TRANSCRIPTIONAL EFFECTS OF PTH AND ESTROGEN TREATMENT DURING ANABOLIC BONE FORMATION

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Introduction:
In a recent study we have demonstrated the synergistic effects of PTH+E2 combination treatment in an OVX Swiss-Webster mouse model (1). To further refine this animal model we carried out transcriptional profiling to explore to what degree there are unique intracellular signaling pathways for E2- and PTH-regulated genes that underlie their separate anabolic effects in bone, or whether these two treatments share similar activation of anabolic pathways in bone.

The purpose of this study was to characterize transcriptional responses during anabolic bone formation in OVX Swiss-Webster mice, having undergone a combination treatment of 17 beta-estradiol (E2) and PTH (1-34). We utilized microarray RNA profiling to acquire detailed profiles of transcriptional regulation at several timepoints.

Material and Methods:
Animals. 90 three-month-old Swiss-Webster mice underwent either OVX or Sham operation. 60 OVX operated mice were randomly assigned to six different groups of 10 mice, 30 sham operated animals to three different groups of 10 mice. Five weeks post-OVX the mice were administered s.c. either 80 mg/kg/d of bPTH (1-34) daily, or 500 mg/kg/once a week of E2, or a combination of E2 and PTH, or vehicle for four weeks. Animals were sacrificed after week 1 ( Sham and OVX), week 5 (Sham and OVX) and after 9 weeks (Sham, or PTH, or E2, or E2 & PTH or vehicle-treated OVX).

At treatment days 4, 11, and 24, femurs were excised from vehicle- E2- or PTH-treated animals (n = 4, per RNA preparation). RNA was subsequently extracted from the diaphysis and the metaphysis of the bone samples by homogenization in TRIzol reagent. (Transitional Profiling). Gene expression profiling was performed using Affymetrix GeneChip arrays. Affymetrix Microarray Suite 4.1 was used to scan each mouse genome U74v2 oligonucleotide array chip and analyze the relative abundance of each gene from the average difference in signal intensities. Real-time quantitative RT-PCR. Gene expression was measured by Taqman real-time PCR. (Applied Biosystems, Foster City, CA). The number of PCR cycles needed to cross a threshold of a statistically significant increase in fluorescence (Ct = threshold cycle) was measured using Applied Biosystems software. Relative target gene expression was determined using the formula: Relative Expression = 2^(-ΔCt) where ΔCt = (Ct*gene – Cref gene in bone cDNA sample) –(Cttarget gene – Cref gene in mock reverse transcribed RNA sample).

Results: Genes regulated by PTH or E2 treatment in bone. For the initial analysis of microarray data, we employed strict criteria for inclusion in this candidate gene list, which were as follows: 1) all candidates had an absolute signal intensity >0.5 (where 1.0 is the median signal intensity on the chip); this criterion excludes low intensity gene signals that may exhibit large changes in regulation due to low baseline signal/noise values, 2) all candidate genes were required to exhibit upregulation at all timepoints and at both the femoral metaphysis and diaphysis; this additional criterion excludes gene candidates that may only be upregulated at either the metaphysis or diaphysis at a specific timepoint–thus our analysis may have dropped several important early-intermediate regulated genes, but also excludes false positives that were not found to be regulated at more than one bone site or experimental timepoint. Among the PTH-regulated genes of interest are e-cfos, vitamin D receptor, Phex, Rank ligand, and Cathepsin K. For the regulated genes, several intriguing candidates were consistently upregulated at all timepoints and in both metaphysis and diaphysis bone sites; this additional criterion excludes gene candidates that may only be upregulated in either the metaphysis or diaphysis at a specific timepoint–thus our analysis may have dropped several important early-intermediate regulated genes, but also excludes false positives that were not found to be regulated at more than one bone site or experimental timepoint. Among the PTH-regulated genes of interest are e-cfos, vitamin D receptor, Phex, Rank ligand, and Cathepsin K. For the regulated genes, several intriguing candidates were consistently upregulated at all timepoints and in both metaphysis and diaphysis, including Be12-associated Bag3 (an anti-apoptotic protein), BMP1 and 8a, cyclin D1, PTH1R, and Cathepsin L. In order to verify the results obtained from this iterative microarray analysis, the mRNA levels of 4 regulated genes were analyzed using real-time PCR and RNA from either PTH- or E2-treated metaphysis at 4d, 11d, and 24d. Gene expression was measured by Taqman real-time PCR. Figures 1 and 2 show Taqman results from PTH and E2 treated metaphysial RNA, respectively. Metaphysial RNA samples from PTH-treated mice consistently showed elevated RNA levels of Rank ligand (RankL) and vitamin D receptor (VDR) at all 3 timepoints tested by real time Taqman PCR, while PTH1R and Rank RNAs showed no altered expression as a result of PTH treatment (Figure 1). Metaphysial RNA samples from E2-treated mice consistently showed elevated RNA levels of PTH1R at all 3 timepoints tested by real time Taqman PCR, while RankL, VDR and Rank RNAs showed no altered expression as a result of E2 treatment (Figure 2).

Discussion:
In this study, transcriptional profiling has identified a short list of important regulatory protein s, and that real-time PCR is in agreement with microarray analysis. Among the PTH-regulated genes listed are efos, vitamin D receptor, Phex, Rank ligand, and Cathepsin K. The protooncogene e-cfos and other activating protein 1 family members are critical transcriptional mediators in bone, and knockout mice (c-fos -/-) fail to respond to intermittent PTH. There is also ample evidence that PTH increases expression of VDR and RANK. For E2 regulated genes, several intriguing candiates were consistently upregulated at all timepoints and in both metaphysis and diaphysis, including Be12-associated Bag3 (an anti-apoptotic protein), BMP1 and 8a, cyclin D1, PTH1R, and Cathepsin L. In primary OBs derived from transgenic rats harboring a dominant negative ER mutant, cyclin D1 and D2 mRNA are downregulated when compared with wildtype littermates, suggesting a role for E2 in regulating cyclins, and potentially OB proliferative rates.

This OVX Swiss-Webster mouse model offers an opportunity to introduce genetic modifications into a valid animal model for osteoporosis, thus enabling investigation of possible molecular mechanisms involved in the effect of anabolic and anti-resorptive agents on the osteoporotic skeleton. By examining site-specific transcriptional regulation at several timepoints over the course of PTH or E2 treatment, we have begun to catalog a more complete description of critical genes regulated during osteoblast and osteoclast differentiation, with the ultimate goal of identifying novel osteoblast-specific genes and critical regulators of the observed anabolic growth of bone. Our profiling results thus far confirm that important bone regulatory molecules, such as Vitamin D receptor (VDR), RANKL, PTH1R, AP-1 and Cathepsin K, exhibit upregulation in vivo in response to these treatments. These findings have been confirmed for a subset of identified genes by subsequent real-time PCR. These data increase our confidence that such a strategy can be used to build a physiologically relevant picture of the multitude of regulatory pathways involved in anabolic bone growth.

Reference: 1. von Stechow et al. ORS 2002, #337
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