The Effects of 1,25 D_3 on the Mouse Osteopontin Promoter

Vitamin D (1,25 D_3) is an important transcriptional regulator of many genes including our gene of interest, osteopontin. Osteopontin is a known regulator of bone calcification and mineralization and is involved in signaling in the immune response. We looked at osteopontin expression in mouse MC3T3-E1 preosteoblast cells in response to 1,25 D_3. We also tested how pathways known to crosstalk with 1,25 D_3 modify mOPN expression. These include MNAR, Wnt3a conditioned media, PTH, and dexamethasone. Additionally, we checked how ZK159222, an antagonist to VDR with partial agonist activity, effects mOPN expression. The osteopontin promoter was cloned into a PGL3 reporter vector and transfected into MC3T3-E1 cells. A luciferase assay normalized by a β-gal assay was used to measure gene expression. 1,25 D_3 induces activity of the mOPN promoter. ZK159222, MNAR, Wnt3a conditioned media, PTH, and dexamethasone were not found to effect mOPN promoter activity in the presence of 1,25 D_3.

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The Effects of 1,25 D_3 on the Mouse Osteopontin Promoter

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Abstract:

Vitamin D (1,25 D_3) is an important transcriptional regulator of many genes including our gene of interest, osteopontin. Osteopontin is a known regulator of bone calcification and mineralization and is involved in signaling in the immune response. We looked at osteopontin expression in mouse MC3T3-E1 preosteoblast cells with and without the addition of 1,25 D_3. We also tested pathways known to crosstalk with 1,25 D_3 to see how they modify OPN expression. These include MNAR, Wnt3a conditioned media, PTH, and dexamethasone. Additionally, we checked the ability of ZK159222, an antagonist to VDR with partial agonist activity, to activate OPN expression. This was done by cloning the osteopontin promoter and upstream region into a PGL3 reporter vector and then transfecting it into MC3T3-E1 cells. A luciferase assay normalized by a β-gal assay was used to measure gene expression. 1,25 D_3 induces activity of the mOPN promoter. ZK159222, MNAR, Wnt3a conditioned media, PTH, and dexamethasone were not found to effect mOPN promoter activity in the presence of 1,25 D_3.

Background:

Osteoporosis is a condition that affects 25 million people in the United States today (Travis 1995). In a properly functioning bone, the bone matrix is continually being built up by osteoblasts and resorbed by osteoclasts. In osteoporosis, however, the osteoclasts are deteriorating bone matrix faster than it is being built up by the osteoblasts,

resulting in thinner, weaker bones (Teilbelbaum 2004). In extremely rare cases, osteopetrosis also occurs. This happens when the bone matrix builds up more rapidly than the osteoclasts break it down resulting in a loss of marrow cavity (Teilbelbaum and Ross 2003). In order to combat osteoporosis and osteopetrosis, it is essential to understand how osteoclasts and osteoblasts function and how they are signaled to work

Vitamin D (1,25 D_3) is important in bone health. It regulates a variety of genes in different ways (Grant 2005). We will be focusing on the way the osteopontin (OPN) gene is regulated by 1,25 D_3 in a preosteoblast cell line called MC3T3-E1. OPN is a regulator of bone calcification and mineralization and is involved in signaling in the immune response (Giachelli 2000). The OPN gene is also turned on in cancer cells.

Vitamin D response elements (VDREs) have been identified on the OPN gene and may play a role in OPN regulation. VDREs are turned on when 1,25 D_3 binds to the vitamin D receptor (VDR) that in turn binds to a VDRE (Stall 1996). The vitamin D receptor can bind to OPN, but VDR binding is stronger when it is combined with retinoid X receptor (RXR) to form the VDR/RXR heterodimer (Nishikawa 1994). It has been shown that mouse OPN expression decreases when the vitamin D receptor is interrupted (Shen 2005). We saw that VDR binds to the mouse OPN promoter increasing OPN transcription.

