Introduction: The mechanism for normal bone mineralization is complex and has not been fully elucidated, however it is known to be influenced by factors such as stress, systemic factors (e.g. estrogen) and local factors such as bone morphogenic proteins and interleukins. Osteomalacia is characterized by defective bone mineralization, with increased undermineralization matrix. The PHEX gene (Phosphate encoding gene with Homologies to Endopeptidases on the X chromosome) is abnormal in X-linked hypophosphatemic rickets (XLH) aka “Phosphate Diabetes.” This manifests as hypophosphatemia, low or inappropriately normal 1,25 (OH)2 vitamin D3 (D3) levels, high serum alkaline phosphatase and osteomalacia.

PHEX is mainly expressed in osteoblasts, and is theorized to regulate anabolic and catabolic mechanisms of mineralization of the extracellular matrix1. D3 has been shown to downregulate PHEX in primary and cultured mouse osteoblasts2. PHEX deficient osteoblasts have an altered response to D3 treatment; further, D3 decreases PHEX mRNA levels and protein synthesis in normal osteoblast culture2, with preliminary evidence indicating that this decrease is at the transcriptional level3. The murine PHEX promoter has been previously cloned by our laboratory and its functionality was demonstrated in rat osteoblast-like (UMR-106) cells4. Examination of this sequence for the presence of putative cis-acting elements identified multiple binding sites, including those for estrogen and glucocorticoid receptors; a putative binding site for vitamin D receptor was not identified.

The present study was undertaken to confirm that D3 regulates PHEX at a transcriptional level both in vitro and in vivo, and to determine the promoter region responsible for this regulation.

Methods: Northern Blot Analysis: UMR-106 rat osteosarcoma cells were treated with 10−7 M D3 or EtOH for 24, 48 and 72 hours. PHEX mRNA was detected by Northern blot analysis with a PHEX specific radiolabeled probe. The blots were then stripped and reprobed with GAPDH specific probes. Hybridization signals were quantified by phosphorimage analysis. 

Real-Time PCR Analysis: 4 to 5 week old C57-BL6 mice received subcutaneous injection of 6 µg/kg body weight D3 in 100 µl of 1:4 ethanol:propylene glycol or equal volume ethanol:propylene glycol and sacrificed 24 hours post-injection. Total RNA was prepared from calvaria. PHEX expression was determined by Real-Time PCR (ABI Prism 7000 sequence detection system), using commercially available probes/primer sets. Applied Biosystems (Fig. 3).

Electrophoretic mobility shift assays (EMSA): Nuclear protein (NP) was prepared from UMR-106 cells. Synthetic oligonucleotides probes were radiolabeled and incubated with nuclear protein. For competition studies, 100x cold probe was added to the reaction. Competitors included: Comp 1, PHEX –133 to –121 bp; comp 2, PHEX –126 to –107 bps and comp 3 PHEX –111 to –87 bps. Mutant oligonucleotides were created abolishing a poly-A region (mut 1), as well as putative binding sites for Myt-1, position –102 to -96 bps, (mut 2), Bcl6, position –111 to –106 bps, (mut 3) and both Myt-1 and Bcl6 (mut 4). The reactions were electrophoresed and then exposed to x-ray film. The wild type oligonucleotide utilized for EMSAs was PHEX –134 to –83 bps.

EMSA analysis shows –133 to –121 bp as a site involved in D3 regulation. The shift seen with D3 (lane 3) was competed by excess cold probe in the regions –126 to –107 bp (lane 5) and –111 to –87/+104 (lane 6). Mutation in the poly A region did not compete (lane 7). Mutations of putative binding sites for MYT-1 and BCL6 abolished the shift (lanes 8, 9 and 10).

Discussion: Function of the PHEX gene is necessary for maintenance of bone mineralization; the Hpy mouse (with a loss of function PHEX mutation) phenotypically has XLH with osteomalacia. D3 is known to decrease PHEX mRNA, and the present study confirms our hypothesis that this decrease is through a transcriptional mechanism. Northern blot analysis and real-time PCR analysis reveal a decrease of PHEX mRNA following treatment with D3. This is evident both in vitro in UMR-106 osteoblast-like cells and importantly is recapitulated in vivo in C57-BL6 mice after D3 treatment.

Previously presented studies by our laboratory have shown that the region responsible for D3 regulation is likely between –133 and +104 bps of the PHEX 5′ flanking region5. Although no putative VDR binding site was identified in this region, EMSA analysis with competition with short or mutated oligonucleotides indicates that the –134 to –83 region may be involved in the D3 responses. Studies are planned to explore this region to determine the nature of the interaction between D3 and this area of the promoter.

Increased understanding of the interaction between PHEX and D3 will improve knowledge of mechanisms and control of bone mineralization. As a modulator of serum calcium, D3 may decrease PHEX transcription to regulate calcium levels. Eventual manipulation of this interaction may allow for control of bone mineralization. This has broad reaching importance to the care of patients with musculoskeletal disorders or injuries, and prevention of bone disease.

References:

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Figure 1. D3 treatment decreases PHEX mRNA expression in UMR-106 cells.

Figure 2. EMSA analysis shows significant decrease in PHEX expression. n=4