# The Grass is Always Greener on the Other Side (of the Basement Membrane): Prostate Cancer Metastasis as Intracorporeal Dispersal

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April 8, 2019

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## Abstract

*'Knowing trees, I understand the meaning of patience. Knowing grass, I can appreciate persistence.'*
*-Hal Borland, 1965*

Dispersal ecology offers a possible explanation for the behavior of prostate cancer (PCa), which disproportionately metastasizes to the bone. Organisms become more likely to leave their home patch when conditions are unfavorable, with the goal of becoming established in more favorable patches. Similarly, PCa cells are pushed from the prostate tumor by hypoxic stress and pulled toward the bone by the presence of bone stromal cells (BSCs), which support PCa growth and proliferation. When PCa settles in the bone, it leads to the formation of a feedforward loop in which PCa enhances osteoblast differentiation; osteoblasts in turn promote PCa proliferation via growth factors. This feedforward loop is mediated by bone morphogenetic proteins (BMPs), which drive bone growth by promoting osteoblast differentiation. In a further ecological parallel, this behavior appears analogous to parasitic manipulation of hosts. To test whether the feedforward loop could be disrupted, and to help determine the origin of the loop, I cultured PCa alone and in co-culture with BSCs with DorsoMorphin Homolog 1, an inhibitor of BMP. I found that monocultures of either PCa or BSCs were largely unaffected by DMH1. However, DMH1 treatment of the co-culture appeared to block excessive PCa proliferation but not osteoblast differentiation; at the same time, PCa, but not BSCs, upregulated BMP production, consistent with PCa driving the feedforward loop. These results suggest that DMH1 is a more effective treatment against metastatic prostate cancer than localized prostate cancer, and that the parasite analogy between cancer and bone cells is appropriate.

## Introduction

*'Cells are organisms, and entire animals and plants are aggregates of these organisms.'*
*-Theodor Schwann, 1839*

In multicellular organisms, cells are tightly regulated and only proliferate (divide into new cells) in response to an appropriate signal (Stearns and Medzhitov 2016). Cancer occurs when, through mutations, these controls fail and a subset of cells become deregulated and insensitive to anti-growth signals, causing the cells to proliferate continuously, (Hanahan and Weinberg 2011). This excess proliferation leads to formation of a tumor, or mass of cells. Tumors eventually outgrow existing blood vessels, and must induce angiogenesis, or formation of new blood vessels, in order to continue growing (Hanahan and Weinberg 2011). In time, cancer cells leave the primary (original) tumor to form distant new tumors in a process called metastasis. Metastasis is a multistep process; cancer cells must first break through the basement membrane (a layer of protein) separating the tumor from the underlying tissue, then enter blood vessels and lymph nodes to travel to distant sites (Egeblad and Werb 2002). To enter blood vessels, cancer cells must first undergo the epithelial-to-mesenchymal transition (EMT), a process that increases motility and decreases the adhesion of cancer cells to each other (Gupta and Massagué 2006). Cancer cells then travel to distant sites through circulation, where they form micrometastases (microscopic metastases) that have the potential to grow into macrometastases, or visible tumors (Hanahan and Weinberg 2011). Different cancer types localize disproportionately to certain organs; for example, colon cancer most commonly metastasizes to the liver, while breast and prostate cancer (PCa) favor bone (Langley and Fidler 2011.)

The public health and clinical import of cancer is significant. Cancer is the second-leading cause of death in the United States, with the American Cancer Society projecting over 1.75 million new cases and over 600,000 deaths in 2019 (Siegel et al. 2019). On the individual level, the progression of cancer leads to a drastic decrease in quality of life. As cancer progresses, patients may experience cachexia (loss of fat and muscle mass), thrombosis (blood clotting), and dyspnea (difficulty breathing), which all cause discomfort and often death (Amend & Pienta 2015, Loberg et al. 2007).

Cancer mortality is disproportionately caused by metastasis, not by the primary tumor. Metastasis is responsible for over 90% of cancer deaths (Rankin and Giaccia 2016). In the cases of breast and prostate cancer, 5-year survival is near 100% for localized (confined to primary site) cancer, but less than 35% for distant (metastatic) cancer (Noone et al. 2018). Therefore, to reduce the mortality and disease burden of cancer, we must improve our understanding of metastasis. However, cancer research so far has focused more on how cancer arises than on how it metastasizes (Gupta and Massagué 2006).

