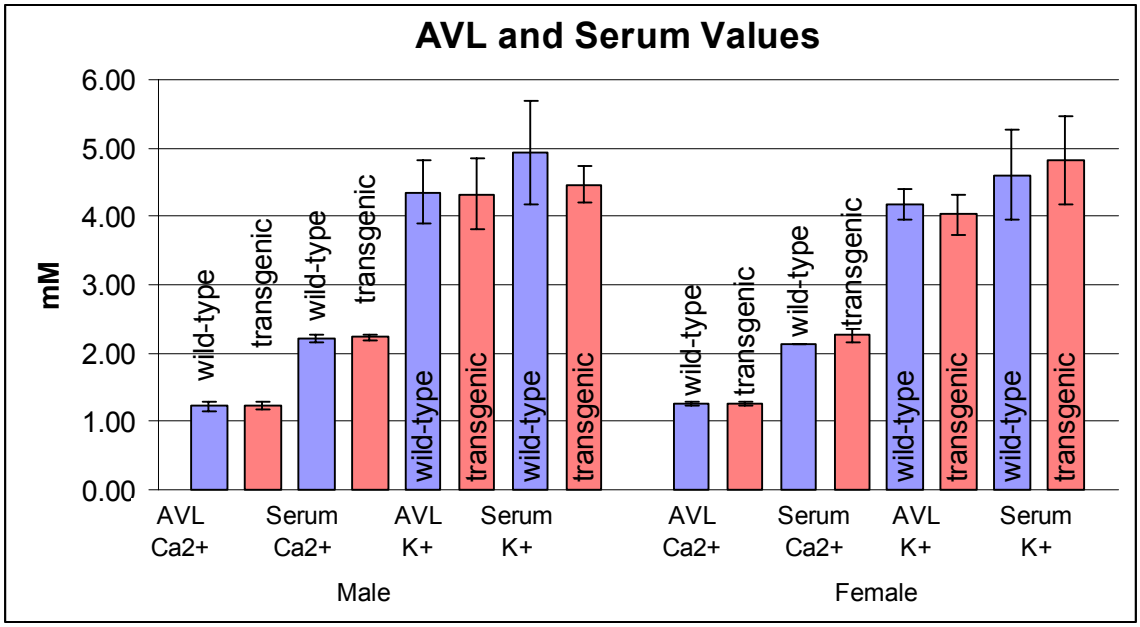
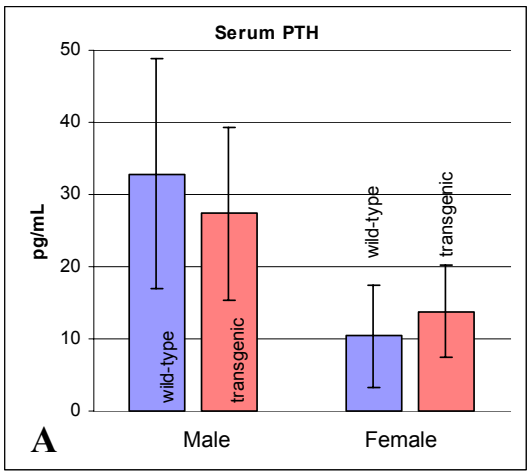
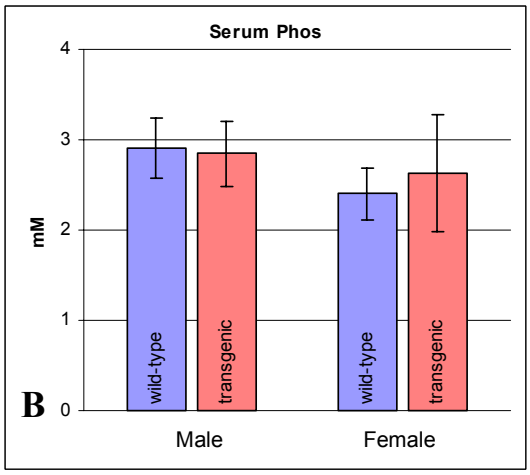


Figure 20
Serum probes taken by retroorbital puncture and cardiac bleed analyzed for ion content. Comparison between wild-type and 237 β-actin PHEX transgenic animals for both genders.





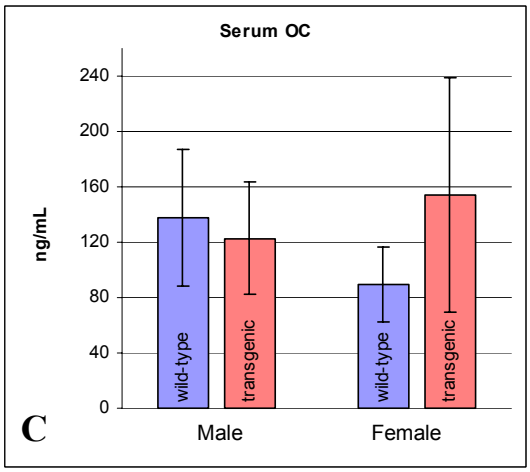


Figure 21: A, B, C
Comparison of serum values between wild-type and 237 β-actin PHEX transgenic animals for both genders.
A) PTH
B) Phosphorus
C) Osteocalcin

Phosphate serum values of female 237 transgenic animals rose ten percent over wild-type over wild-type, but due to high standard deviation in the groups, the findings were not significant. PTH and osteocalcin remained, likewise, little changed between sample groups of like gender (figure 21: A,B,C).

Overnight collections of urine were analyzed for ion content and displayed no significant variation in sodium, calcium or potassium, corrected by creatinine content (table 6). In the overall analysis of the 237 biochemical data, there were no significant changes in any of the measured parameters (tables 4 – 6).

4.4.2 Rescue mice display normalization of parameters

In order to examine whether the expression of the transgene in varied tissues, including bone, could rescue the phenotypical abnormalities of the Hyp mice, a cross of the two lineages was established. Only β-actin 236 mice were used for crossing into the Hyp mice due to the higher expression of hPHEX compared to the β-actin 237 lineage. The offspring from the crossing of hPHEX transgenic mice and Hyp affected mice were labeled rescue mice if they carried the transgene and hyp mutation simultaneously. Three different offspring combinations were possible: Hyp positive and hPHEX negative (+/-, Hyp mice), Hyp positive and hPHEX positive (+/+, rescue mice) or Hyp negative and hPHEX negative (-/-, wild-type). C57/Bl6 mice were used as control mice. The most significant phenotypical or

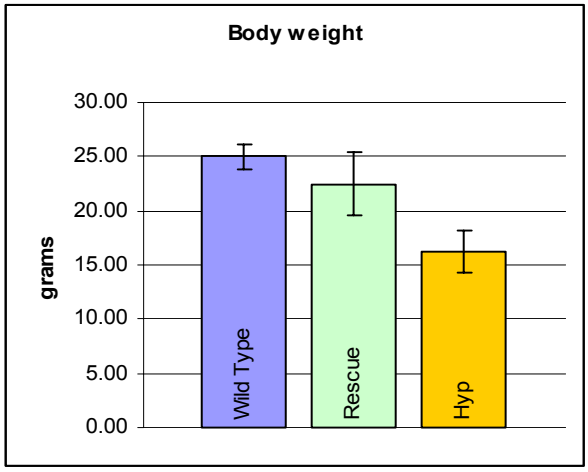


Figure 22
Rescue mice display a significant recovery of body weight over the hyp mutant mice.

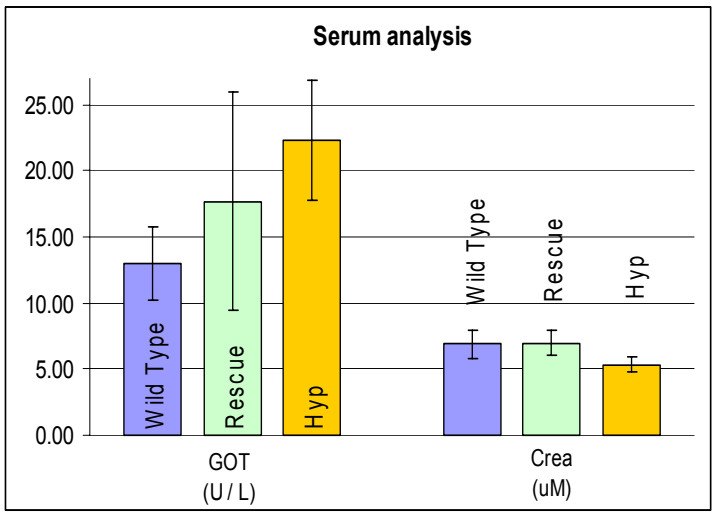


Figure 23
Serum values for GOT and Crea obtained from analysis of cardiac puncture serum/

biochemical result from the crossing of the hPHEX transgenic mice and Hyp mice, was the almost normalization of body weight for the rescue mice and the increase in bone mineral density.