A coactivator may be working with 1,25 D_3 to activate the vitamin D receptor. ZK159222 is a vitamin D analog that has partial agonist activity and is also an antagonist of VDR. ZK15922 has been shown to behave differently on different gene targets. ZK159222 negates the effect 1,25 D_3 has on the 25-hydroxyvitamin D3-24-hydroxylase (Cyp24) gene. (Kim 2004)

Some steroid receptors, like vitamin D, activate Src via the Modulator of Nongenomic Actions of the estrogen Receptor (MNAR) (Haas 2005). The Wnt3a signaling pathway is used by osteoblast cells (Spencer 2005). Glucocorticoids like parathyroid hormone (PTH) and dexamethasone are involved in bone formation. We tested how MNAR, Wnt3a conditioned media, PTH, and dexamethasone affect OPN promoter activity alone and in combination with 1,25 D_3 treatment.

Methods:

The mouse osteopontin (mOPN) gene sequence was found on genome.ucsc.edu and analyzed in consite to find potential VDREs. PCR was used to amplify the region upstream of the mOPN start site that contains the VDREs from mouse genomic DNA and the product was ran out on a 1% TAE gel. The DNA was extracted from the gel using the Qiagen Gel Extraction Kit and eluted into 30uL water.

The promoter sequence was inserted into PGL3 and PGL4.14 plasmids and ligated together using T4 DNA liagase. The plasmid was set up so that the cloned mOPN promoter region drives the expression of firefly luciferase. If the mOPN promoter is active, the firefly luciferase will be turned on and the cell lysate will glow. The plasmid also contains ampicillin resistance to allow for selection of transformed bacterial hosts. DH5α cells were transformed with the plasmid and grown on LB-ampicillin plates. PCR was used to verify that the DH5α cells had taken up the mOPN promoter and then the Qiagen Miniprep Kit was used to isolate the DNA. The plasmid was sent to IDT for sequencing and once the correct sequence was confirmed the Qiagen Maxiprep Kit was used to create a stock of the PGL3 and PGL4.14 vectors containing the mOPN promoter.

The plasmid was inserted into pre-osteoblast MC3T3-E1 cells via transfection with 3.0:1.5 μL PLUS:Lipofectamine. Each treatment was done in triplicate. Cells were treated with the hormone overnight and then lysed the next day for 20 minutes with Promega lysis buffer. A luciferase assay (10 μL cell lysate: 25 μL luciferase substrate) read for 5 seconds was used to determine activity. Transfection efficiency was normalized through a β-gal assay (201 μL 0.1 M sodium phosphate buffer (pH 7.5), 66 μL 1x ONPG, 3 μL Mg solution (0.1 M $MgCl_2$, 4.5 M β-Mercaptoethanol): 30 μL cell lysate).

Results:

The mouse osteopontin gene contains two potential VDREs and two start sites. The mOPN promoter from –1571 to +1123 base pairs was cloned into a PGL3 and PGL4.14 reporter plasmid. This section contains only one of the potential VDREs but both start sites. A PGL3 empty, PGL3 with mOPN promoter, PGL4.14 empty, or PGL4.14 with mOPN promoter plasmid was transfected into MC3T3-E1 cells treated with varying concentrations of 1,25 D_3: 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, or ethanol vehicle for a control. A luciferase assay was done to check for induction and a β-gal assay was used to normalize all of the values. The data showed that this section of the mOPN promoter region is induced by 1,25 D_3. Induction was greater in the PGL3 plasmid so this was the plasmid chosen for use in future experiments. The PGL3 and PGL4.14 empty plasmids showed no response to 1,25 D_3. (Figure 1)

The mOPN promoter was again transfected into MC3T3-E1 cells and treated varying concentrations of 1,25 D_3: 10^{-9}, 10^{-8}, 10^{-7}, or ethanol vehicle. This time the

Cyp24 gene was added as a positive control because 1,25 D_3 has been shown to induce Cyp24 activity (Kim 2004). A dose dependent increase in activity was seen with mOPN and Cyp24 treated with 1,25 D_3. The PGL3 empty plasmid showed no response. (Figure 2)