A number of researchers have noted parallels between metastasis and the ecological concept of dispersal (Amend et al. 2016, Chen et al. 2011, Marco et al. 2009). Dispersal occurs when organisms move from where they were born, the natal site, to a new location where they reproduce (Matthysen 2012). Dispersal ecology evaluates the factors that influence organisms to move and the consequences of doing so (Starfelt and Kokko 2012). Because the expected fitness (reproductive success) of organisms varies in time and space, the ability to disperse is an evolutionary adaptation allowing organisms to move to new locations and persist in heterogeneous environments (Starfelt and Kokko 2012). However, the potential benefits of dispersal are counterbalanced by potential costs, such as the

energy required to disperse, the increased risk of mortality and loss of fecundity during transit, and the possibility of not finding a suitable new habitat (Bonte et al. 2012). Because of the risks, dispersal is most advantageous when local environmental conditions are adverse, which increases the probability of finding a new patch elsewhere that is more favorable than the original patch (Stamps 2001). Therefore, organisms often respond to stressors by increasing their dispersal phenotype, or characteristics (Stamps 2001).

Similarly, cancer cells face potential risks during metastasis. Breaking down the basement membrane and undergoing EMT require energy; furthermore, cancer cells are less fecund while in the mesenchymal state and risk destruction by immune cells or shear forces in the bloodstream (Amend et al. 2016).

The benefits of dispersal can include access to resources and escape from natural enemies at the home site. Both of these apply to organismal dispersal, and may provide insight into metastasis by analogy.

Nutrient stress is thought to be an impetus for dispersal, with an organism's dispersal trait inversely related to nutrient availability in the natal environment (Matthysen 2012). Many plant species have multiple seed and achene (single-seed fruit) phenotypes, including one with a greater propensity towards dispersal (e.g., greater aerodynamicity for greater flight time in wind dispersal, or bristles that stick to animal fur). When grown under nutrient-deficient conditions, these plants produce greater proportions of dispersal-favoring achenes (Imbert and Ronce 2001, Martorell and Martínez-López 2013, Teller et al. 2014). An important caveat comes from the silver spoon effect, the tendency for organisms that would benefit least from dispersal to be the most competitive in dispersing, and vice-versa (Stamps 2006). The latter author suggested that stressors that

increase dispersal tendency also decrease the physical strength of the organism; conversely, a lack of stressors reduces dispersal tendency while increasing the organism's physical strength (Stamps 2006). This idea was supported by Martorell and Martínez-López (2013), who found that increased water stress in the annual forb *Heterosperma pinnatum* led to a smaller overall number of achenes produced, with a greater proportion of dispersive achenes; conversely, reduced stress led to a larger overall number of achenes produced, with a lower proportion of dispersive achenes.

Nutrient stress has noteworthy parallels with hypoxia (low-oxygen conditions). Hypoxia is a consequence of cancer; because tumors outgrow the ability of existing vasculature to support them, they induce new blood vessel growth (Amend and Pienta 2015). This allows tumors to increase in mass, but often fails to prevent hypoxia, as the new blood vessels have an abnormal structure that cannot deliver adequate oxygen to all areas of the tumor (Brown and Giaccia 1998, Jain 2003). Just as nutrient stress induces a more dispersal-oriented phenotype in plants (Martorell and Martínez-López 2013), hypoxia is strongly associated with increased invasiveness and EMT in cancer (Amend et al. 2016, Rankin and Giaccia 2016).

Cancer may even exhibit a silver spoon effect, with certain drug treatments shrinking tumors even as they encourage metastasis. Given the importance of angiogenesis in tumor growth, angiogenesis inhibitors are often used in clinical treatment, with some promising results (Kerbel and Folkman 2002). However, by blocking blood vessel formation, angiogenesis inhibitors increase hypoxia in tumors (Pàez-Ribes et al. 2009) and have been associated with increased metastasis, even as they reduce the mass of the primary tumor (Pàez-Ribes et al. 2009, Ebos et al. 2009). From an ecological perspective,

there is no contradiction in this observation: stressors such as hypoxia reduce the physical strength of the organism (debulking the primary tumor) while also encouraging the organism to disperse (increasing metastasis). The theory of dispersal ecology can thus help explain what promotes metastasis, and perhaps discourage clinical use of angiogenesis inhibitors, which only increase survival by months, without offering a persistent cure (Loges et al. 2009).