The rescue mice showed a recovery of seventy-one percent of lost body weight due to the hyp mutation. Statistical analysis gives a p-value of less than 0.001, making the findings highly significant (figure 22). In gross examination of rescue mice compared to hyp mice there is less extreme curvature of the spinal column, which is commonly seen in hyp mice. Calcium, potassium and sodium values as measured in AVL and cardiac serum probes showed no significant variation. Other unaffected parameters in the rescue mice were serum values for T-bili, CK, ALB and Hast, all with p-values greater than 0.05. Values displaying a trend toward normalization included GOT, creatin, phosphate and PTH (figures 23 - 25) . Urine values corrected by creatine content were not significantly different between hyp, rescue and wild-type mice (tables 7 – 9).

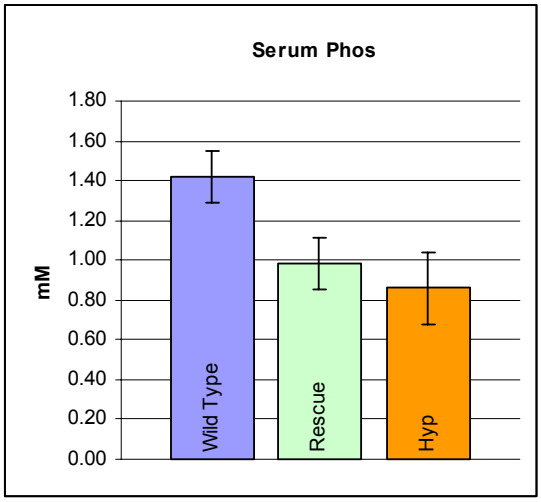


Figure 24
Serum phosphate levels from cardiac puncture.

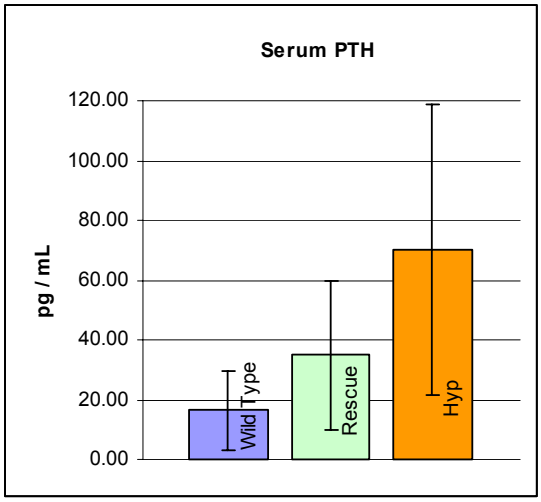


Figure 25
Serum PTH levels from cardiac puncture.

4.4.3 Biochemical data tables

β-actin-hPHEX 236

Abbreviations

BW – Body Weight
Ca – Calcium
Na – Sodium
K – Potassium
SD – Standard deviation
AP – Alkaline Phosphatase
T-bili – T billirubin
CK – Creatine kinase
Alb – Albumin
HAST – N-hydroxyarylamine sulfotransferases
Crea – Creatinine
Phos – Phosphorous
OC – Osteocalcin

Table 1
BW & AVL serum values for female β-actin 236

	BW *g*	*Ca* *mM*	*Na* *mM*	*K* *mM*
Transgenic				
n=	6	6	6	6
mean	23.63	1.22	138.17	3.38
SD	1.48	0.02	2.32	0.28
Wild Type				
n =	8	8	8	8
mean	23.24	1.24	140.00	3.28
SD	1.12	0.03	3.16	0.23

Table 2
Serum values for female β-actin 236

	Na *mM*	*K* *mM*	*Ca* *mM*	*AP* *U / l*	*T-Bili* *µM*	*CK* *U / l*	*Alb* *g / l*	*HAST* *mM*	*Crea* *µM*	*Phos* *mM*	*PTH* *pg / ml*	**OC** **ng/ml**
Transgenic												
n=	6	6	6	6	6	6	6	6	6	6	5	6
mean	148.22	3.90	2.19	131.83	1.90	82.00	30.83	9.62	12.17	2.75	5.92	103.40
SD	1.70	0.39	0.08	15.69	0.41	31.28	1.99	1.47	1.17	0.33	3.03	17.46
Wild Type												
n =	8	8	8	8	8	7	8	8	8	8	2	8
mean	146.94	3.59	2.18	133.25	1.80	92.00	31.56	9.21	11.88	2.79	2.83	112.75
SD	0.84	0.22	0.04	9.51	0.30	23.16	2.03	0.72	0.99	0.47	3.60	31.86

Table 3
Urine values for female β-actin 236

	Na / Crea **mM / mM**	**K / Crea** **mM / mM**	**Ca / Crea** **mM / mM**	**Crea** **mM**	**Phos / Crea** **mM / mM**
Transgenic					
n =	6	6	6	6	6
mean	48.04	104.57	1.29	4.29	19.63
SD	10.40	61.35	0.58	1.85	5.86
Wild type					
n =	8	8	8	8	8
mean	54.26	115.49	1.49	3.97	20.86
SD	21.84	87.52	0.43	1.32	5.76

β-actin-hPHEX 237

Abbreviations

BW – Body Weight
Ca – Calcium
Na – Sodium
K – Potassium
SD – Standard Deviation
GOT – Glutamate Oxaloacetate Transaminase
AP – Alkaline Phosphatase
T-bili – T billirubin
CK – Creatine kinase
Alb – Albumin
HAST – N-hydroxyarylamine sulfotransferases
Crea – Creatinine
Phos – Phosphorous
PTH – Parathyroid hormone
OC – Osteocalcin

Table 4
BW & AVL serum values for β-actin 237

Transgenic Male	BW g	Ca mM	K mM	Transgenic Female	BW g	Ca mM	K mM
n =	8	7	7	n =	7	7	7
mean	22.95	1.24	4.33	mean	18.02	1.26	4.03
SD	2.35	0.06	0.51	SD	1.12	0.04	0.29
WT Male				**Wild type Female**			
n =	6	6	6	n =	3	3	3
mean	25.13	1.23	4.35	mean	20.68	1.25	4.17
SD	1.65	0.07	0.46	SD	1.05	0.03	0.23

Table 5
Serum values for β-actin 237

	Na	*K*	*Ca*	*GOT*	*AP*	*CK*	*Alb*	*HAST*	*Crea*	*Phos*	*PTH*	OC
Trans. Male	mM	mM	mM	U / l	U / l	U / l	g / l	mM	µM	mM	pg / ml	ng/ml
n =	7	7	7	7	7	7	7	7	7	7	7	7
mean	157.01	2.23	1.12	39.71	75.57	64.00	14.60	5.18	10.29	1.42	27.35	11.16
SD	2.62	0.13	0.04	30.11	31.64	122.74	0.53	0.94	0.76	0.18	11.99	3.70
WT Male												
n =	6	6	6	6	6	6	6	6	6	6	6	6
mean	156.10	2.47	1.11	25.00	60.83	27.83	13.59	4.95	10.17	1.46	32.85	12.50
SD	2.49	0.38	0.02	16.71	20.70	39.92	0.71	1.62	1.72	0.16	15.91	4.46
Trans. Female												
n =	7	7	7	7	7	7	7	7	7	7	7	7
mean	157.73	2.41	1.13	30.57	77.14	16.14	16.32	4.65	12.00	1.32	13.80	13.96
SD	2.38	0.32	0.05	17.08	29.11	8.84	1.74	1.65	1.91	0.32	6.41	7.65
WT Female												
n =	3	3	3	3	3	3	3	3	3	3	2	3
mean	158.47	2.31	1.07	35.00	52.67	19.33	14.93	4.72	10.00	1.20	10.39	8.13
SD	1.97	0.33	0.01	18.68	11.93	18.58	1.34	0.30	1.00	0.15	7.09	2.42

β-actin-hPHEX 237

Table 6					
Urine values for β-actin 237					
	Na / Crea	K / Crea	Ca / Crea	Crea	*Phos / Crea*
	mM/mM	mM/mM	mM/mM	mM	*mM/mM*
male transgenic					
n =	8	8	8	8	8
mean	39.09	47.76	1.21	4.38	21.54
SD	23.95	16.98	0.62	3.55	12.49
male wild type					
n =	6	6	6	6	6
mean	38.33	58.69	1.42	5.26	19.07
SD	20.32	22.75	0.90	4.88	13.24
female transgenic					
n =	7	7	7	7	7
mean	44.73	54.84	1.77	2.36	23.77
SD	16.39	14.54	0.34	0.98	7.59
female wild type					
n =	3	3	3	3	3
mean	31.90	70.80	1.34	2.76	11.14
SD	11.17	66.51	1.59	1.21	5.52

