Antagonist Minigenes Identify Genes Regulated by Parathyroid Hormone Through G Protein-Selective and G Protein Co-regulated Mechanisms in Osteoblastic Cells

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Abstract

Parathyroid hormone (PTH) is the major hormone regulating bone remodeling. Binding of PTH to the PTH1 receptor (PTH1R), a heterotrimeric G protein coupled receptor (GPCR), can potentially trigger multiple signal transduction pathways mediated through several different G proteins. In this study, we employed G protein antagonist minigenes inhibiting $G\alpha_s$, $G\alpha_q$ or $G\alpha_{12}$ to selectively dissect out which of these G proteins were responsible for effects of PTH(1–34) in targeted signaling and osteogenesis arrays consisting of 159 genes. Among the 32 genes significantly regulated by 24 hr PTH treatment in UMR-106 osteoblastic cells, 9 genes were exclusively regulated through G_s, 6 genes were solely mediated through G_q, and 3 genes were only controlled through G_{12}. Such findings support the concept that there is some absolute specificity in downstream responses initiated at the G protein level following binding of PTH to the PTH1R. On the other hand, 6 PTH-regulated genes were regulated by both G_s and G_q, 3 genes were regulated by both G_s and G_{12}, and 3 genes were controlled by G_s, G_q and G_{12}. These findings indicate potential overlapping or sequential interactions among different G protein-mediated pathways. In addition, two PTH-regulated genes were not regulated through any of the G proteins examined, suggesting additional signaling mechanisms may be involved. Selectivity was largely maintained over a 2 – 48 hour time period. The minigene effects were mimicked by downstream inhibitors. The dissection of the differential effects of multiple G protein pathways on gene regulation provides a more complete understanding of PTH signaling in osteoblastic cells.

Keywords

Parathyroid hormone; Osteoblast; Gene array; G protein; Minigene

1.1 Introduction

Parathyroid hormone (PTH), an endocrine factor secreted by the parathyroid glands, is the major hormone regulating bone remodeling. PTH has both anabolic and catabolic effects on bone *in vivo* mediated through its activation of the PTH1 receptor (PTH1R) expressed on osteoblastic cells. PTH1R mediates intracellular responses largely through heterotrimeric guanine nucleotide binding proteins (G proteins) and thus is a member of the superfamily of G protein coupled receptors (GPCRs). As is observed with many GPCRs, PTH1R may signal through several different G proteins in parallel, thus activating multiple signal transduction pathways [1]. The heterotrimeric G proteins are composed of three subunits (alpha (α), beta (β), and the smaller gamma (γ) subunits). Four subfamilies of G protein have been identified in humans and they are classified according to their α subunits: G_s, $G_{q/11}$, $G_{i/o}$ and $G_{12/13}$.

The best defined signaling pathway activated by PTH in osteoblastic cells is the protein kinase A (PKA) pathway, by which PTH stimulates the formation of cyclic 3′,5′-adenosine monophosphate (cAMP) through the action of the stimulatory G_s protein. PKA activated by cAMP subsequently phosphorylates transcription factors such as the activator protein-1 (AP-1) family (c-jun, c-fos), cAMP-response element-binding (CREB) protein, and Cbfa1/Runx2, thereby regulating transcription of a number of genes important to bone formation including those genes that contain an AP-1 promoter element (e.g., matrix metallopeptidase 13) or the runt domain promoter element (e.g., Bcl-2, osteocalcin, osteopontin, collagen I). Studies on gene expression profiles of PTH-regulated genes in UMR-106 cells showed that PTH(1–34) regulated several genes (transcription factor CEBP, interferon γ receptor, metallothionein-1, lumican, selenoprotein P) in the same direction as occurs during osteoblast differentiation [2]. The G_s-cAMP-PKA pathway is thought to be the dominant mechanism for the anabolic actions of intermittent PTH(1–34) on bone, these actions being mediated through increased osteoblast survival and differentiation [3]. There is also evidence for sustained activation of cAMP, mediated through an internalized PTH(1–34)/PTH1R/Gs ternary complex [4].

In addition to the G_s-cAMP-PKA pathway, binding of PTH to PTH1R also activates phospholipase C (PLC) β through G_q, leading to the formation of diacylglycerol and 1,4,5-inositol trisphosphate, which go on to activate protein kinase C (PKC) and increase intracellular free Ca^{2+}. Only a small number of genes have been found to be regulated by PTH partially or completely through the PKC pathway in osteoblastic cells, and these include insulin-like growth factor binding protein 5 (IGFBP5) and transforming growth factor (TGF) β1 [5,6]. Treatment with low concentrations of PTH promoted proliferation of UMR106 cells as a consequence of PKC-dependent stimulation of ERK and MAPK signaling and regulation of cyclin D1 [7]. Such actions suggest that the G_q-PLCβ-PKC mediated signaling pathway could be involved in PTH-induced cell proliferation.

Besides the well-defined G_s-driven PKA and G_q-driven PKC pathways, our earlier studies showed that PTH was able to activate a $G_{12/13}$-mediated signaling pathway, which stimulated RhoA/Rho kinase and phospholipase D (PLD) activities in osteoblastic cells [8,9]. Importantly, RhoA, Rho kinase and phosphatidic acid phosphatase were shown to be essential for PTH effects on PKCα translocation in UMR-106 cells [10,11]. Recently we have shown that disruption of RhoA signaling in osteoblastic cells results in loss of actin cytoskeletal elements [12] and increased osteoblastic cell apoptosis [13].

The activation of multiple signaling pathways by PTH may constitute a complex system of regulation, through crosstalk between these G protein pathways. Although the G_s-cAMP-PKA pathway is considered to be the major mechanism for transducing PTH signals, the

pathways mediated through G_q-PLC-PKC and G_{12}-RhoA-PLD may also play important roles in PTH-mediated anabolic and catabolic effects, with each pathway independently regulating unique sets of transcription factors and genes. The dual anabolic and catabolic effects of PTH in osteoblasts would thus be a sum of actions resulting from the diverse signaling cascades initiated by the different G proteins. In this study, we use selective inhibition by G protein antagonist minigenes to identify genes that are regulated specifically by one G protein-mediated pathway or another, as well as genes that are regulated by two or more G proteins, suggesting that some overlapping or sequential signaling may exist. The ability to dissect out differential effects of G protein signaling initiated by activation of PTH1R contributes to a more complete understanding of how PTH can mediate its observed opposing effects on bone.

2.1 Materials and Methods

2.1.1 Gα C-terminal minigene vectors

Gα C-terminal minigene vectors were used to block the signaling mediated by G_s, G_q, or G_{12} on gene expression. Gα C-terminal minigene vectors encode the unique COOH-terminal sequence for each G protein α subunit. Since the C-terminal sequences of Gα subunits are critical for binding to their cognate GPCRs, the minigene vectors selectively block signal transduction through a given G protein while leaving other G protein signaling pathways intact. This system is a powerful tool for dissecting out which G protein mediates a given biochemical or physiological function [9,14–17], and has been used previously in other models in which a receptor couples to multiple G proteins [18,19].