Apart from escaping nutrient stress, organisms may disperse to escape predation (Matthysen 2012). For example, pea aphids, *Acyrthosiphon pisum*, produce an alarm pheromone, (E)-β-farnesene (EBF), in the presence of predators such as ladybird beetles; EBF causes aphids to walk or drop off of the host plant, and leads to greater proportions of winged offspring (Kunert et al. 2005). In the case of prostate and breast metastases, the bone may offer a refuge from immune attack, which may partially explain why prostate and breast cancer so frequently metastasize to bone (Baschuk et al. 2015).

Escape from hypoxia and refuge from immune attack may help explain why PCa localizes to bone, but not how it becomes established as macrometastases. There is evidence that PCa becomes established in part through associations with bone stromal cells (BSCs, which make up connective tissues supporting outer cell layers) such as bone-producing osteoblasts and bone-absorbing osteoclasts (Dai et al. 2015). Osteoblast activity increases in the presence of PCa, leading to the formation of excess bone; in turn, PCa proliferation increases in the presence of BSCs (Buenrostro et al. 2016).

The interactions between PCa and BSCs are mediated by bone morphogenetic proteins, or BMPs. BMPs are part of the larger transforming growth factor β (TGFβ) protein superfamily, and include several subclasses with many targets and functions; however, a

common function is to promote bone growth by inducing osteoblast precursors (osteoprogenitors) to differentiate into osteoblasts (Chen et al. 2004, Yang et al. 2005).

BMP-4 prompts the osteoblastic PCa line, LNCaP, to express sonic hedgehog (SHH), a gene involved in organ development; SHH in combination with BMP-4 in turn drive the osteoprogenitor line MC3T3.E1 to differentiate into osteoblasts (Nishimori et al. 2012). When MC3T3.E1 was co-cultured with LNCaP and BMP-4, MC3T3.E1 expressed higher levels of growth factors such as fibroblast growth factor-2 (Fgf2) and epidermal growth factor (Egf), which increased the proliferation of LNCaPs relative to LNCaP monoculture (Nishimori et al. 2012). Together, these interactions appear to constititute a feedforward loop in which PCa drives BSC differentiation, which in turn drives PCa proliferation, and so on. It is unclear how this loop is initiated. Nishimori et al. (2012) suggest that the BMPs involved in the loop originate from the bone itself, not from PCa cells. On the other hand, at least one BMP, BMP-6, is more likely to be expressed in metastatic PCa than in benign PCa, or non-cancerous prostate tissue (Barnes et al. 1995, Bentley et al. 1992, Hamdy et al. 1997).

Complicating the picture is the fact that BMP's effect on cancer is dependent on context. Nishimori et al. (2012) found that BMPs promoted PCa growth in the presence of BSCs, but had the opposite effect in the absence of BSCs. The same authors also found low baseline expression of BMPs in PCa monoculture. Other studies suggest that BMPs can be tumor-suppressing in isolation, but can become cancer-promoting by making non-cancer cells in the tumor microenvironment more conducive to cancer growth (Owens et al. 2015). For example, Ye et al. (2009) found that overexpression of BMP-9 in PCa grown in monoculture resulted in decreased proliferation and increased apoptosis (programmed cell

death). Owens et al. (2013) found that mammary fibroblasts stimulated with BMPs secrete factors that promote breast cancer proliferation and metastasis.