Rescue Mice

Abbreviations

BW – Body Weight
Ca – Calcium
Na – Sodium
K – Potassium
SD – Standard deviation
GOT – Glutamate Oxaloacetate Transaminase
AP – Alkaline Phosphatase
T-bili – T billirubin
CK – Creatine kinase
Alb – Albumin
HAST – N-hydroxyarylamine sulfotransferases
Crea – Creatinine
Phos – Phosphorous
PTH – Parathyroid hormone
OC – Osteocalcin

Table 7
BW and AVL serum values for rescue mice

Genotype	BW g	Ca mM	K mM
Hyp			
n =	4	4	4
mean	16.30	1.19	3.70
SD	1.92	0.12	0.42
rescue			
n =	8	8	8
mean	22.49	1.22	4.10
SD	2.86	0.09	0.60
wild-type			
n =	8	8	8
mean	24.97	1.22	4.15
SD	1.07	0.03	0.41

Table 8
Serum values for rescue mice

Genotype	*Na* mM	*K* mM	*Ca* mM	*GOT* U/l	*T-Bili* µM	*CK* U/l	*Alb* g/l	*HAST* mM	*Crea* µM	*Phos* mM	*PTH* pg/ml	OC ng/ml
Hyp												
n =	3	3	3	3	3	3	3	3	3	3	3	4
mean	143.17	2.16	1.05	22.33	1.56	37.67	16.36	3.27	5.33	0.86	70.17	15.25
SD	1.40	0.13	0.13	4.51	0.35	27.54	1.21	0.41	0.58	0.18	48.66	6.55
rescue												
n =	7	7	7	7	7	7	7	7	7	7	7	10
mean	143.34	2.35	1.05	17.71	1.33	47.29	16.05	4.47	7.00	0.98	34.80	14.89
SD	3.78	0.53	0.02	8.24	0.59	65.18	1.30	0.91	1.00	0.13	25.19	9.43
wild-type												
n =	8	8	8	8	8	8	8	8	8	8	8	8
mean	144.93	2.02	1.05	13.00	1.53	20.00	15.35	3.86	6.88	1.42	16.33	11.33
SD	3.39	0.15	0.03	2.73	0.30	6.93	0.63	0.48	1.13	0.13	13.31	2.56

Rescue mice

Table 9
Urine vales for rescue mice

Genotype	Na / Crea mM/mM	K / Crea mM/mM	Ca / Crea mM/mM	Crea mM	P / Crea mM/mM
Hyp					
n =	3	3	3	3	3
mean	53.55	69.83	1.44	1.97	22.89
SD	5.86	6.90	0.36	0.83	9.72
rescue					
n =	5	5	5	5	5
mean	48.00	54.91	0.78	3.06	27.07
SD	7.99	18.44	0.33	0.76	8.44
wild-type					
n =	8	8	8	8	8
mean	63.08	68.76	1.36	1.96	11.95
SD	24.04	10.15	0.73	0.66	4.26

4.5 Assay of bone mineral density

An analysis of the bone mineral density revealed insignificant effects of hPHEX transgene in both the 236 and 237 lineages. Measurements of the femoral shaft and femoral metaphysis showed the mineralized bone densities, areas and cortical thickness remained constant (Tables 10 - 11 and figures 26 - 29).

4.5.1 Female β-actin-hPHEX 236

Table 10

Femoral shaft

		total density	total area	cortical density	cortical area	cortical thickness
	mean	699.37	1.772	1193.98	0.910	0.228
wild type	SD	26.58	0.069	17.32	0.042	0.010
	mean	700.30	1.784	1199.31	0.914	0.227
transgenic	SD	27.90	0.067	21.11	0.032	0.008

Femoral Metaphysis

		total density	cortical subcort. density	trabecular density	total area	trabecular area	Cortical subcort. area
	mean	558.08	792.82	224.97	3.706	1.391	2.314
wild type	SD	23.40	23.91	25.66	0.103	0.171	0.141
	mean	556.51	803.34	215.84	3.637	1.407	2.228
transgenic	SD	23.35	26.92	25.65	0.126	0.214	0.223

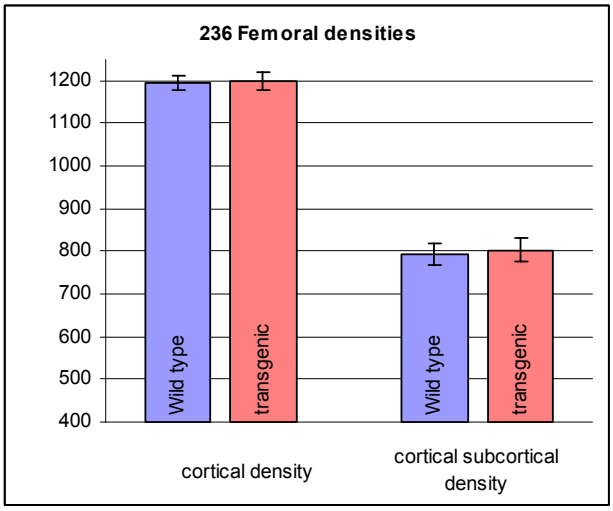


Figure 26
Shaft cortical density and metaphysis cortical sub-cortical density comparisons.

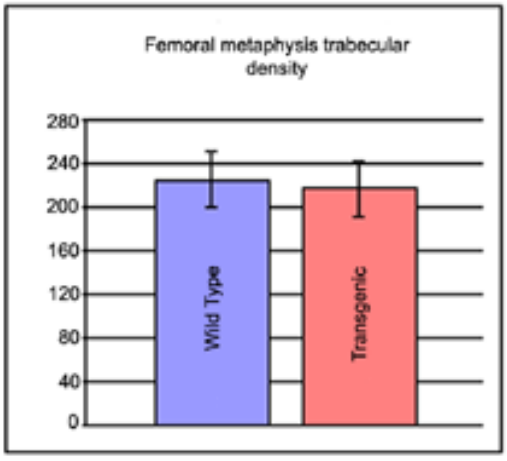


Figure 27
Metaphysis trabecular density.

4.5.2 β-actin-hPHEX 237

Table 11

Femoral shaft

		total density	total area	cortical density	cortical area	cortical thickness
Female wild type	mean	678.20	1.813	1171.80	0.920	0.226
	SD	34.14	0.076	41.57	0.035	0.007
Female transgenic	mean	639.51	1.687	1120.10	0.821	0.207
	SD	59.47	0.151	72.42	0.099	0.020
Male wild type	mean	605.67	1.881	1112.93	0.870	0.207
	SD	39.24	0.058	41.14	0.059	0.014
Male transgenic	mean	584.85	1.912	1104.85	0.848	0.198
	SD	17.35	0.099	28.47	0.053	0.009

Femoral Metaphysis

		total density	cortical subcort. density	trabecular density	total area	trabecular area	Cortical subcort. area
Female wild type	mean	563.46	832.26	214.18	3.620	1.504	2.119
	SD	29.10	23.45	9.79	0.166	0.123	0.161
Female transgenic	mean	508.11	750.04	219.56	3.473	1.535	1.939
	SD	44.02	82.20	36.94	0.218	0.124	0.202
Male wild type	mean	470.26	680.86	263.18	3.982	1.883	2.101
	SD	17.48	42.86	24.77	0.165	0.153	0.198
Male transgenic	mean	436.36	695.27	226.16	3.879	2.068	1.812
	SD	20.66	32.84	24.86	0.192	0.125	0.194

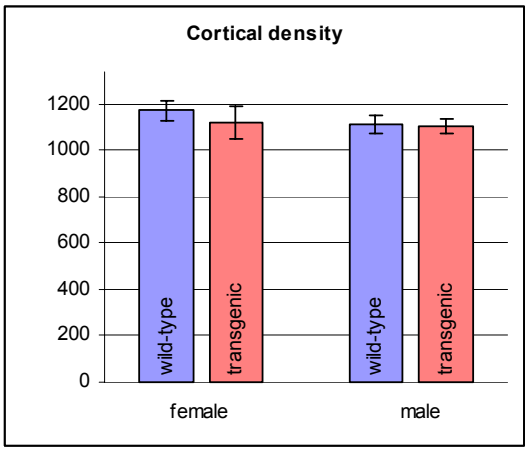


Figure 28
Femoral shaft cortical density for both genders

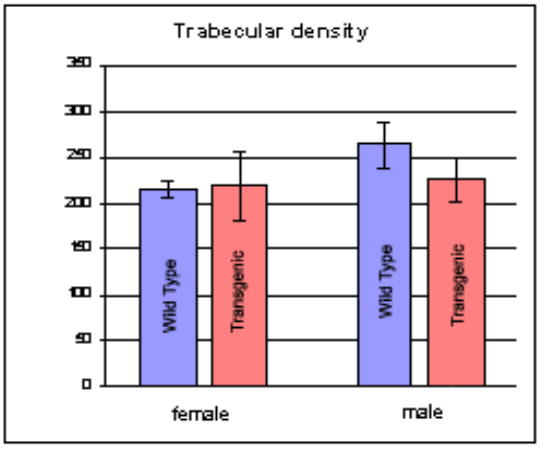


Figure 29
Femoral metaphysis trabecular density for both genders

4.5.3 Bone density is increased in rescue mice

Once again the analysis of the bone mineral density of the rescue mice showed significant normalization from the Hyp mutant toward wild-type mice. Femoral shaft measurements for total density increased in rescue mice while total area was insignificantly changed (figures 30 - 31). Values for cortical density, area and thickness all displayed significant changes toward normalization (table 12 and figures 32 - 33).