2.1.2 Cell culture, transfection and treatments

Rat osteoblastic UMR-106 cells (American Type Culture Collection, Manassas, VA) and rat osteoblastic ROS 17/2.8 cells (kindly provided by Dr. Peter Friedman, University of Pittsburgh) were transiently transfected with minigene vectors expressing C-terminal peptides of G_s, G_q, and G_{12} respectively, using Lipofectamine Plus reagents (Invitrogen, Carlsbad, CA). The parental vector pcDNA3.1, was used as an empty minigene vector (EV) control. After 24 hours, the cells were treated with PTH(1–34) at 10 nM for an additional 24 hours. The cells were then collected to extract total RNA for gene expression analysis. Transfection with GFP-tagged EV showed close to 100% transfection efficiency in both the UMR-106 and ROS 17/2.8 cells (data not shown). Pharmacological inhibitors were the PKA inhibitor protein kinase A inhibitor 14–22 amide (PKI) (Calbiochem, San Diego, CA), the PLC inhibitor U-73122 (Calbiochem), and the geranylgeranyl transferase I inhibitor GGTI-2133 (Calbiochem).

2.1.3 Gene expression profiling using qPCR gene array

Total RNA was extracted from the cells using a RNeasy kit (Qiagen, Valencia, CA). Genomic DNA contamination was eliminated and the first strand cDNA was synthesized from 1 μg total RNA using a RT^2 first strand kit (SABiosciences, Frederick MD). Gene expression profiles in the cells were studied using two types of targeted arrays purchased from SABiosciences, an osteogenesis PCR array consisting of 84 key genes related to bone formation and bone resorption and a signal transduction PathwayFinder PCR array containing 84 key genes representative of 18 different signal transduction pathways. Each of the arrays also contains 5 different housekeeping genes as loading controls for normalization. In addition to the housekeeping genes, nine genes appear on both arrays, these being Bmp2, Bmp4, Fn1, Icam1, Mmp10, Nfkb1, Tnf, Vcam1, and Vegfa. By using the two types of arrays, we were able to study the expression of 159 different target genes. The cDNA samples mixed with qPCR master mix (containing SYBR green) were loaded in 96-well PCR arrays and qPCR reactions were performed on a BioRad (Hercules, CA) iQ

real-time PCR detection system. After denaturing the template and activating the HotStart DNA polymerase at 95°C for 10 minutes, the two-step cycling program was run for 40 cycles at 95°C for 15 seconds, and 60°C for 60 seconds. The PCR array data were analyzed using Excel-based data analysis templates (SABiosciences), performing $\Delta\Delta C_t$ based fold-change calculations from raw threshold cycle data. Pair-wise comparisons between test samples and control samples were performed. Any genes that were increased or decreased more than two fold with a P value less than 0.05 were considered to be significantly modified.

2.1.4 RT-PCR

cDNA was synthesized using 1 μg total RNA and a reverse transcription system (Promega, Madison, WI). PCR was performed in a 25-μl reaction solution containing Platinum Taq DNA polymerase and cDNA template. After denaturing the template and activating the polymerase at 94°C for 2 minutes, the PCR reaction was run for 30–40 cycles at: 94°C for 15 seconds, 55–60°C for 30 seconds, and 72°C for 60 seconds.

3.1 Results

3.1.1 PTH regulates genes related to multiple signaling pathways and osteogenesis in UMR-106 osteoblastic cells

Based on the criteria described in the Methods (section 2.1.3), twenty genes were found to be regulated by PTH(1–34) on the signaling pathway array (Table 1A), and fifteen genes were found to be regulated by PTH on the osteogenesis array (Table 1B). Three genes (Tnf, Fn1,Vegfa) were regulated by PTH on both the signaling pathway array and the osteogenesis array. Thus, 32 different genes were regulated by PTH out of the 159 genes studied.

3.1.2 PTH effects on gene expression are differentially regulated by antagonist minigenes to G_s, G_q and G_{12}

After the cells were transfected with G_s, G_q or G_{12} minigenes to block receptor signaling through the respective individual G protein (other G protein signaling remains intact), the effects of PTH on gene expression were studied and compared with control (EV-transfected) cells. Among the thirty-two genes that were significantly regulated by PTH, several groups of genes showed different patterns of antagonism dependent on the specific minigene transfected, as shown in Table 2.

Group A—Gene expression antagonized only by the G_s minigene: As shown in Table 2A, PTH up-regulated the expression of Myc, Egf, Pdgfa. Igfbp3, Cyp19a1, and Rbp1, and these changes were blocked in cells expressing the G_s minigene but not cells transfected with the G_q or G_{12} minigene. PTH down-regulated the expression of Fasn, Flt1 and Fgf1, and expression of the G_s minigene selectively reversed these effects. The results indicate that the expression of this group of 9 genes is regulated by PTH through G_s signaling.

Group B—Gene expression antagonized only by the G_q minigene: As shown in Table 2B, PTH up-regulated the expression of Tgfb1, Egr1, Igf1, and Tnf, while the hormone down-regulated the expression of Bmp3 and Bmp7. The effects of PTH on these genes were antagonized by the G_q minigene but not by the G_s or G_{12} minigene, indicating that expression of this group of 6 genes is regulated by PTH through G_q signaling.

Group C—Gene expression antagonized only by the G_{12} minigene: Three genes were found to be selectively regulated by PTH through G_{12} signaling (Table 2C). PTH treatment

increased the expression of Mmp 8 and Gadd45a, and decreased the expression of Foxa2, and these effects were inhibited by the G_{12} minigene, but not by the G_s or G_q minigene.

Although a number of genes were exclusively blocked by only one Gα C-terminal minigene, and presumably were initiated solely through signaling downstream of that G protein, other genes were found to be blocked by two or more G protein minigenes (Group D, E, F). The regulation of some genes by multiple G protein minigenes suggests the possibility of crosstalk among the three different G proteins studied (G_s, G_q, G_{12}).

Group D—Gene expression antagonized by the G_s or G_q minigenes but not by the G_{12} minigene: Expression of six genes (Hsf1, Fgf4, Cdh1, Mmp7, Col3a1 and Fn1) was found to be up-regulated by PTH and blocked to a similar extent by either the G_s minigene or the G_q minigene (Table 2D), indicating that PTH may regulate expression of these genes through both G_s and G_q pathways.

Group E—Gene expression antagonized by G_s or G_{12} minigenes but not by the G_q minigene:

Up-regulation of Cebpb and Col14a as well as down-regulation of Itgav by PTH treatment were eliminated by transfection of either the G_{12} minigene or the G_s minigene (Table 2E), which indicated that the PTH-stimulated expression of these three genes could be regulated by both signaling pathways.