The observation that PCa induces abnormal bone growth via excess osteoblast differentiation, and that these osteoblasts promote PCa proliferation and metastasis, raises another intriguing ecological parallel. If PCa changes the behavior of bone cells, promoting its own spread, is it acting like a behavior-manipulating parasite? Manipulation of host behavior by many parasites benefits parasitic reproduction and dispersal. For example, the tapeworm *Schistocephalus solidus* uses the threespine stickleback fish *Gasterosteus aculeatus* as an intermediate host, and predatory birds as the definitive host in which reproduction occurs (Ness and Foster 1999). Infected *G. aculeatus* exhibit unusual behavior in which they swim slower and closer to the surface than uninfected fish, making them more likely to be eaten by a bird and thus allowing the tapeworm to reach its definitive host (Ness and Foster 1999). However, it has been difficult to demonstrate that the behavior is truly manipulation on the part of the parasite; to do so requires showing that the behavior modification improves the parasite's fitness and is directed by the parasite, not an incidental side effect of parasitic infection (Adamo and Webster 2013, Hafer-Hahmann 2019). In the context of prostate cancer, the behavior modification would be the osteoprogenitor's elevated rate of differentiation and excess secretion of growth factors. The "parasite's" fitness is the successful proliferation of prostate cancer, which was already shown to be increased by interacting with BSCs (Nishimori et al. 2012). To demonstrate directed rather than incidental behavior modification would require showing that the feedforward loop is initiated by PCa rather than being an incidental effect of combining PCa and BSCs. My study aimed to test for such an effect.

The role of BMP in the progression and establishment of prostate cancer metastases in bone presents a potential target for cancer therapeutics. In the present study, I evaluated the ability of DorsoMorphin Homolog 1 (DMH1), a BMP inhibitor, to disrupt the feedforward loop between PCa and BSCs. Secondarily, I wanted to determine whether the loop is initiated by PCa, and also what the effects of DMH1 are on PCa and BSCs individually. To do so, I cultured PCa cells of human origin and BSC cells of murine, or mouse origin in monoculture, as well as a co-culture of PCa and BSC, then treated each combination with DMH1. I measured response to DMH1 by measuring expression levels of BMPs -2, -4, and -6, as well as the following osteoblast differentiation markers: parathyroid hormone-related protein (PTHRP), receptor activator of nuclear factor kappa-B ligand (RANKL), and runt-related transcription factor 2 (RUNX2). Use of human PCa and mouse BSC allowed me to distinguish the origin of genes in co-culture (i.e., from PCA or from BSC).

## Hypotheses

**Hypothesis 1:** Prostate cancer in isolation is indifferent to DMH1

In isolation, BMP inhibits the proliferation of PCa, and thereby its fitness. Per the theory of dispersal ecology, lowered expected fitness would increase dispersal tendency, but given that PCa already has a low baseline expression of BMP (Fig. 1A), inhibition wouldn't lower BMP function by much. I hypothesized that DMH1 would not have an effect on BMP expression in PCa (Fig. 1B).


**Figure 1A - PCa has low baseline expression of BMP**

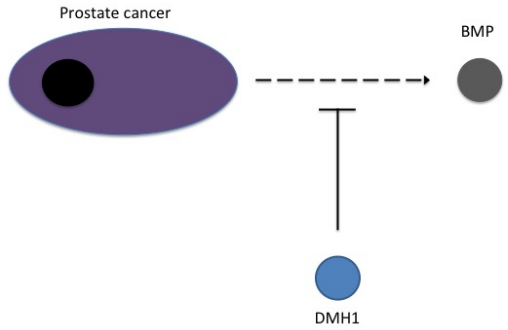



**Figure 1B - DMH1 will have no effect, as baseline expression is already low**

**Hypothesis 2:** In isolation, the differentiation of osteoprogenitors into osteoblasts is blocked by DMH1

BMP signaling drives osteoprogenitors towards differentiation (Fig. 2A). In the absence of PCa, there won't be an overwhelming push towards differentiation, and the action of any stray BMPs will be inhibited by DMH1 (Fig. 2B). Therefore, treated cells were expected to show lower expression of BMPs and of differentiation markers (PTHRP, RANKL, and RUNX2), consistent with inhibited differentiation.