Next, the mOPN promoter with both potential VDREs, -2793 to +1123 base pairs, was cloned into the PGL3 plasmid. It was then transfected into MC3T3-E1 cells and treated with varying concentrations of 1,25 D_3: 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, or ethanol vehicle. This promoter was induced weakly, if at all, by 1,25 D_3. Because of its low activity, the -2793 to +1123 mOPN promoter was abandoned and the rest of the experiments were carried out using the PGL3 -1571 to +1123 promoter plasmid. (Figure 3)

Once we knew that this mOPN promoter was induced by 1,25 D_3, we tested to see how coregulators would effect the activity. The mOPN promoter was transfected into MC3T3-E1 cells and treated with ZK159222: 10^{-8}, 10^{-7}, 10^{-6}, or ethanol vehicle alone and with 1,25 D_3: 10^{-8}. We saw that ZK15922 alone does not change activity in the Cyp24 gene, but when cells are treated with both 1,25 D_3 and ZK159222, ZK159222 antagonizes the 1,25 D_3 activity. When the mOPN promoter is treated with ZK159222 alone no change in activity is observed. Unlike in Cyp24, ZK159222 does not antagonize 1,25 D_3 activity in the mOPN promoter. (Figure 4)

Next, transfected cells were treated with MNAR (50 ng) and 1,25 D_3: 10^{-9}, 10^{-8}, 10^{-7}, or ethanol vehicle. PGL3 basic had no change in activity with the addition of both 1,25 D_3 and MNAR. Cells containing the mOPN promoter treated with both 1,25 D_3 and MNAR had the same induction as the mOPN promoter treated only with 1,25 D_3.

MNAR does not change this mOPN promoter activity. Cyp24 was again used as a positive control. (Figure 5)

MC3T3-E1 cells transfected with the mOPN promoter were treated with conditioned media that contains Wnt3a: 0%, .5%, 1%, 5%, 10%, or 30%. Wnt3a activates stabilization of β-catenin and activation of TCF/LEF transcription factors (Spencer 2005). TopFlash is induced by Wnt3a conditioned media and was used as a positive control (Warner 2005). TopFlash was induced by Wnt3a conditioned media but not by 1,25 D_4. The mOPN promoter was only moderately induced by Wnt3a conditioned media alone and not better than 1,25 D_3 alone or 1,25 D_3 with Wnt3a conditioned media. (Figure 6)

PTH works by activating cAMP and consequently CREB (Tyson 2002). It has been shown that osteopontin can inhibit PTH suppression of bone formation (Kitahara 2003). PTH: 10^{-7} was added to MC3T3-E1 cells transfected with the mOPN promoter and treated with Wnt3a conditioned media: 0%, .5%, 1%, 5%, 10%, or 30%. PTH with Wnt3a conditioned media decreased the overall activity of the osteopontin promoter compared to cells treated with Wnt3a conditioned media only. When 1,25 D_3 was added along with PTH in Wnt3a conditioned media, 1,25 D_3 overrode the PTH effect. PTH no longer suppressed mOPN promoter activity. (Figure 7)

Dexamethasone activates GR and has been previously shown to suppress osteopontin (Kirton 2006). Dexamethasone: 10^{-7} was added to MC3T3-E1 cells transfected with the mOPN promoter and treated with Wnt3a conditioned media: 0%, .5%, 1%, 5%, 10%, or 30%. Dexamethasone with Wnt3a conditioned media knocked down the overall activity of the mOPN promoter compared to cells treated with Wnt3a

conditioned media only. When 1,25 D_3 was added along with dexamethasone in Wnt3a conditioned media, mOPN activity was similar to acitivty with 1,25 D_3 alone. Dexamethasone no longer suppressed mOPN promoter activity. (Figure 7)

Discussion:

We saw that 1,25 D_3 has a dose dependent increase on the activity of the -1571 to +1123 mOPN promoter. When the promoter is increased in size to -2793 to +1123 to include a second VDRE this dose dependent induction of the promoter is lost. The reason for this is unclear. It is possible that with the addition of about 1,000 base pairs other effects are appearing that may or may not be due to the addition of a second VDRE.