table 12 **Femoral shaft**

		total density	total area	cortical density	cortical area	cortical thickness
Hyp	mean	369.10	2.060	835.30	0.620	0.130
	SD	24.51	0.070	28.63	0.040	0.010
Rescue	mean	529.15	2.310	1027.46	0.980	0.210
	SD	59.79	0.310	76.01	0.210	0.030
Wild-type	mean	598.13	1.99	1116.94	0.91	0.21
	SD	20.79	0.09	24.8	0.04	0.01

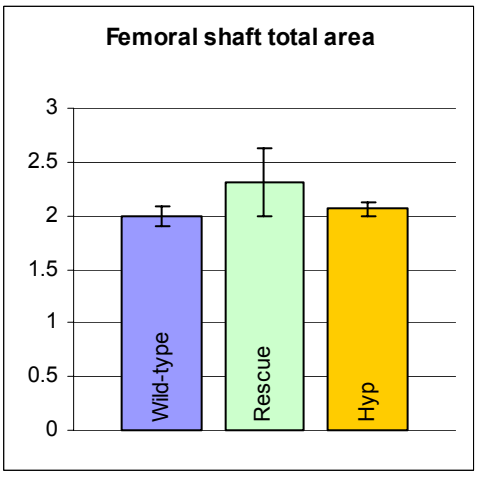


Figure 30
Femoral shaft total area

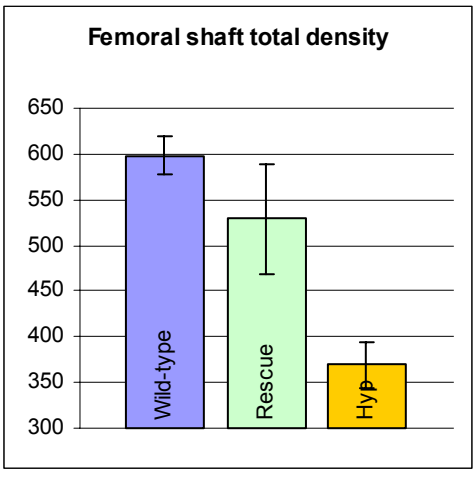


Figure 31
Femoral shaft total density

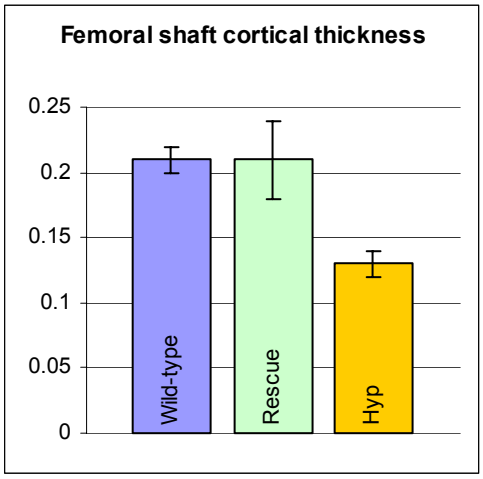


Figure 32
Femoral shaft cortical thickness

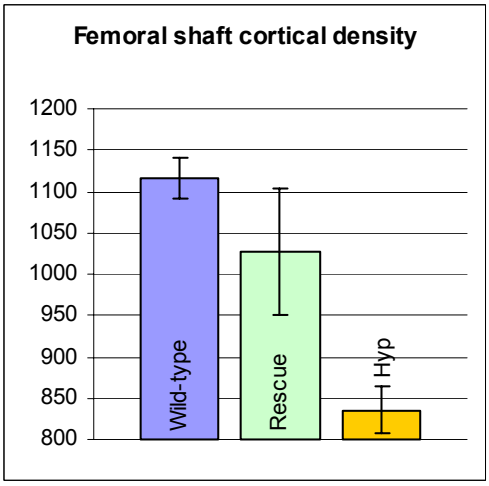


Figure 33
Femoral shaft cortical density

Rescue Mice Femoral Metaphysis

In an examination of the metaphysis, significant changes were noted in the total density, cortical-subcortical density and trabecular density (table 13 and figures 34 - 36)

Table 13

Femoral Metaphysis

		total density	cortical subcort. density	trabecular density	total area	trabecular area	Cortical subcort. area
Hyp mice	mean	288.68	584.41	110.69	3.752	2.323	1.430
	SD	4.76	26.58	20.30	0.633	0.385	0.283
Rescue mice	mean	383.06	644.15	180.01	4.616	2.530	2.085
	SD	51.52	27.23	42.45	0.656	0.199	0.501
Wild-type	mean	424.09	696.72	221.20	3.645	2.060	1.584
	SD	15.40	17.12	13.46	0.093	0.099	0.117

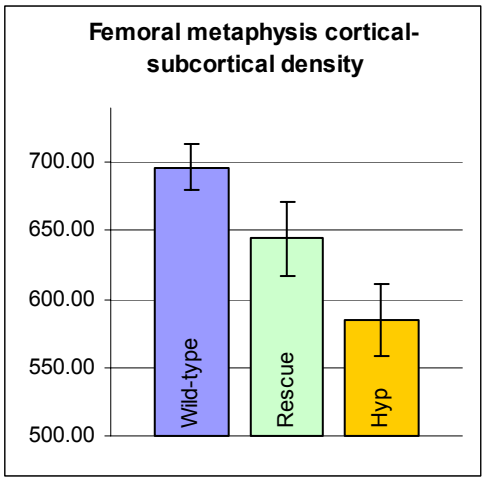


Figure 34
Comparison of rescue mice with Hyp and wild-type mice metaphysis cortical sub-cortical density

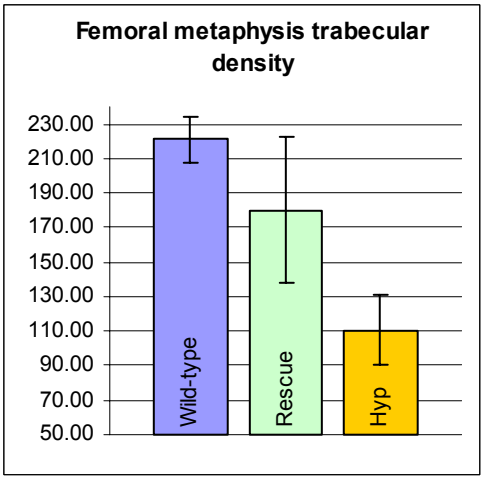


Figure 35
Comparison of rescue mice with Hyp and wild-type mice metaphysis trabecular density

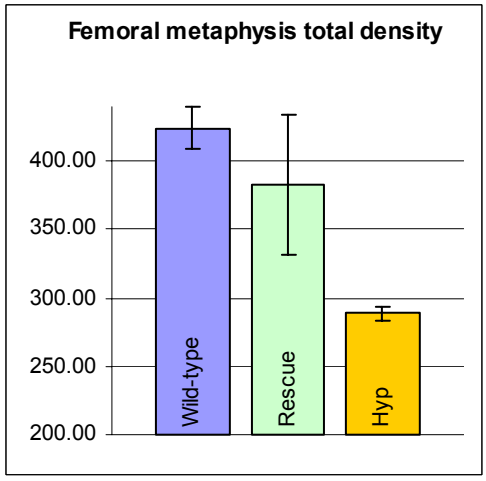


Figure 36
Comparison of rescue mice with Hyp and wild-type mice metaphysis total density

4.6 Histological examination of femurs

The femoral epiphysis of the 236, 237 was stained for mineralized bone (black in figures 37,40) with the Van Kossa/McNeal technique. Gross examination of the sections reveals no difference between wild-type and transgenic samples. TRAP

staining of sections from 236 β-actin hPHEX mice and wild-type littermates indicate no difference in osteoclast proliferation (figure 38, arrows). Calcein staining of sections with a time delay of three days between injections allowed the rate of bone remodeling to be visualized. Sections from the 236 and 237 transgenic animals appear identical to wild-type sections, indicating a similar remodeling capacity (figures 39, 41 - 42).

Staining of rescue mice sections for mineralized bone in the growth plate and the secondary spongiosa clearly reveals better, though still somewhat abnormal, organization of chondrocytes and the growth plate compared to Hyp sections (figure 43 -44). Fluorochrome staining (figure 45) displays an improvement of bone remodeling in rescue mice over Hyp mice, but clearly not as defined as seen in the wild-type femur.