Group F—Gene expression antagonized by G_s, or G_q, or G_{12} minigenes:

The effects of PTH on c-Fos (Fos), Birc3 and Vegfa were antagonized by all three minigenes (Table 2F), indicating that these genes could be regulated through all three G protein-mediated pathways.

Group G—PTH-regulated gene expression not antagonized by G_s, G_q, or G_{12} minigenes: In addition to the PTH effects mediated through G_s, G_q, or G_{12}, there were some responses not altered by any minigenes. PTH treatment increased the expression of Pmepa1 while decreasing the expression of Hk2. Neither of these effects were altered by the presence of any of the minigenes tested (Table 2G). This result indicates that PTH-mediated changes in Pmepa1 and Hk2 gene expression may be regulated through pathways independent of G_s, G_q and G_{12}.

3.1.3 RT-PCR confirmation of differential antagonism by minigenes on selected PTH-regulated genes

In order to verify the results from gene array experiments, RT-PCR studies were carried out. UMR-106 cells were transfected with G protein minigenes or empty vector (pcDNA) and then treated with PTH (10 nM) for 24 hr. Representative genes from groups A to G as identified by the gene array experiments (Myc, Egr1, Mmp8, Col3a1, Col14a1, Fos and Hk2) were examined using RT-PCR. In order to assess whether the observed effects on gene expression were the result of constitutively inhibiting G protein signaling or were only noted following PTH treatment, the expression level of genes in cells transfected with the minigenes but which did not receive PTH treatment was also examined.

As evidenced in Figure 1, the basal expression of the seven selected genes was not significantly affected by the presence of the Gα minigenes. With PTH treatment, the expression of Myc, Egr1, Mmp8, Col3a1, Col14a1, and Fos was increased, while the expression of Hk2 was decreased, as was observed with the gene array experiments. Although the results were consistent with those observed in the gene array experiments, the

magnitudes of the increases or decreases were different. This could be due to variation between independent experiments or to differences in sensitivity obtained with qRT-PCR versus conventional RT-PCR. As was detected in the qRT-PCR arrays, PTH-stimulated up-regulation of Myc (from group A), Egr1 (from group B) and Mmp8 (from group C) were exclusively inhibited by G_s, G_q and G_{12} minigenes, respectively. The effects of PTH on Col3a1 (from group D) were blocked by G_s and G_q minigenes, while the effects on Col14a1 (from group E) were blocked by G_s and G_{12} minigenes. The PTH-stimulated increase of Fos (from group F) was inhibited by all three G protein minigenes. The PTH-induced decrease of Hk2 (from group G) was not affected by any of the minigenes.

3.1.4 Replication of the effects in the ROS 17/2.8 cell line

To determine if the results observed were found only in UMR-106 cells or could be replicated in another osteoblast cell line, ROS 17/2.8 cells were transfected with the G protein minigenes and treated with PTH. RT-PCR was carried out on the same representative genes as were examined as in section 3.1.3. In PTH treated ROS 17/2.8 cells (Figure 2), the responses were remarkably similar to those that had been seen in the UMR-106 cells, with 24 hour treatment leading to an increased gene expression of Myc, Egr1, Mmp8, Col3a1, Col14a1, and Fos, while the expression of Hk2 was decreased. Moreover, Myc, Egr1, and Mmp8 were exclusively inhibited by G_s, G_q, and G_{12} minigenes respectively, which Col3a1 was blocked by G_s and G_q minigenes, Col14a1 was antagonized by G_s and G_{12} minigenes, and Fos was inhibited by all three G protein minigenes. Finally, the PTH-induced decrease of Hk2 was not affected by any of the minigenes. As was the case with the UMR-106 cells, it is worth noting that none of the minigenes had marked effects in the absence of PTH. The results indicated that G protein interactions with PTH1R and subsequent signaling pathways appear consistent between ROS 17/2.8 and UMR-106 osteoblast cell lines.

3.1.5 Effects of minigenes on the PTH-responsive genes over a range of time points

More extensive time course studies were carried out on the representative genes examined in the studies shown in Figure 1. UMR-106 cells were treated with 10 nM PTH for 2, 8, 24, or 48 hr (Figure 3). Although there were differences in the regulation of gene expression by the minigenes at different time points, the regulation by a specific G protein was always consistent with the results observed previously. Of those genes uniquely regulated through a single G protein pathway, Myc was selectively antagonized by the G_s minigene at 8, 24, and 48 hr. PTH-mediated upregulation of Myc gene expression did appear to be regulated by G_q at the 2 hr time point. This finding was confirmed in a replicate analysis (data not shown). Egr1 was selectively antagonized by the G_q minigene at the 8 and 24 hr time points, but was not affected at the 2 and 48 hr time points. Mmp8 was selectively antagonized by the G_{12} minigene at 24 and 48 hr, while no inhibition was seen at the 2 hr or 8 hr time points. Although the PTH effect on Col3a1 at 8 hr was the largest effect noted by any of the genes at any time point, there was not a clear minigene effect on the response at that time point. The inhibition by G_s and G_q minigenes on Col3a1 gene expression was noted for both the 24 hr and 48 hr time points. The selective effect of G_s and G_{12} minigenes on Col14a1 was clear at 24 and 48 hr, with effects detectable at 8 hr as well. In the case of Fos, we found that at 24 hr, PTH-stimulated Fos expression was antagonized by all three minigenes, reproducing our previous experimental findings. However, at 2 hr, inhibition of Fos gene expression was only noted for the G_{12} minigene, and at 8 hr there was an effect by G_s or G_{12} minigenes, but not with the G_q minigene. Hk2 was not affected by any of the minigenes at any of the time points examined. Thus, the findings at 24 hr appear to reflect a pattern that was largely consistent over a period of time.

3.1.6 Effects of downstream signaling inhibitors of the G_s, G_q and G_{12} pathways on the PTH-responsive genes

Pharmacological downstream inhibitors of the three pathways were used to validate the effects noted with minigene transfection on the 7 selected genes shown in Figures 1–3 (Figure 4). UMR-106 cells were treated for 24 hr with 10 nM PTH alone or in the presence of inhibitors. The PKA inhibitor PKI (10 μM) was used to validate the effects of the G_s minigene, the PLC inhibitor U73122 (4 μM) was used to validate the effects of the G_q minigene, and the geranylgeranyl transferase I inhibitor GGTI-2133 (30 μM) to validate the effects of the G_{12} minigene. We found that the effects of the downstream inhibitors mimicked those observed with the minigenes, with PKI inhibiting PTH-mediated increased Myc gene expression, U73122 blocking PTH-mediated increased Egr1 gene expression, and GGTI-2133 reducing PTH-mediated increased Mmp8 gene expression. We also observed that the PTH effect on Col3a1 was blocked by both PKI and U73122, the PTH effect on Col14a1 was antagonized by both PKI and GGTI-2133, and the PTH effect to increase Fos gene expression was inhibited by all three compounds. As was noted with the minigenes, the PTH-induced decrease of Hk2 was not affected by either PKI, U73122, or GGTI-2133. Thus, the findings with the pharmacological downstream inhibitors were consistent with our results obtained following transfection of the minigenes.