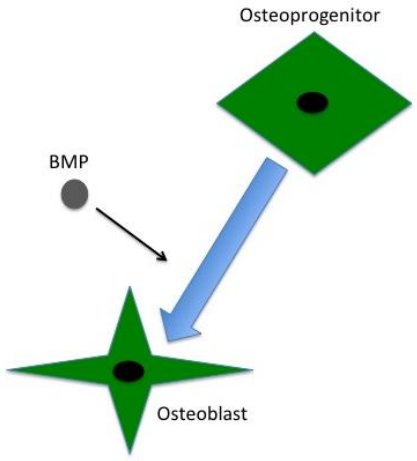



**Figure 2A - BMPs enhance maturation of osteoprogenitors into osteoblasts; mature osteoblasts produce BMPs**

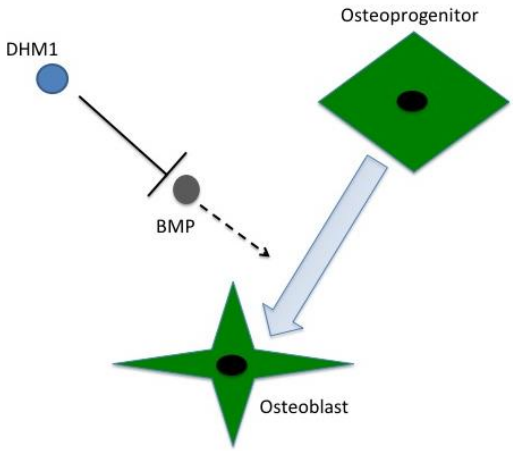



**Figure 2B - Inhibiting BMPs will inhibit osteoblast maturation**

**Hypothesis 3a:** DMH1 disrupts interactions between PCa and BSCs

The mutual promotion of PCa proliferation and osteoblast differentiation in co-culture is mediated by BMPs (Fig. 3A). DMH1 inhibits the action of BMPs. I therefore hypothesized that disrupting the action of BMPs would disrupt both PCa proliferation and osteoblast differentiation (Fig 3B).

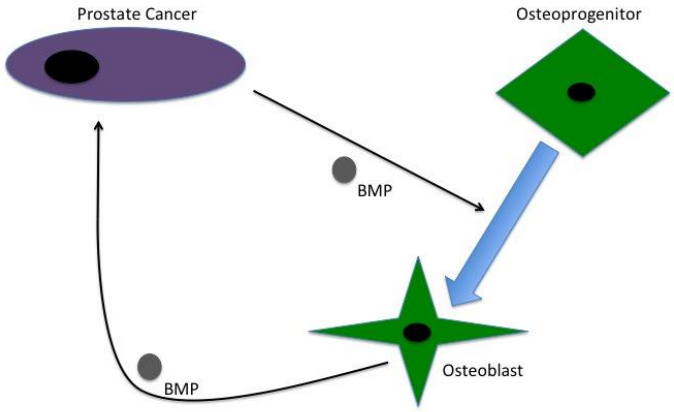



**Figure 3A - Prostate cancer/osteoprogenitor interactions, no intervention**

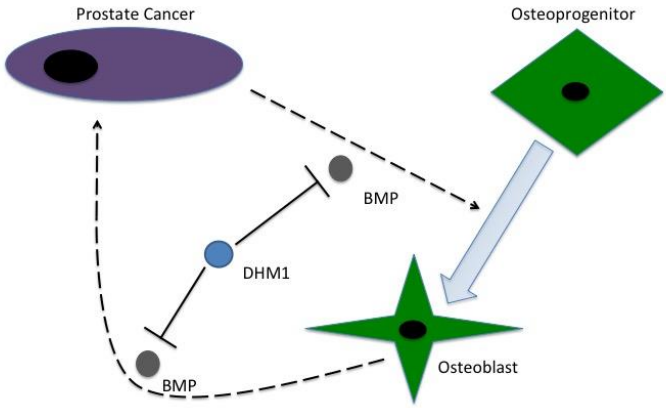



**Figure 3B - Hypothesized effect of DMH1 on prostate cancer/osteoprogenitor interactions**

**Hypothesis 3b:** Prostate cancer's interactions with bone cells are analogous to parasitism

The introduction of PCa to bone creates a feedforward loop involving abnormal bone growth via osteoblasts and promotion of PCA proliferation and metastasis. This appears to be analogous to parasitic manipulation of hosts, in which a parasite alters the typical behavior of the host for its own benefit.

One criterion for parasitic manipulation is that the abnormal behavior is driven by the parasite. BMPs are deleterious to PCa in isolation, but beneficial in the presence of BSCs. I had hypothesized earlier that BMPs in PCa/BSC co-culture would eliminate this beneficial effect. If so, PCa may attempt to restore the beneficial effect by producing more BMPs. I therefore hypothesized that the addition of DMH1 to PCa/BSC co-culture would induce compensatory upregulation of BMPs by PCa (Fig 3C).