β-actin-hPHEX 236

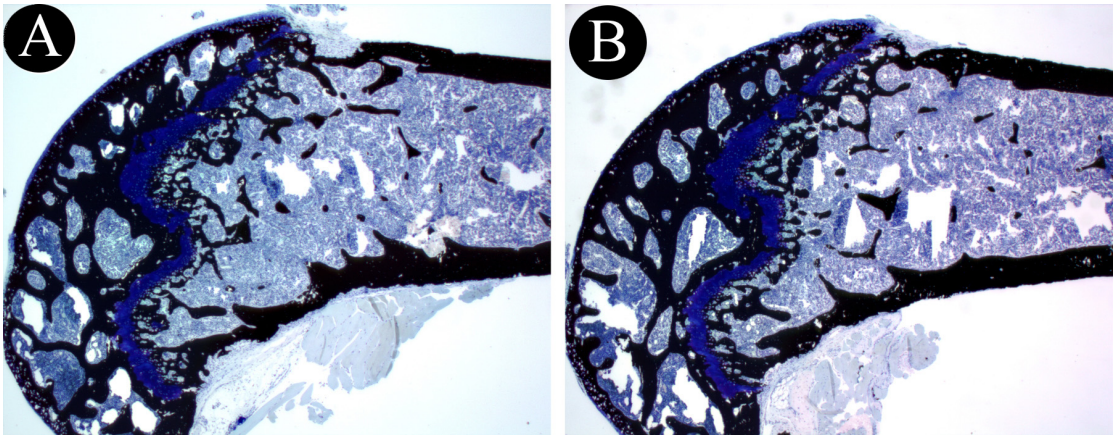


Figure 37: 12 week old mouse femur epiphysis stained for mineralized bone using von Kossa/Mac Neal protocol.
2.5X. A)wild-type B)transgenic

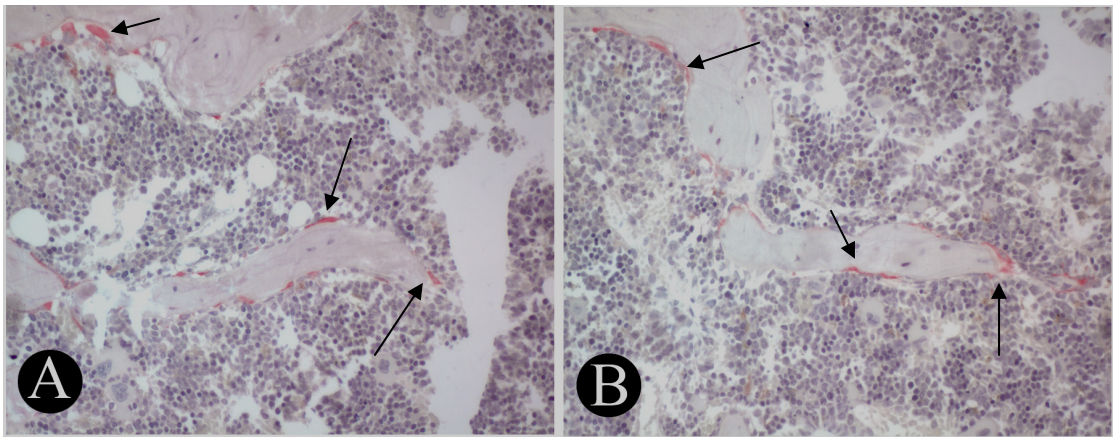


Figure 38: 12 week old mouse femur stained using TRAP protocol (osteoclasts indicated by arrows). 20X. A)wild-type B)transgenic

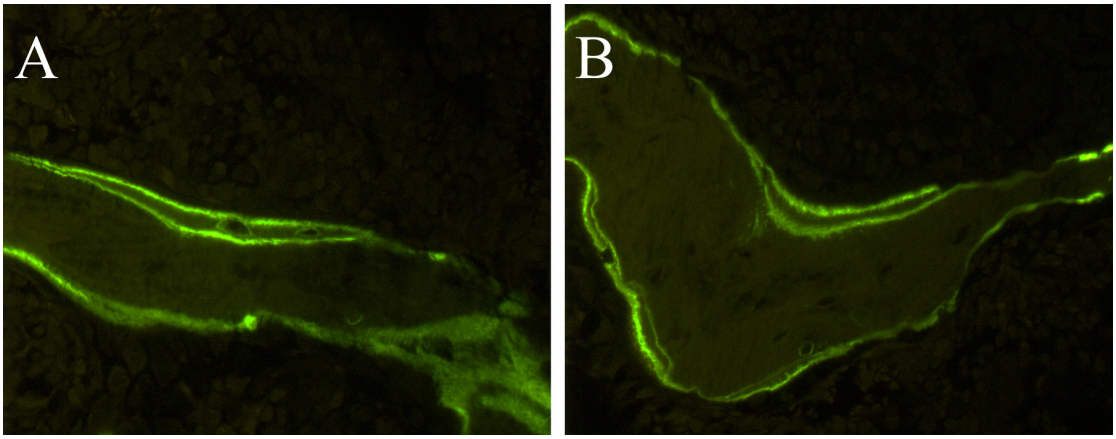


Figure 39: Fluorochrome (calcein) labeling in femor of 12 week old mouse, viewed under blue excitation. 40X. A) wild-type B) transgenic

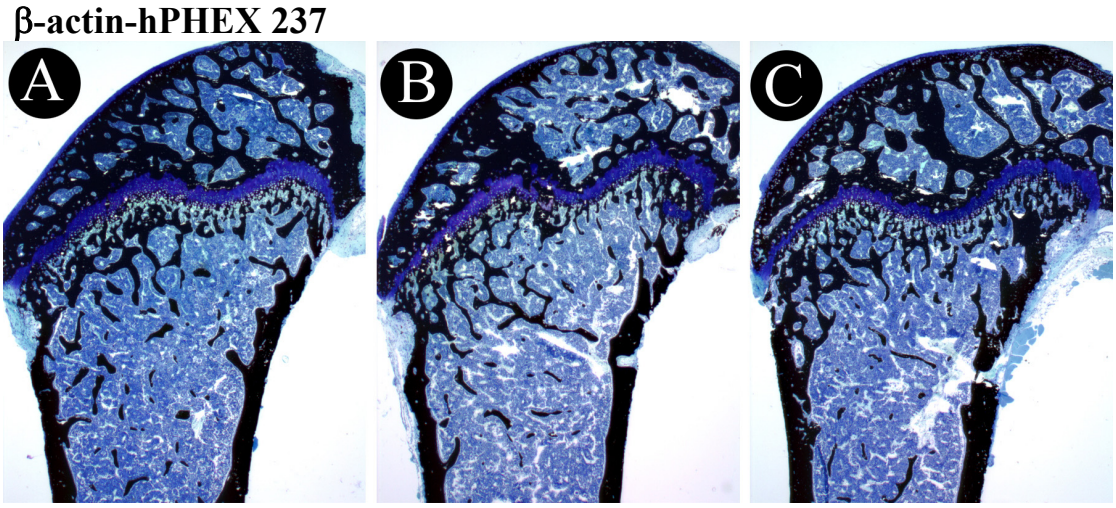


Figure 40: 12 week old mouse femur epiphysis stained for mineralized bone using von Kossa/Mac Neal protocol.
2.5X. A)male wild-type B) male transgenic C) female transgenic

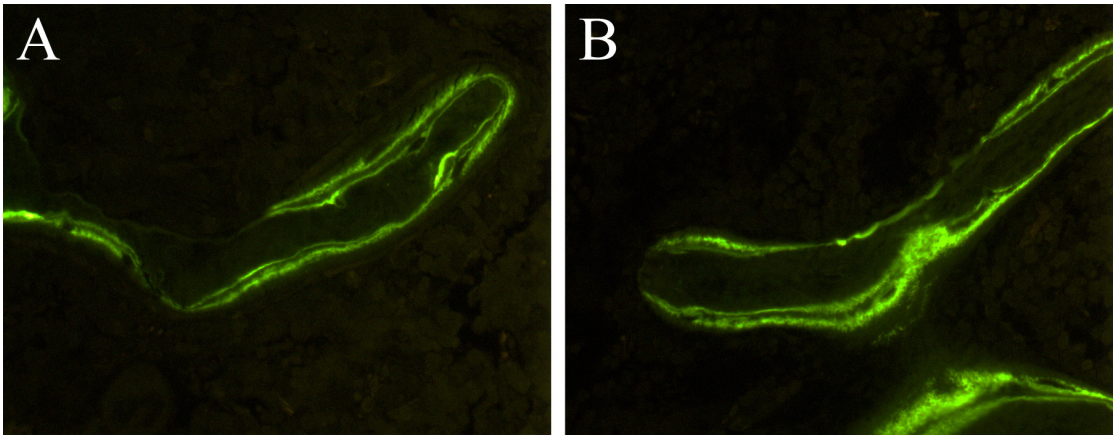


Figure 41: Fluorochrome (calcein) labeling in femor of 12 week old mouse, viewed under blue excitation.
40X. A) male wild-type B) male transgenic