4.1 Discussion

The current studies have used a novel approach, treatment with Gα C-terminal minigene vectors to selectively inhibit PTH signaling through G_s, G_q and G_{12}, in an effort to obtain further insight into the roles of specific G proteins in PTH-mediated gene expression. The results support the concept that activation of different signaling pathways initiated at the level of the G protein could result in distinct responses to PTH in osteoblasts. In addition, our resuts suggest that it is not uncommon for multiple G proteins to be involved in the effect of the hormone on a single gene.

As depicted in Figure 5, PTH regulated 32 of the 159 genes examined. Some PTH- regulated genes were exclusively antagonized through one class of G protein, indicating that there may be absolute specificity at the G protein level for some downstream responses. Other genes were regulated through multiple G proteins, with some genes being regulated through G_s and G_q pathways, others G_s and G_{12} pathways, and some through G_s, G_q and G_{12} pathways. That two or more G proteins can control expression of a common gene suggests that crosstalk may occur between different G protein-mediated pathways. Taken together, the results provide evidence for the involvement of multiple signaling pathways and show a role for all three G proteins tested (G_s, G_q, G_{12}) in PTH action. Although we did not find any common genes regulated through G_q and G_{12} pathways, pathways often linked in their signaling responses [20], such genes could exist, as only a limited number of genes were examined. It is also notable that we identified two PTH-regulated genes that were not affected by any of the G protein minigenes that we tested. This suggests that some PTH effects in osteoblastic cells might be mediated through alternative G proteins [21] or could be completely independent of G protein signaling such as effects mediated through beta-arrestin [22].

A small subset of genes was selected from the 32 affected genes for more in-depth studies. The subset (Myc, Egr1, Mmp8, Col3a1, Col14a1 and Fos) included genes regulated either through individual G proteins (Myc, Egr1, Mmp8) or by multiple G proteins (Col3a1, Col14a1, Fos). Analyses of the time course response of these genes showed that the 24 hr time point was not the only time at which effects of the minigenes could be observed; the inhibition of PTH effects by specific minigenes observed at the 24 hr time point was often noted at the other time points examined. One exception that we observed was an apparent

regulation of Myc at 2 hr through G_q, at 8 hr by both G_q and G_s minigenes, and at 24 and 48 hr by only the G_s minigene. There also appeared to be some time-dependent differences in the G protein regulation of Fos, which was down-regulated earlier and longer by the G_{12} minigene. The finding that multiple minigenes are involved in PTH regulation of Fos at the 24 hr time point is noteworthy in view of the evidence that Fos expression at time points earlier than 2 hr is dependent on PKA and independent of PKC [23,24]. The later PKC-dependent response that we observed could be a secondary effect reflecting a signaling cascade. The current findings reveal that early expression of Fos may be regulated through G_{12} signaling and that signaling through G_{12} is sustained. It is perhaps not surprising that Fos could be regulated through multiple signaling pathways, as Fos is critical for the differentiation of osteoblasts [25] and *in vivo* studies have shown impairment of PTH-induced osteoblast differentiation in association with a decrease in c-Fos mRNA [26]. Other work has revealed that c-Fos is also relevant to osteoclasts and thus may have a crucial role in bone formation in response to PTH [27,28].

Using the same small subset of genes, the antagonism shown with the G protein minigenes was validated with pharmacological downstream inhibitors. PKI, an inhibitor of PKA, U73122, an inhibitor of PLC and GGTI-2133, which inhibits the geranylgeranylation of RhoA [29], a downstream effector of G_{12} signaling in UMR-106 cells [9], mimicked the effects of the of the G_s, G_q and G_{12} minigenes. Additionally, PTH-mediated G protein signaling appeared to be consistent between UMR-106 cells and another osteoblast cell line, ROS 17/2.8, indicating the applicability of this novel approach to dissect out relevant G protein signaling pathways on PTH-mediated gene expression.

Studies of PTH signaling have largely focused on G_s, and the clinical relevance of this G protein in bone pathophysiology has been established. Mutations in G_s that lead to constitutive activation are present in fibrous dysplasia of bone and McCune-Albright syndrome, while loss-of-function mutations lead to Albright hereditary osteodystrophy. However, some important PTH-regulated genes may be regulated through other G proteins. The involvement of multiple G proteins in the response to agonists is characteristic of class II G protein coupled receptors, of which the PTH1R is a member [30]. Yet, the evidence for PTH-mediated signaling via diverse G proteins, as well as crosstalk occurring between Gα signaling pathways, has previously been obtained from indirect approaches including the use of inhibitors and activators of downstream effectors such as adenylyl cyclase, PKA and PKC. As an example of crosstalk, PTH activation of PLC (a $G_{q/11}$-mediated event) was enhanced when cells were treated with the PKA inhibitor H-89 [31] presumably working downstream of G_s. PTH fragments have also been useful in not ony highlighting the ability of the PTH1R to signal multiple G proteins, but also in dissecting the pathways. For example, Li et al [32] found that PTH 3–34, which does not increase cAMP (unless very high concentrations are used) regulated very few genes, consistent with the concept that cAMP signaling through G_s is the major pathway for PTH actions on bone. However, PTH(3–34), like PTH(1–34), was found to increase IGFBP5 mRNA, suggesting that there could be dual regulation of this important regulatory protein which may potentiate anabolic effects of PTH. Wu and Kumar [6] utilized the PTH(29–32) fragment, which activates PKC but not PKA, to show that the TGFβ1 isoform, but not TGFβ2, is regulated by a PKC-dependent pathway. They used agonists and antagonists selective for PKA or PKC to demonstrate that the G_s-cAMP-PKA pathway is involved in PTH-mediated activation of the TGFβ2 isoform. Agents that act more proximal to PTH1R, such as the G protein selective minigenes used in the current studies, will help to definitively establish the contributions of a given G protein(s) in PTH actions.

The present findings on PTH effects on gene expression in UMR-106 cells can be compared with the previous studies by Qin et al. [2], in which UMR-106 cells were treated with

PTH(1–34) at 10^{-8}M for 4, 12 or 24 hr, and gene expression profiles examined using a hybridization-based microarray. Out of the 8799 transcripts examined, 125 genes were found to be regulated by PTH treatment. Among the 32 genes regulated by a 24 hour PTH treatment in our studies, ten genes were shown to be regulated by PTH in their study, including Bmp3 (12 hr), Bmp7 (24 hr), Fos (4 hr, 12 hr), Cebpb (4 hr), Gadd45a (12 hr), Rbp1 (4 hr, 12 hr), Hk2 (24 hr), Col3a1 (24 hr), Fn1 (24 hr), and Myc (4 hr). Qin et al. also found several additional genes regulated by PTH after 4 hr treatment, including Bmp4, Vegfb, and Ccnd1. Although these genes were on our array, we did not find significant changes in their expression after PTH treatment for 24 hr. A number of other microarray studies have examined effects of PTH on gene expression in bone or bone cells, however because of the models used or the gene sets examined, the results were not directly comparable to the present work [33–36].