1,25 D_3 may not be working alone to activate the osteopontin promoter. We saw that ZK159222, MNAR, and Wnt3a conditioned media have no effect on mOPN acitivty. PTH and dexamethasone appear to knock down the level of mOPN activity without 1,25 D_3 and with 1,25 D_3 they have no effect on activity. In all of these cases, 1,25 D_3 is the stronger signal for regulation. 1,25 D_3 is able to override the suppression of PTH and Dexamethasone. PTH and dexamethasone were tested in combination with Wnt3a conditioned media and 1,25 D_3 because this better represents what is present in the bone marrow environment. More coregulators would need to be tested to determine what is working with 1,25 D_3 to activate the mouse osteopontin promoter.

Figures:

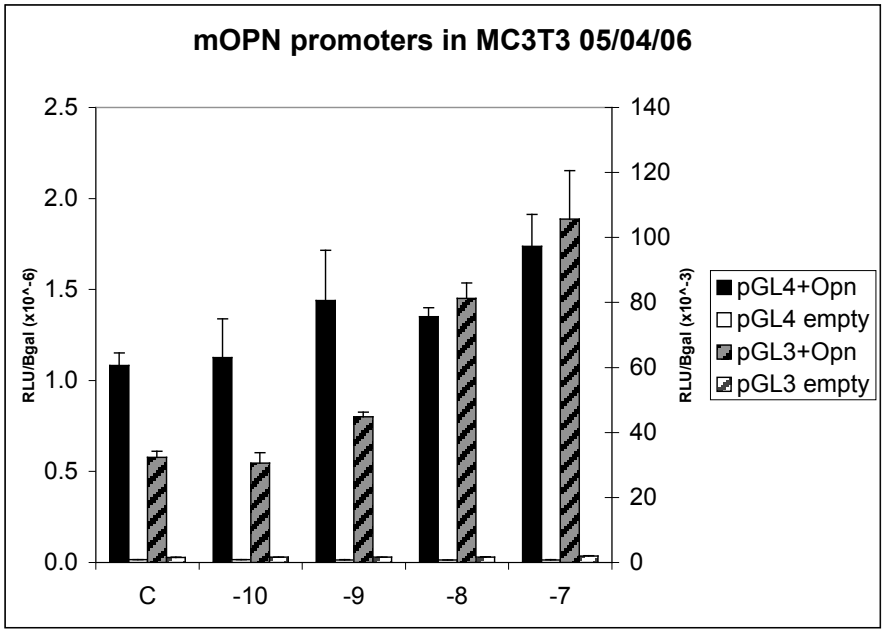

Figure 1. Induction of the mOPN+PGL3 and mOPN+PGL4 plasmids in MC3T3-E1 cells treated with varying concentrations of 1,25 D_3.