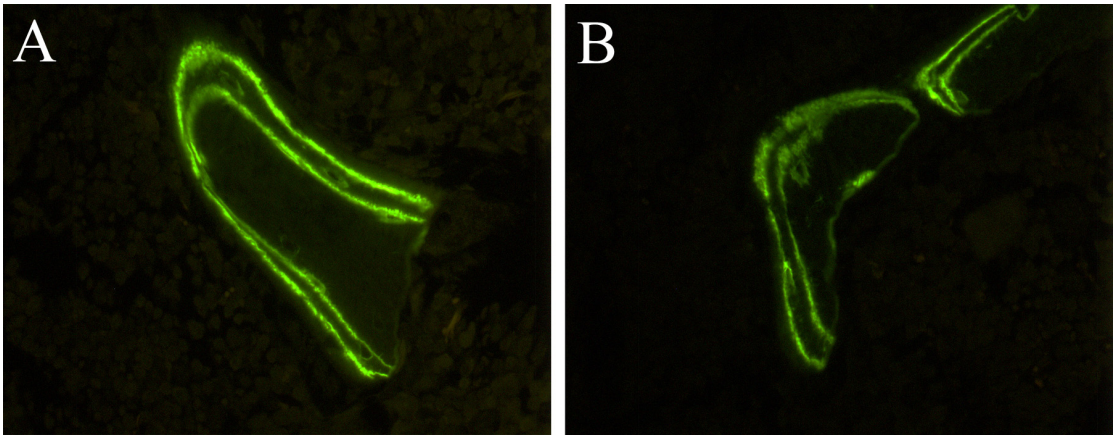


Figure 42: Fluorochrome (calcein) labeling in femor of 12 week old mouse, viewed under blue excitation.
40X. A) female wild-type B) female transgenic

Rescue mice

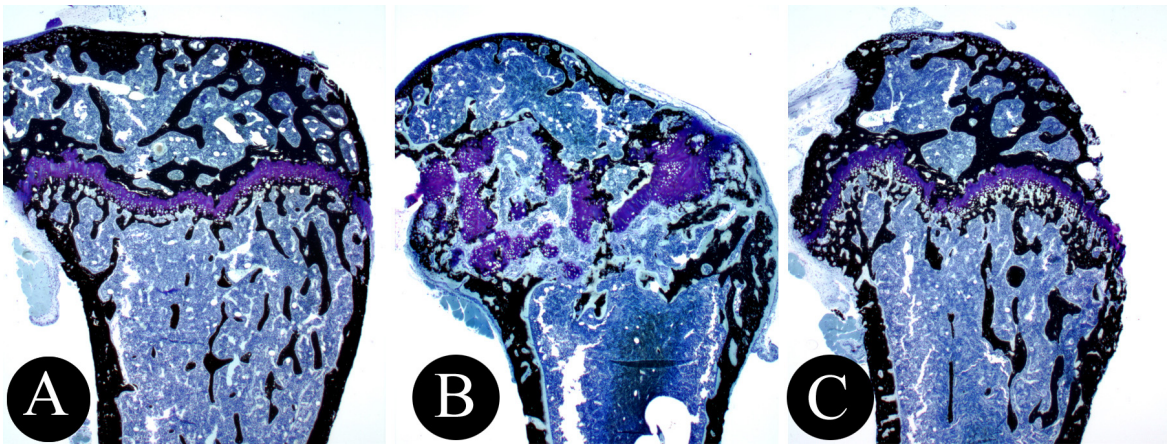


Figure 43: 12 week old mouse femur epiphysis stained for mineralized bone using von Kossa/Mac Neal protocol.
2.5X. A)wild-type B) Hyp C) rescue

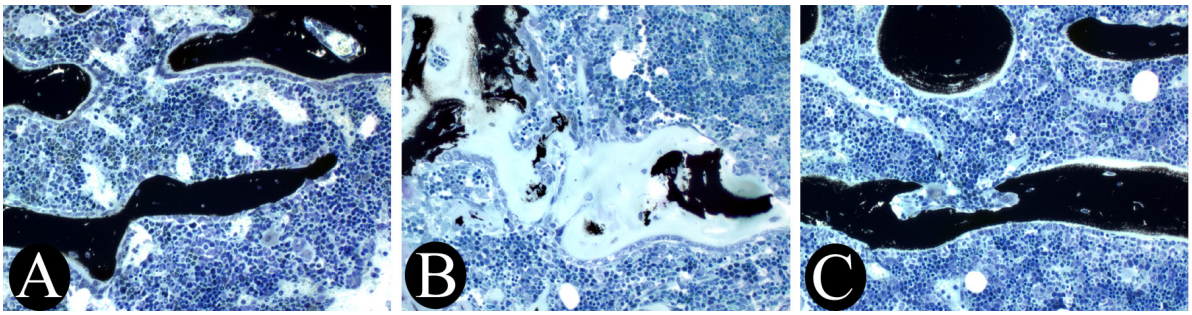


Figure 44: 12 week old mouse femur secondary spongiosa stained for mineralized bone using von Kossa/Mac Neal protocol.
20X. A)wild-type B) Hyp C) rescue

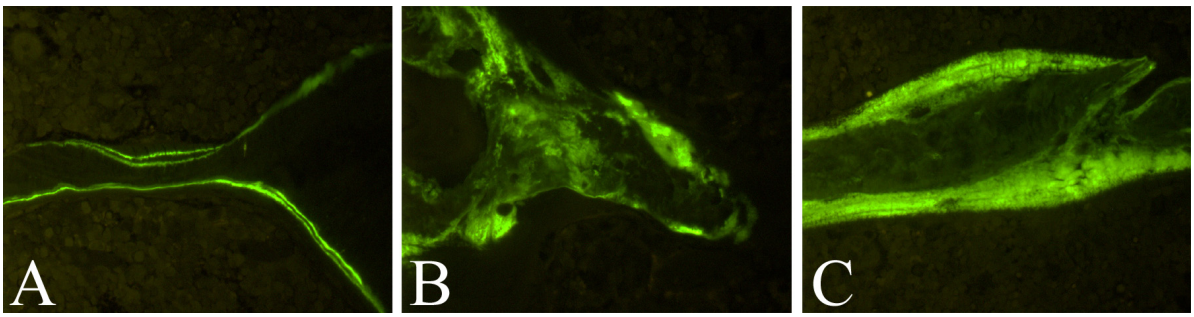


Figure 45: Fluorochrome (calcein) labeling in femor of 12 week old mouse, viewed under blue excitation.
40X. A)wild-type B) Hyp C) rescue

5 Discussion

The pathways leading to phosphate homeostasis are affected by many factors and involve varied and complicated feedback loops all of which insure a stable concentration of phosphate available throughout the microcellular landscape. The aim of this doctoral work has been to characterize mice overexpressing the phosphate regulating gene with homologies to endopeptidases on the X chromosome (PHEX). We also analyzed the outcome of a cross between the newly created human PHEX (hPHEX) transgenic mice and Hyp mice carrying a spontaneously mutated copy of the mouse PHEX (mPHEX)gene.

PHEX has 95% DNA similarity between human and mouse genomes and shares the same exon organization. PHEX is a gene crucial to renal phosphate reabsorption and phosphate homeostasis in general, as well as playing a role in the mineralization of bone. It has been identified as the causative factor in the human disorder X-linked hypophosphatemia (XLH), a disordered primarily defined by its low serum phosphate levels. Patients with XLH suffer from hypophosphatemia as well as other phenotypical abnormalities (rickets, shortened stature, etc.) due to deletions or nonsense mutations in the carboxy terminal end of the PHEX gene. These mutations lead to the ablation of the extracellular catalytic domain. A spontaneous mutation in mouse has been characterized and shares the same physiological traits as human XLH patients. The abnormalities inherent in this mutant mouse, labeled Hyp for hypophosphatemia, have also been attributed to the (mPHEX) carboxy terminus being truncated between exon 15 and 16. Hypophosphatemia studies involving parabiosis between Hyp and wild-type (WT) mice compared to WT with WT pairings indicated

a circulating factor is involved in the phosphate regulating pathway (Meyer et al, 1989). This factor was named phosphatonin. Further hypophosphatemia studies involving renal transplant between Hyp and WT mice proved there was no intrinsic defect in the kidneys of Hyp mice (Nesbitt et al.,1992). It has been shown that mRNA and protein levels of the Na^+ dependent phosphate transporter type II (Npt2), which is expressed in the proximal tubule of the kidney, are compromised by 50% in Hyp mice (Tenenhouse et al, 1994). This reduction is ultimately responsible for hyperphosphaturia (Levi, 2000).