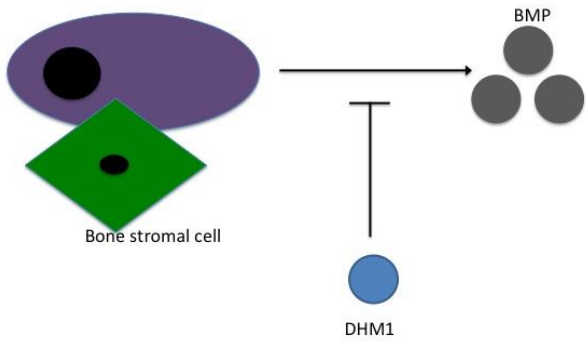



**Figure 3C – Treatment of prostate cancer/bone stromal cell co-culture with DMH1 is hypothesized to induce higher expression of BMP in prostate cancer**

## Materials and Methods

### Cell Culture and DMH1 Treatment

PC3 luc, C4-2B and C3H10T½ cells were grown in DMEM medium with 10% Fetal Bovine Serum (FBS) and antibiotic. MC3T3.E1 cells were grown in αMEM medium with 10% Fetal Bovine Serum (FBS) and antibiotic. Monocultures of PC3 luc, C4-2B, C3H10T½ and MC3T3.E1 were prepared by placing 2 x $10^5$ of each cell line into each well of separate ultra-low adhesion culture 6-well plates with their respective medium. The monocultures received treatment with DMH1 after 36 hours before being allowed 36 additional hours to grow. A co-culture of PC3 and MC3T3.E1 was prepared by first transferring PC3 luc to αMEM for 36 hours, then combining $10^5$ each of PC3 luc and MC3T3.E1 in each well of an

ultra-low adhesion culture 6-well plate with αMEM. The co-culture was allowed 36 hours to grow before receiving DMH1 treatment and an additional 36 hours thereafter to continue growing. DorsoMorphin Homolog 1 was used at 20 μM for all experiments, while an equal quantity of DMSO was used as a vehicle control.

**RNA Isolation, cDNA synthesis, qPCR and primer selection**

RNA isolation was performed with RNeasy purification including DNAseI treatment (Qiagen). Equal quantities of RNA were synthesized into cDNA using the VILO cDNA synthesis kit (Invitrogen). SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) was combined with 1 μM of both a forward and reverse primer sequence into 20 μL reactions and cycled from 95° for 10s to 60° for 30s for 40 cycles followed by a melting curve. Bio-Rad CFX96 was used and instrument-provided software was used to determine relative normalized expression to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression; GAPDH was chosen as a control because its expression is expected to be stable (Barber et al. 2005).