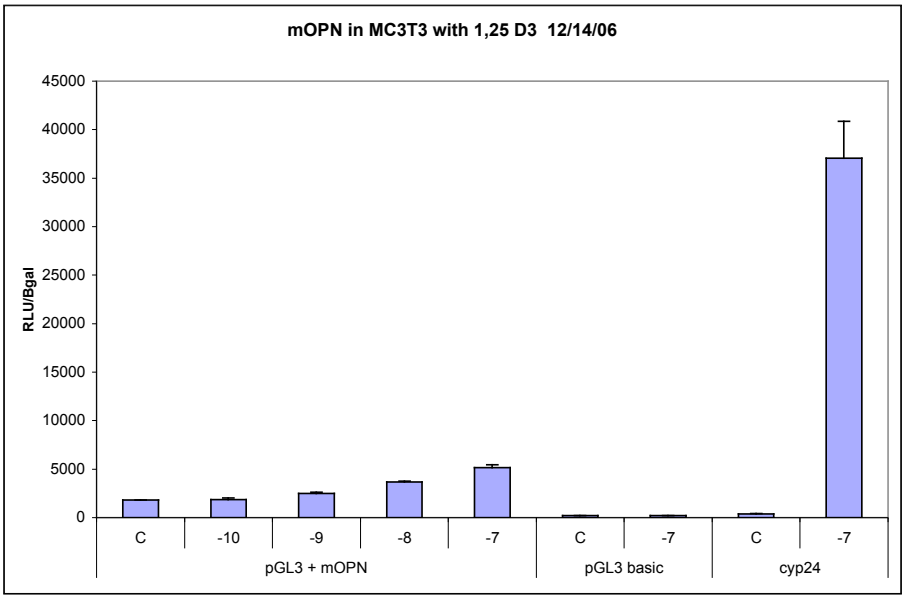

Figure 2. Induction of mOPN promoter in MC3T3-EI cells treated with varying concentrations of 1,25 D_3. Cyp24 was used as a positive control.

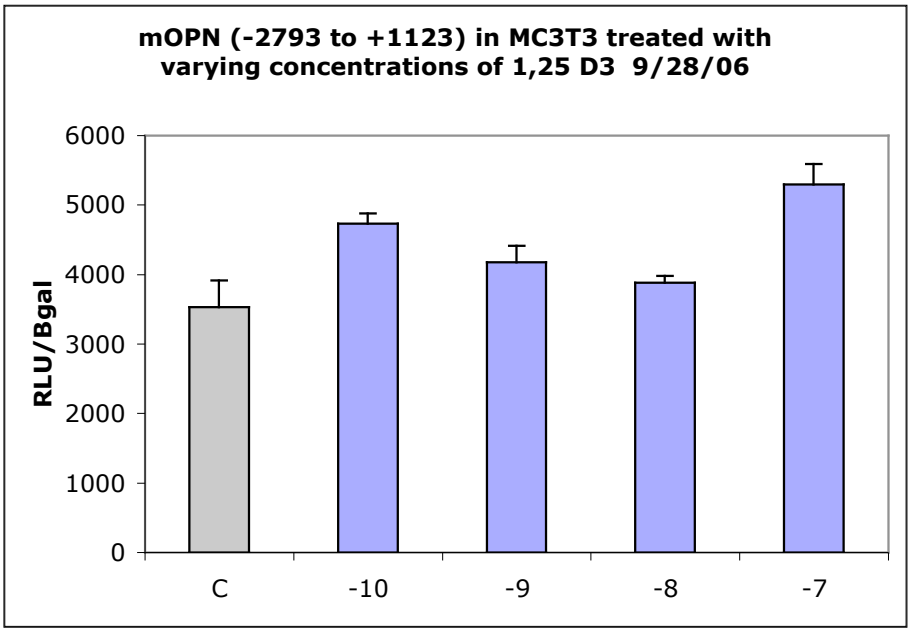

Figure 3. Induction of mOPN (-2793 to +1123 bp) in MC3T3-E1 cells treated with varying concentrations of 1,25 D_3.