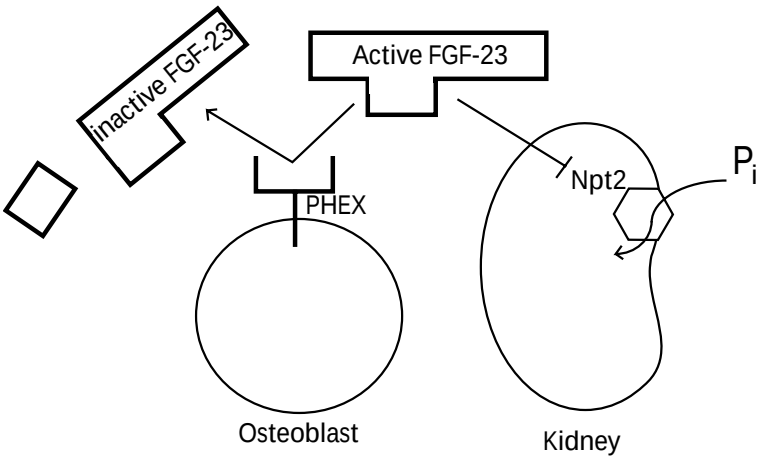
Secondary to hypophosphatemia, osteoblasts in Hyp mice exhibit an intrinsic mineralization defect. Ecarot-Charrier et al. (1988) were able to show that the ability of the Hyp osteoblasts to mineralize osteoid (unmineralized bone matrix) is seriously compromised *in vivo*. Hyp periosteal cells produced a matrix with an increased osteoid thickness when transplanted in normal gluteal muscle compared to wild-type periosteal cells transplanted into the same environment. In addition, the secondary mouse PHEX (mPHEX) defect allows for the accumulation of an unknown factor that inhibits osteoid mineralization *in vitro*. These studies indicate the production of a mineralization inhibitory factor by Hyp osteoblasts (Xiao et al., 1998).

PHEX expression has been identified predominantly in osteoblasts, and odontoblasts (Ruchon et al., 1998). Expression levels are seven fold higher in bone than found in lung, muscle or ovary, with no expression detected in the kidney. The specific substrate(s) for PHEX endopeptidases activity have thus far not been fully identified and/or characterized. PHEX endopeptidase activity has been verified by its ability to cleave several non-specific substrates to include PTH (1-34), PTH (1-38), FGF-23,

ZAAL-pNA and [Leu]enkephalin, of which the latter two are specific substrates of related endopeptidases. It remains to be conclusively determined, but FGF-23 could be a PHEX specific substrate. FGF-23 has been recently identified as the causative factor in the disease autosomal dominant hereditary rickets (ADHR). FGF-23 is a 32kDA circulating protein whose expression has only been identified in tumors causing tumor induce osteomalacia (TIO) and the forebrain (Shimada et al., 2001). In ADHR patients, FGF-23 contains a mutation (ΔFGF-23) in its cleavage site which negates endopeptidase activity. Cleavage studies indicate PHEX is unable to cleave ΔFGF-23. If PHEX tertiary folding is similar to that of the related and already crystallized NEP structure, then with its small size, FGF-23 would likely fit into the catalytic pocket formed by PHEX (Oefner, 2000) In further studies FGF-23 was cleaved by PHEX and demonstrated to inhibit renal phosphate cotransport (Bowe et al., 2001)

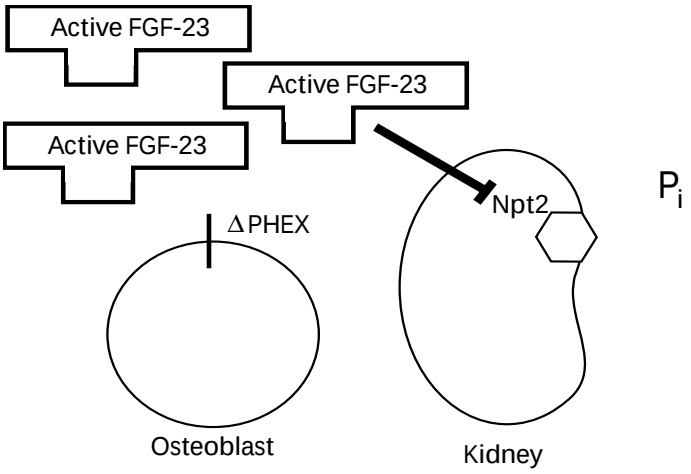
From the evidence presented so far, the following models for regulation of phosphate resorption were established.

Figure 46: Normal phosphate resorption



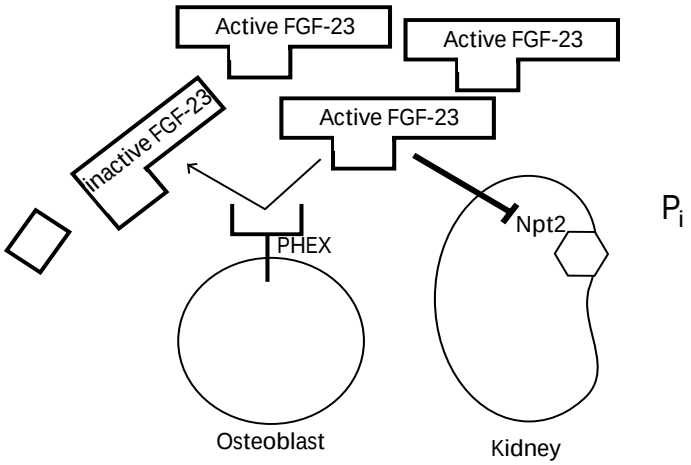
Normal : Phosphate is resorped principally through the actions of Npt2 in the proximal tubules of the kidney. Npt2 is negatively regulated through a pathway dependent on the circulating levels of active FGF-23. The levels of active FGF-23 are regulated by the inactivating endopeptidase activity of PHEX (figure 46).

Figure 47: XLH / Hyp disorder



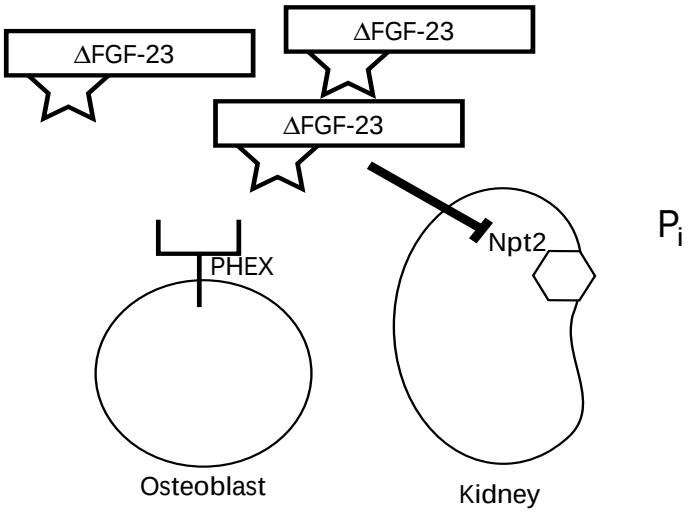
XLH / Hyp : In Hyp mice and XLH patients, the catalytic domain of PHEX is destroyed, and the resultant buildup of active FGF-23 causes a drastic reduction in Npt2 levels which results in the development of hypophosphatemia (figure 47).

Figure 48: TIO / OHO disorder



TIO / OHO : The supraphysiological levels of active FGF-23 caused by tumors overwhelm the endopeptidase activity of PHEX, which depletes Npt2 levels and allows hypophosphatemia to develop. The complete removal of the tumors returns the active FGF-23 levels to normal and hypophosphatemia is alleviated (figure 48).

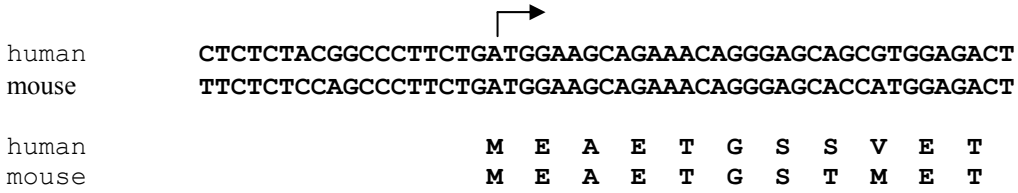
Figure 49: ADHR disorder



ADHR : FGF-23 in mutated in such a way as to obstruct cleavage by the endopeptidase while retaining its activity in the regulatory pathway of Npt2. Npt2 expression levels are again reduced and hypophosphatemia results (figure 49).

In an examination of the PHEX translation start site, it is unusual to find a pyrmidine instead of a purine residue at position -3. In higher vertebrates 97% of translation start sites conform to the classic Kozak sequence (Kozak, 1991). The other three percent, to include PHEX, are believed to be strongly regulated genes that involve tight post-transcriptional control (figure 50).

Figure 50 Translational start site of human and mouse PHEX.