5.1 Conclusions

5.1.1

The study has used a novel approach, transfection of antagonist minigenes specifically targeting selected G proteins, to provide insights into the roles of these proteins in PTH-mediated gene expression.

5.1.2

The findings indicate that there are responses that appear to be selectively controlled by a single G protein, and responses resulting from co-regulation or sequential regulation, suggesting crosstalk between G proteins, as well as responses that may be G protein independent.

5.1.3

The studies raise interesting possibilities for the dynamic regulation of PTH responses at the G protein level. Such diversity in response may be the result of ligand-induced changes in the conformational state of the receptor [37], consistent with biased signaling by fragments and analogs of parathyroid hormone.

5.1.4

Given that PTH promotes both anabolic and catabolic effects, determining the role for each of the G proteins will assist researchers as they endeavor to design drugs that selectively activate specific downstream signaling pathways which provide therapeutic potential while minimizing unwanted side effects.

Acknowledgments

7.1 References

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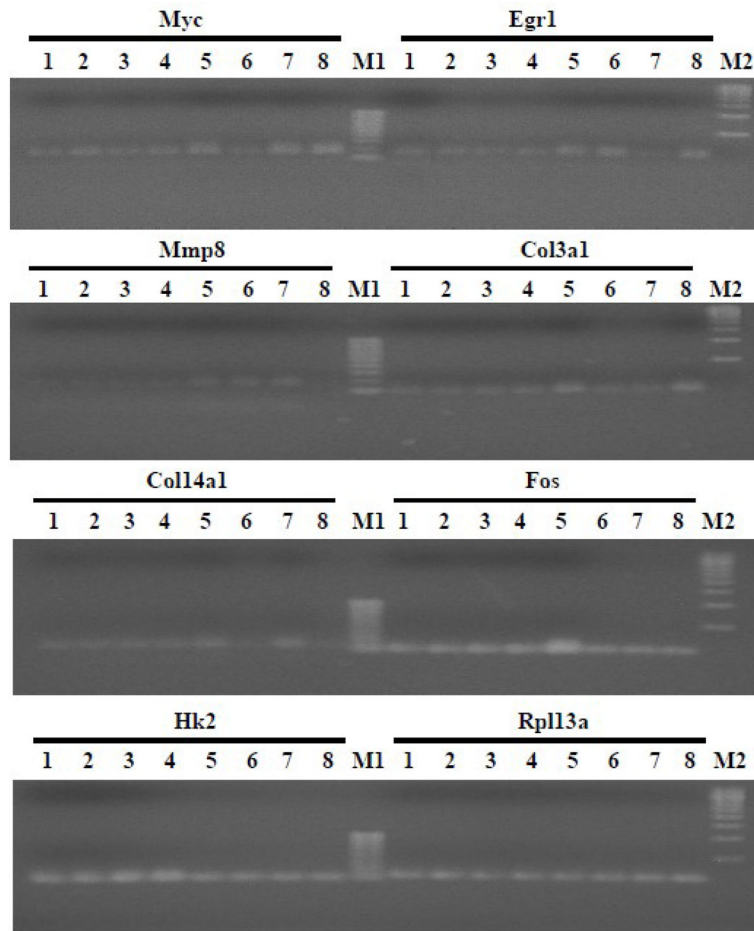
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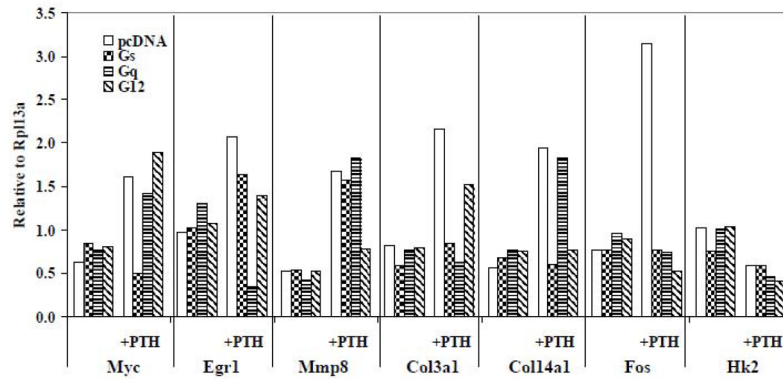
Figure 1B

Figure 1.
(A) RT-PCR of representative genes that were regulated by PTH and affected by different G protein minigenes in UMR-106 cells. The cells were transfected with G protein minigenes or empty vector (pcDNA), and then were treated with PTH (10 nM) for 24 hr. Lane 1, pcDNA transfected; Lane 2, G_s minigene transfected; Lane 3, G_q mingene transfected; Lane 4, G_{12} minigene transfected; Lane 5, pcDNA transfected + PTH; Lane 6, G_s minigene transfected +

PTH; Lane 7, G_q mingene transfected + PTH; Lane 8, G_{12} minigene transfected + PTH; M1, 100bp molecular ruler; M2, 500bp molecular ruler. (B) The relative expression levels of genes were analyzed and normalized against the housekeeping gene Rpl13a. The bands shown in Figure 1A were scanned and quantified for the analysis.

Figure 2A

Figure 2B

Figure 2.

(A) RT-PCR of the effects of PTH on representative genes affected by different G protein minigenes in ROS 17/2.8 cells. The cells were transfected with G protein minigenes or empty vector (pcDNA), and then were treated with PTH (10 nM) for 24 hr. Lane 1, pcDNA transfected; Lane 2, G_s minigene transfected; Lane 3, G_q mingene transfected; Lane 4, G_{12} minigene transfected; Lane 5, pcDNA transfected + PTH; Lane 6, G_s minigene transfected + PTH; Lane 7, G_q mingene transfected + PTH; Lane 8, G_{12} minigene transfected + PTH. (B) The relative expression levels of genes were analyzed and normalized against the housekeeping gene Rpl13a. The bands shown in Figure 2A were scanned and quantified for the analysis.