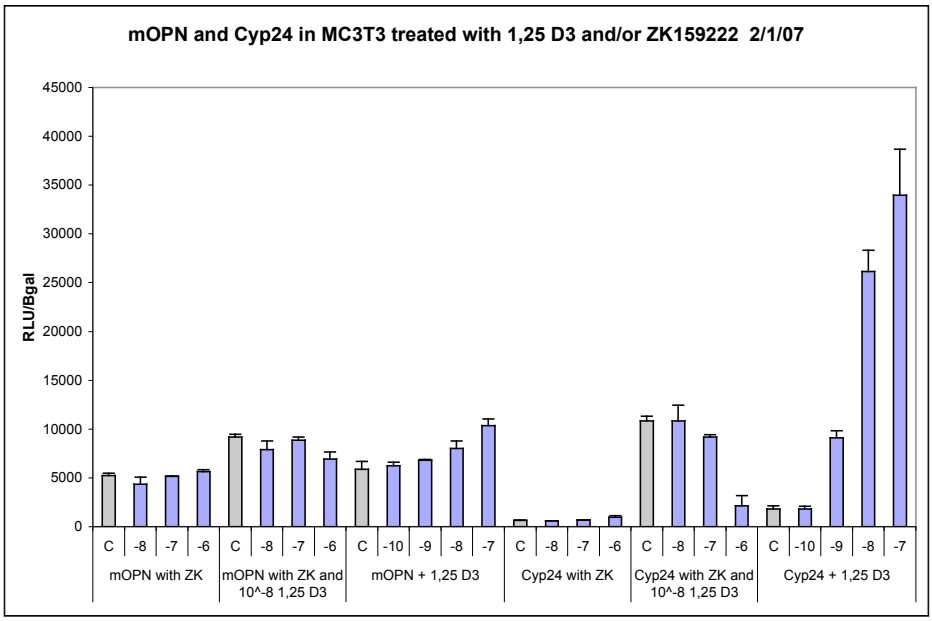

Figure 4. Induction of the mOPN promoter in MC3T3-E1 cells treated with varying concentrations of ZK159222 and/or 1,25 D_3. Cyp24 was used as a control.

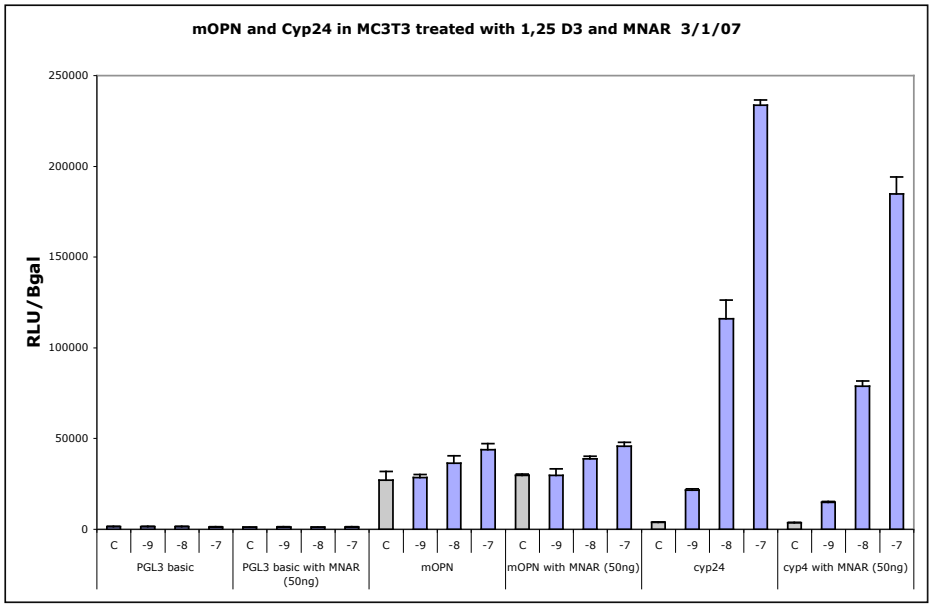

Figure 5. Induction of the mOPN promoter in MC3T3-E1 cells treated with varying concentrations of 1,25 D_3 and MNAR (50ng). PGL3 basic and Cyp24 were used as controls.