A post-tranlational modification consensus sequence, CXXX box motif, is located at position 746 – 749 (CRLW) of the carboxy terminus in both human and mouse sequences. This consensus sequence has been identified as a possible site of prenylation, which is involved in a number of different processes to include regulation of protein function and protein-protein interactions. (Lipmann et al., 1998).

To date there have been few transgenic or knockout mouse models that are characterized by a biochemical change in the phosphate homeostasis. Since the lack of PHEX leads to such a significant drop in serum phosphate levels of XLH patients and in the Hyp mouse, it would be reasonable to expect that an increase in the expression of PHEX could also alter the serum phosphate levels.

Many endopeptidases point of effect is within the tissues of greatest expression. Therefore, the osteoblast specific promoter for the osteocalcin gene (OG2) was used in the creation of the first transgenic construct. Osteocalcin is the only gene thus far identified as being expressed exclusively in mature osteoblasts and odontoblasts. The 1.3kb of mOG2 5'flanking region contains two osteoblast nuclear protein binding sites that act as cis-acting transcriptional elements for increased expression (Ducy and Karsenty, 1995). The osteoblast specific transgene was successfully shown to produce a protein *in vitro* and the pronuclear injection of this transgene created three

different transgenic lines of mice carrying the hPHEX gene. These transgenic lineages displayed between one and four integration sites of hPHEX. Expression studies in all three transgenic lineages showed negative results for the transcription of mRNA species or the subsequent translation of the hPHEX protein. The transgenic construct may be inhibited from being expressed due to its site of integration, or some form of genomic transcriptional control elements around the sites of integration.

Liu et al. (2002) were able to produce multiple PHEX transgenic lineages using the same principle transgene construction strategy. Male PHEX were bred with heterozygous female Hyp mice and the offspring analyzed. The Hyp/PHEX offspring obtained from the matings displayed persistent hypophosphatemia but a significant improvement in the osteoblast mineralization defect was detected (Liu et al., 2002), similar to what we found in our β-actin-hPHEX 236 /Hyp offspring.

β-actin is a ubiquitously expressed gene with relatively high expression levels in all non-muscle cell types. The β-actin promoter has been used successfully to create transgenic mice with ubiquitous expression patterns of multiple genes. The β-actin 5' flanking region as well as 5' untranslated region and intervening sequence I (IVSI) was used to provide the promoter activity for the transgene. The inclusion of these sequences allows for gene expression in muscle cells as well as non-muscle cell types. The exclusion of muscle cells types for the expression of β-actin is conferred by sequence 3' of the IVSI region and was not included in the transgene construct (Gunning et al., 1987). A transgene was constructed using four kilobases of 5' β-actin flanking region as a promoter for hPHEX and a SV-40 poly-A tail. After

confirming transcription and translation of the transgene *in vitro*, two lines of transgenic mice were created, designated β-actin 236 and β-actin 237. The expression of the hPHEX mRNA species was confirmed by RT-PCR in multiple tissues for both lineages. Although the expression of the transgene could be seen by RT-PCR, Northern blot analysis for both lineages was negative. RT-PCR can detect much lower transcript copy number than Northern blot. Development of Northern blots of total RNA at four weeks exposure to BioMax film showed no hybridization at all (results were confirmed by multiple investigators). This was unexpected, as the β-actin gene is highly expressed as were other transgenes using this promoter. If PHEX is indeed a strongly regulated gene, then some form of pre- or post-transcriptional control could be responsible for the low hPHEX transcript numbers being detected. The makeup of the transgene or PHEX cDNA itself may have attracted inhibitory control elements or there may have been a degradation of the majority of transcripts shortly after completion of transcriptional events.

Due to the relatively low expression levels of hPHEX, it was not surprising to find that the biochemical phenotype of the transgenic mice was almost unchanged from that of the wild-type controls. If hPHEX is indeed an inactivating endopeptidase as we hypothesize, an increase in serum phosphate levels would have been expected. The largest increase in serum phosphate levels (0.12 mM) seen in β-actin-hPHEX 237 female mice was not statistically significant. Increased expression of PHEX in osteoblasts of the β-actin-hPHEX 236 and β-actin-hPHEX 237 transgenic lineages did not affect the bone mineralization as measured in bone mineral density studies (pQCT). Histological sections of the distal femur also did not show differences

between transgenic animals and corresponding controls. One or more of the other factors in the phosphate regulatory pathway may compensate for the slightly increased endopeptidase activity of hPHEX in our transgenic lineages. It is also possible that the levels of overexpression found in the β-actin-hPHEX 236 and β-actin-hPHEX 237 lineages were not sufficient to produce a physiological response. The transcription factor Sox-9 is crucial to chondrogenesis and has been shown to influence PHEX promoter activity with up to 6 fold increases of PHEX expression in co-culture experiments. Also identified in the 2736 base pair promoter region of the mPHEX gene were 32 consensus cis-acting elements that are conserved between mouse and human (Liu et al., 2001). By replacing the hPHEX promoter with the OG2 and β-actin promoters we may have disrupted potential binding by these elements that could be essential for the correct transcription or function of the hPHEX protein in vivo.

To further investigate if there is a possible effect in the re-expression of hPHEX in the Hyp mouse where serum phosphate levels are reduced, the β-actin-hPHEX 236 transgenic lineage was crossed with Hyp mice and the offspring (rescue mice) characterized. Rescue mice were the result of a male hPHEX transgenic animal mated with a homozygous Hyp female.

From gross examination it was obvious that the rescue mice had improved body weight and long bone formation. However, in the analysis of the biochemical data, there was no significant change in the different values that were examined. Phosphate was increased from the low levels found in Hyp mice, but not at a significant level. Body weight from the Hyp mice was 65% of the wild-type mice whereas the rescue

mice body weight was 89% of the wild-type controls. The increase in body weight was associated with an increase in bone mineral density as measured by pQCT.. The rescue mice femurs displayed a marked increase in the longitudinal growth and mineralization of femurs. pQCT data indicated that bone mineral density in both the femoral shaft and the metaphysis was significantly improved relative to Hyp mice. Von Kossa/MacNeal stained histological sections of rescue mice compared to Hyp and wild-type sections clearly show the reorganization of the rescue growth plate (figure 43 - 44). Fluorochrome labeling of rescue mice sections displayed more organized bone formation, but compared to the wild-type sections still abnormal. Hyp sections contrarily reveal a very disorganized or jumbled calcein marking (figure 45). These results indicate that although the deletion mutation of PHEX in the Hyp mouse accounts for two major phenotypical changes, they are partially separate events. Hypophosphatemia caused by the lack of functional PHEX is not solely responsible for the mineralization defects in the osteoblasts. The overexpression of hPHEX within the microenvironment of the osteoblasts in our transgenic mice has an effect on a substrate or pathway that to date has not been identified. However, it can not be discounted that the ubiquitous expression of hPHEX in the β-actin-hPHEX 236 lineage has influenced the mineralization of the osteoid from somewhere other than the osteoblast microenvironment.

5.1 Conclusion

Our creation of osteoblast specific hPHEX transgenic mice has been unsuccessful using the current transgene construct therefore a different strategy must be developed. The future strategy may be to use multimers of the cis-acting transcriptional elements identified in the mOG2 5' flanking region. Using multimers of these two regions has produced greater than 200 fold increased expression *in vitro* compared to single copy constructs (Ducy et al., 1995).

β-actin-hPHEX 236 and β-actin-hPHEX 237 transgenic lineages expressed the hPHEX gene across multiple tissues as is consistent with the β-actin promoter region used. The transgenic overexpression of hPHEX produced surprisingly low levels of hPHEX. This minor **over**expression was unable to influence serum phosphate levels or significantly change any of the other biochemical parameters involved in phosphate homeostasis. However, the transgene was able to partially rescue the intrinsic defect identified in Hyp osteoblasts, and mirrored in XLH patients, as seen in rescue mice created through a crossing of the 236 β-actin hPHEX lineage with Hyp mice. The growth plate of femurs from rescued mice displayed better chondrocyte organization than Hyp controls. Despite persistent hypophosphatemia there was greater mineralization measured in both distal femur and femur shaft of rescue mice. The increased mineralization of the osteoid in the rescue mice over the Hyp mice was measured as, metaphysis total density 70%, metaphysis trabecular density 62.7% and femoral shaft cortical density 68.2% with all p values less than 0.05. It would appear that PHEX not only affects the pathway or regulatory elements for NPT2 expression and therefore phosphate resorption in the kidney, but in a separate regulatory pathway affects the mineralization of osteoid.

Future direction should be to study the osteoblast function of the rescue mice *in vitro* and *in vivo*. Further attempts for the creation of a highly overexpressing hPHEX mouse through the use of other promoters or under the influence of Sox-9 may reveal more significant variations in the biochemical data and/or abnormalities in bone growth and density.

6 Literature

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Sprachkenntnisse

Englisch, Muttersprache
Deutsch, sehr gut in Wort und Schrift

München, den 09. Mai, 2005