Figure 3.
(A) RT-PCR of the time course of effects of PTH on representative genes affected by G protein minigenes in UMR-106 cells. The cells were transfected with pcDNA, G_s minigene, G_q minigene, or G_{12} minigene, and then treated with 10nM PTH for 0 hr, 2 hr, 8 hr, 24 hr, or 48 hr. (B) The relative expression levels of genes were analyzed and normalized against the housekeeping gene Rpl13a. The bands shown in Figure 3A were scanned and quantified for the analysis.
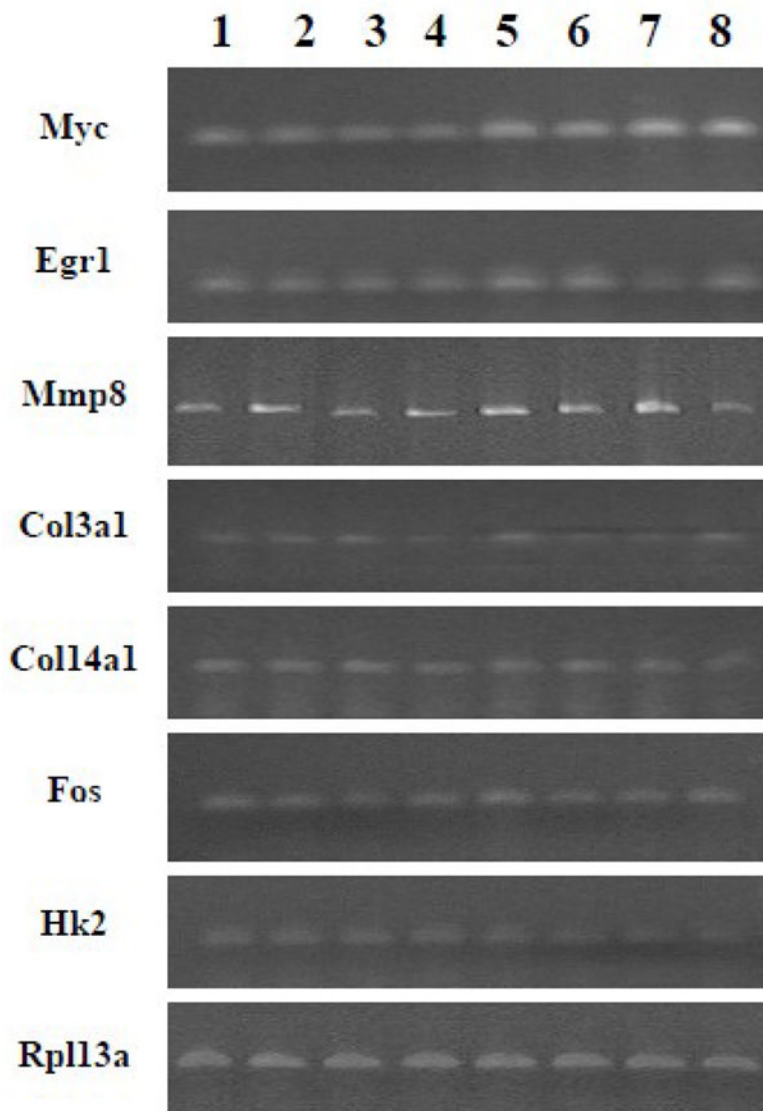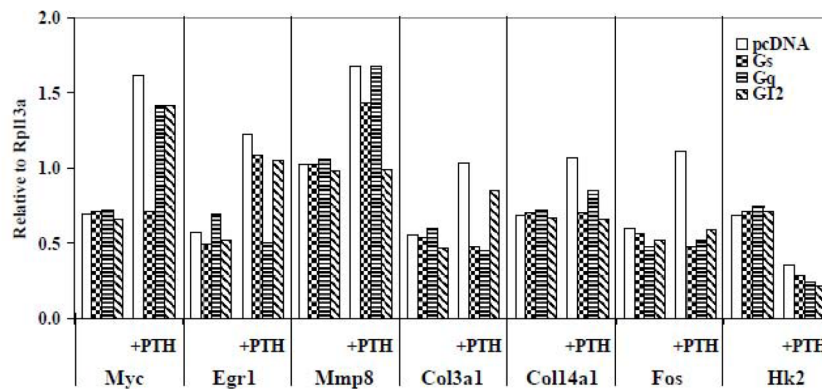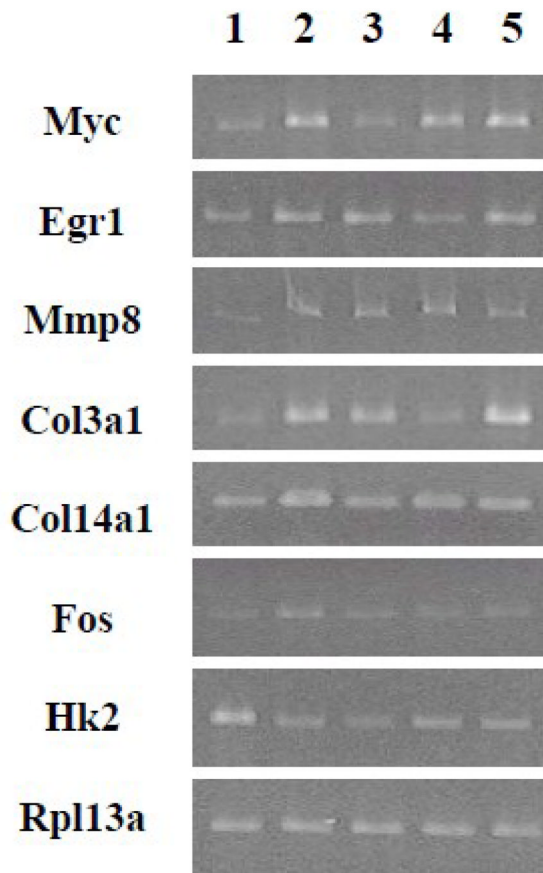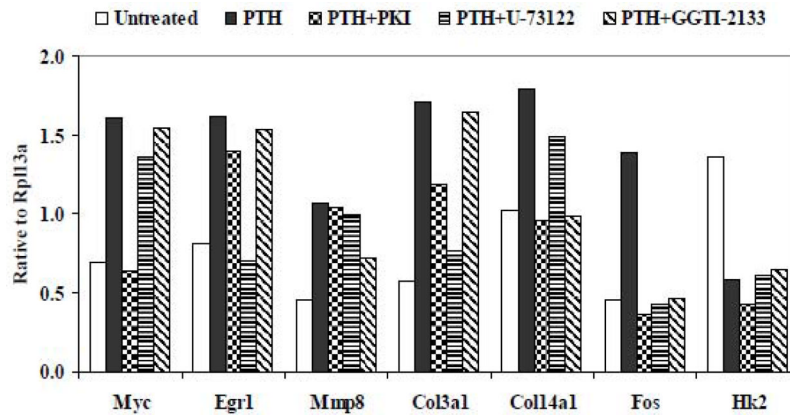
Figure 4.
(A) RT-PCR following treatment with pharmacological downstream inhibitors of signaling pathways mediated by G_s, G_q and G_{12}. UMR-106 cells were treated simultaneously with PTH (10 nM) and the PKA inhibitor PKI (10 μM), the PLC inhibitor U73122 (4 μM) or the geranylgeranyltransferase I inhibitor GGTI-2133 (30 μM) for 24 hr. Lane 1. No treatment; Lane 2. PTH; Lane 3. PTH + PKI; Lane 4. PTH + U-73122; Lane 5. PTH + GGTI-2133. (B) The relative expression levels of genes were analyzed and normalized against the