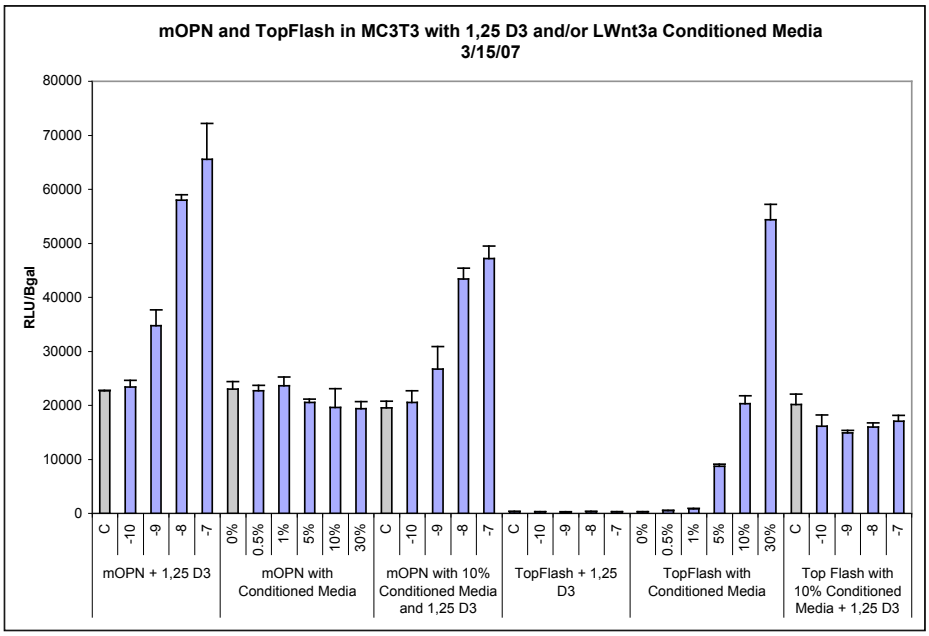

Figure 6. Induction of the mOPN promoter in MC3T3-E1 cells treated with varying concentrations of 1,25 D_3, varying concentrations of Wnt3a conditioned media, or varying concentrations of Wnt3a conditioned media and 1,25 D_3. TopFlash was used as a control.

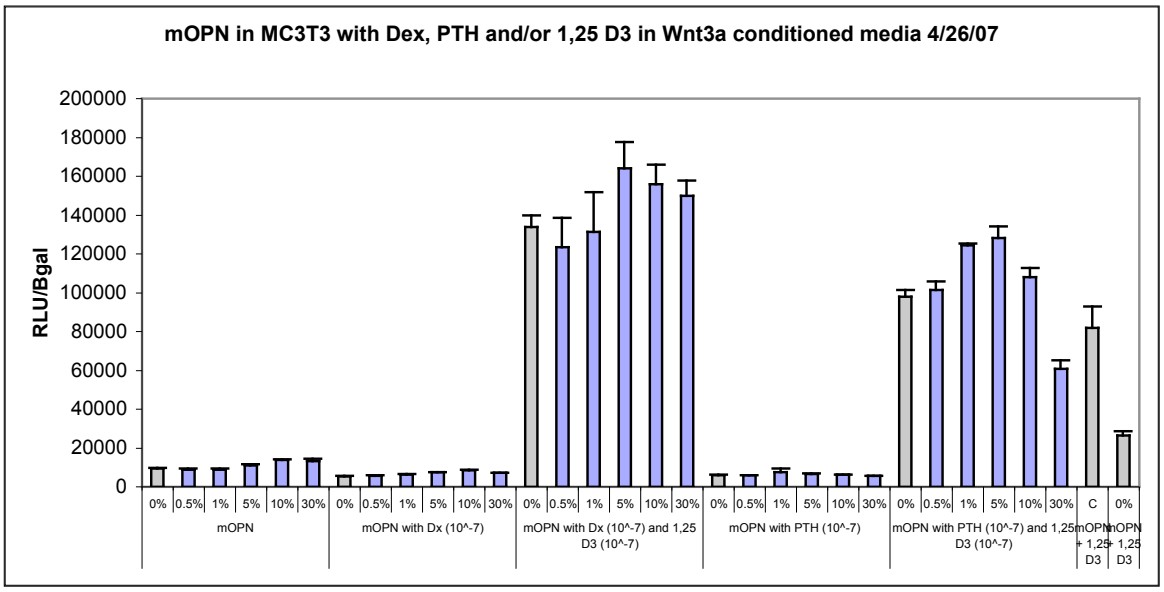

Figure 7. Induction of the mOPN promoter in MC3T3-E1 cells treated with varying concentrations of Wnt3a conditioned media and/or 1,25 D_3, PTH, and Dexamethasone.

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