housekeeping gene Rpl13a. The bands shown in Figure 4A were scanned and quantified for the analysis.
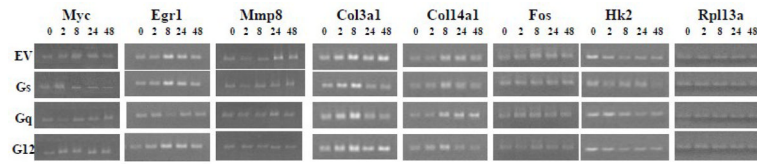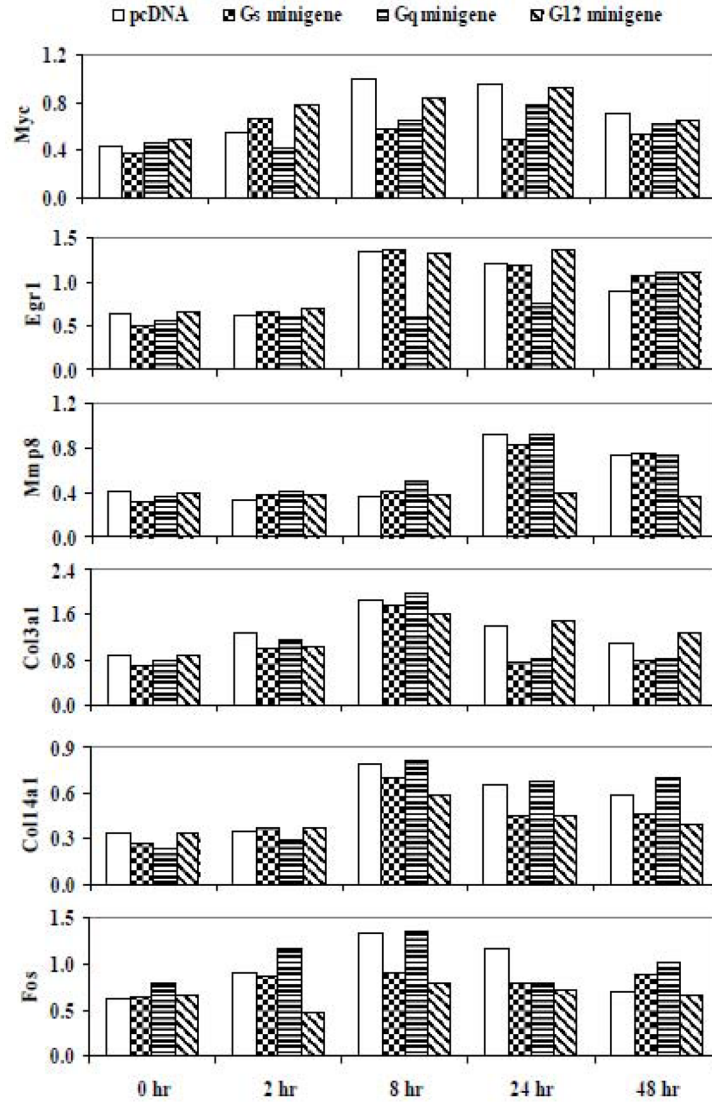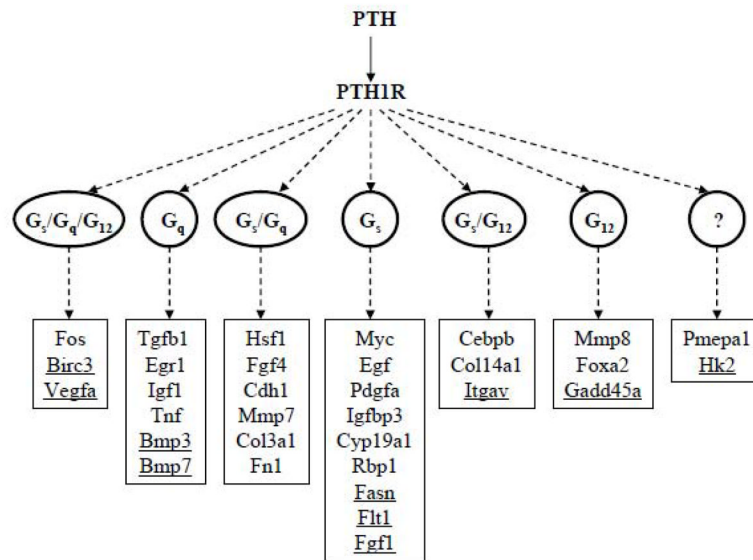
Figure 5.
Diagram of gene regulation by PTH signaling mediated through different G proteins (G_s, G_q, G_{12}). The combination of G proteins (G_s/G_q, G_s/G_{12}, $G_s/G_q/G_{12}$) shows possible crosstalk between different pathways and their downstream genes. The question mark (?) indicates that the mediator of signaling is unknown. The underlined genes were down-regulated by PTH.

Table 1

Genes regulated by PTH treatment (10^{-8}M for 24 hr)

A. Signaling Pathway Array

| Gene Symbol	Gene Name	Accession Number	Fold Difference	P Value
Fos	FBJ osteosarcoma oncogene	NM_022197	14.6	0.026
Hsf1	Heat shock transcription factor 1	XM_001061027	8.7	0.008
Myc	Myelocytomatosis oncogene	NM_012603	7.8	0.017
Fgf4	Fibroblast growth factor 4	NM_053809	6.8	0.048
Cdh1	Cadherin 1	NM_031334	5.0	0.044
Igfbp3	Insulin-like growth factor binding protein 3	NM_012588	4.2	0.012
Mmp7	Matrix metallopeptidase 7	NM_012864	3.9	0.045
Egr1	Early growth response 1	NM_012551	3.8	0.014
Tnf	Tumor necrosis factor	NM_012675	3.5	0.021
Pmepa1	Prostate transmembrane protein, androgen induced 1	XM_230899	3.1	0.048
Cyp19a1	Cytochrome P450, family 19, subfamily a, polypeptide 1	NM_017085	2.6	0.030
Cebpb	CCAAT/enhancer binding protein, beta	NM_024125	2.5	0.027
Gadd45a	Growth arrest and DNA-damage-inducible, alpha	NM_024127	2.3	0.036
Rbp1	Retinol binding protein 1, cellular	NM_012733	2.2	0.011
Fn1	Fibronectin 1	NM_019143	2.2	0.039
Foxa2	Forkhead box A2	NM_012743	−2.0	0.023
Fasn	Fatty acid synthase	NM_017332	−2.0	0.014
Hk2	Hexokinase 2	NM_012735	−2.0	0.035
Birc3	Baculoviral IAP repeat-containing 3	NM_023987	−2.9	0.017
Vegfa	Vascular endothelial growth factor A	NM_031836	−5.2	0.002

B. Osteogenesis Array

Gene Symbol	Gene Name	Accession Number	Fold Difference	P Value
Egf	Epidermal growth factor	NM_012842	5.8	0.019
Tgfb1	Transforming growth factor, beta 1	NM_021578	5.5	0.037
Pdgfa	Platelet derived growth factor, alpha	NM_012801	4.4	0.040
Tnf	Tumor necrosis factor	NM_012675	4.0	0.008
Igf1	Insulin-like growth factor 1	NM_178866	3.8	0.019
Mmp8	Matrix metallopeptidase 8	NM_022221	3.7	0.007
Col3a1	Procollagen, type III, alpha 1	NM_032085	3.5	0.038
Fn1	Fibronectin 1	NM_019143	2.6	0.028
Col14a1	Procollagen, type XIV, alpha 1	XM_235308	2.0	0.026
Flt1	FMS-like tyrosine kinase 1	NM_019306	−2.0	0.017
Itgav	Integrin alpha V	XM_230950	−2.1	0.010
Bmp3	Bone morphogenetic protein 3	NM_017105	−3.1	0.003
Bmp7	Bone morphogenetic protein 7	XM_342591	−3.1	0.004
Vegfa	Vascular endothelial growth factor A	NM_031836	−7.1	0.008
Fgf1	Fibroblast growth factor 1	NM_012846	−9.5	0.023

Table 2

Genes regulated by PTH and antagonized by G protein minigenes

A. PTH-regulated genes antagonized only by G_s minigene

Gene Symbol	G_s minigene + PTH vs EV+PTH		G_q minigene + PTH vs EV + PTH		G_{12} minigene + PTH vs EV + PTH	
	Ratio	P value	Ratio	P value	Ratio	P value
Myc	0.1	0.018	0.6	0.392	0.8	0.358
Egf	0.4	0.011	1.2	0.226	1.6	0.109
Pdgfa	0.2	0.003	1.3	0.407	1.2	0.307
Igfbp3	0.3	0.026	0.7	0.435	0.8	0.089
Cyp19a1	0.3	0.019	1.8	0.070	0.7	0.195
Rbp1	0.5	0.041	1.0	0.986	1.7	0.742
Fasn	2.1	0.010	0.5	0.397	0.6	0.580
Flt1	4.5	0.039	0.7	0.134	0.9	0.849
Fgf1	4.2	0.010	0.9	0.135	0.9	0.072

B. PTH-regulated genes antagonized only by G_q minigene

Gene Symbol	G_s minigene + PTH vs EV+PTH		G_q minigene +PTH vs EV + PTH		G_{12} minigene + PTH vs EV+PTH	
	Ratio	P value	Ratio	P value	Ratio	P value
Tgfb1	1.4	0.267	0.2	0.006	1.3	0.274
Egr1	08	0.708	0.4	0.021	1.0	0.991
Igf1	0.6	0.211	0.2	0.008	1.0	0.961
Tnf*	0.7	0.600	0.5	0.004	2.6	0.259
Tnf**	0.8	0.123	0.2	0.013	0.9	0.849
Bmp3	1.2	0.895	2.2	0.008	1.0	0.961
Bmp7	0.9	0.870	3.3	0.026	1.4	0.464

C. PTH-regulated genes antagonized only by G_{12} minigene

Gene Symbol	G_s minigene + PTH vs EV+PTH		G_q minigene +PTH vs EV+PTH		G_{12} minigene + PTH vs EV+PTH	
	Ratio	P value	Ratio	P value	Ratio	P value
Mmp8	1.2	0.365	1.3	0.573	0.1	0.008
Gadd45a	0.8	0.492	0.7	0.346	0.3	0.022

C. PTH-regulated genes antagonized only by G$_{12}$ minigene

Gene Symbol	G$_s$ minigene + PTH vs EV+PTH		G$_q$ minigene +PTH vs EV + PTH		G$_{12}$ minigene + PTH vs EV+PTH	
	Ratio	P value	Ratio	P value	Ratio	P value
Foxa2	0.6	0.027	0.6	0.024	2.0	0.012

D. PTH-regulated genes antagonized by either G$_s$ or G$_q$ minigene

Gene Symbol	G$_s$ minigene + PTH vs EV+PTH		G$_q$ minigene + PTH vs EV + PTH		G$_{12}$ minigene + PTH vs EV+PTH	
	Ratio	P value	Ratio	P value	Ratio	P value
Hsf1	0.4	0.002	0.5	0.003	1.0	0.574
Fgf4	0.2	0.032	0.3	0.044	0.6	0.133
Cdh1	0.2	0.004	0.2	0.006	1.0	0.744
Mmp7	0.3	0.042	0.2	0.016	1.5	0.253
Col3a1	0.2	0.014	0.5	0.017	1.1	0.675
Fn1 *	0.4	0.012	0.4	0.015	0.7	0.252
Fn1 **	0.3	0.035	0.3	0.025	1.5	0.079

E. PTH-regulated genes antagonized by either G$_2$ or G$_{12}$ minigene

Gene Symbol	G$_s$ minigene + PTH vs EV+PTH		G$_q$ minigene +PTH vs EV + PTH		G$_{12}$ minigene + PTH vs EV+PTH	
	Ratio	P value	Ratio	P value	Ratio	P value
Cebpb	0.1	0.044	1.0	0.883	0.3	0.010
Coll4a1	0.1	0.012	0.8	0.200	0.4	0.009
Itgav	5.4	0.012	0.9	0.495	8.9	0.004

F. PTH-regulated genes antagonized by either G$_s$, G$_q$ or G$_{12}$ minigene

Gene Symbol	G$_s$ minigene + PTH vs EV+PTH		G$_q$ minigene +PTH vs EV + PTH		G$_{12}$ minigene + PTH vs EV+PTH	
	Ratio	P value	Ratio	P value	Ratio	P value
Fos	0.04	0.0002	0.1	0.0001	0.1	0.0002
Birc3	5.1	0.0015	5.3	0.0014	12.9	0.0006
Vegfa *	5.1	0.0015	2.5	0.0047	6.9	0.0010
Vegfa **	4.5	0.0192	2.5	0.0194	4.1	0.0090

G. PTH-regulated genes antagonized by neither G$_s$, G$_q$ nor G$_{12}$ minigene

Gene Symbol	G$_s$ minigene + PTH vs EV+PTH		G$_q$ minigene +PTH vs EV + PTH		G$_{12}$ minigene + PTH vs EV+PTH	
	Ratio	P value	Ratio	P value	Ratio	P value
Pmepa1	0.7	0.238	0.7	0.272	0.8	0.423
Hk2	0.8	0.038	0.7	0.083	1.1	0.103

*
on signaling pathway array;

**
on osteogenesis array