**Visualization**

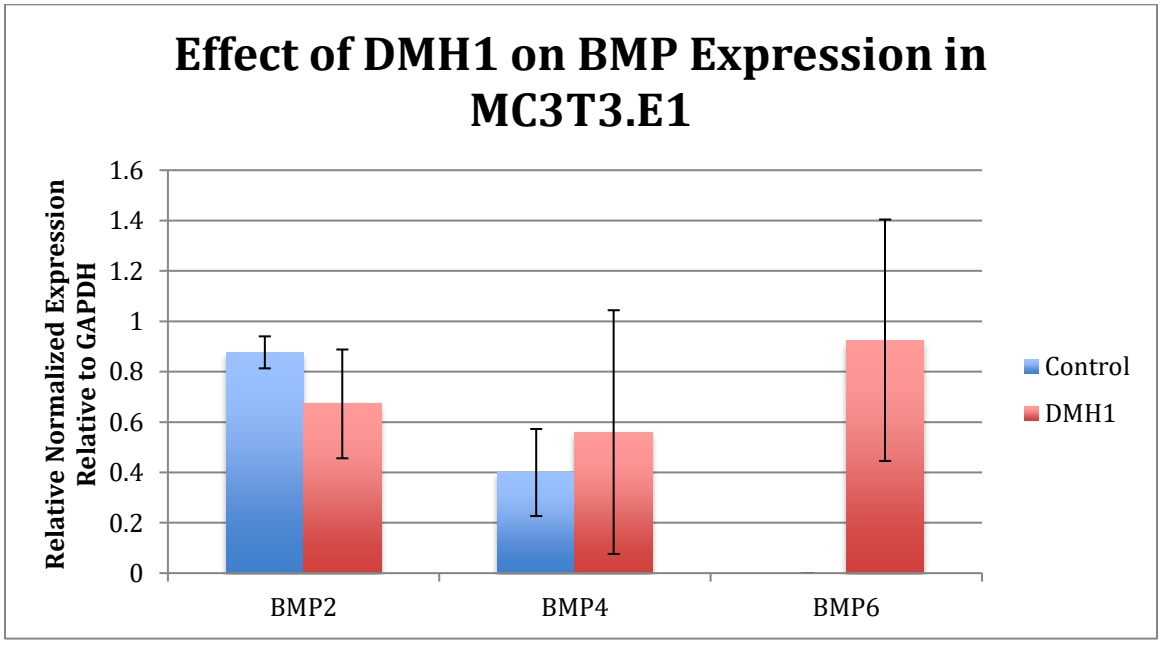
An additional set of experiments was performed to visualize the effects of DMH1 treatment. PC3, C4-2B, and MC3T3.E1 monocultures and C4-2B/MC3T3.E1 and PC3/ MC3T3.E1 co-cultures were placed in 8-well chamber slides. They were allowed to grow for 36 hours before and after receiving treatment with DMH1. Four different combinations were used to stain the cells: 1) $Ecad^{594}$ and $Vim^{488}$, 2) $\alpha SMA^{488}$ and $KS^{594}$, 3) Ki67 and $Phalloidin^{488}$, and 4) pSmad $\frac{1}{5}^{594}$ and $Phalloidin^{488}$.

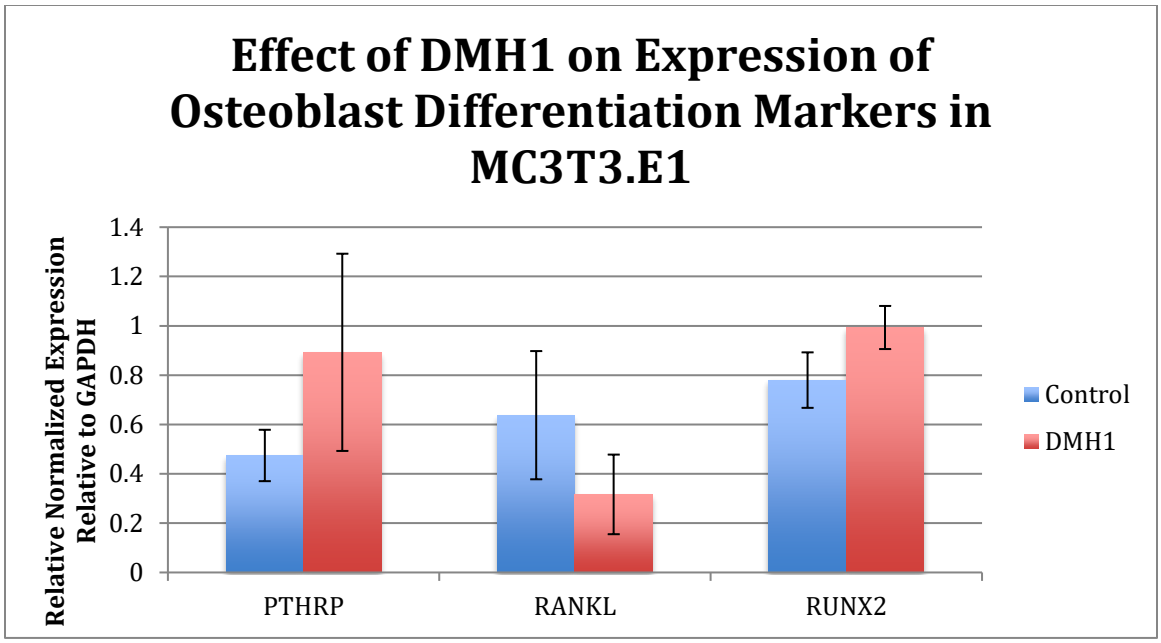
## Results

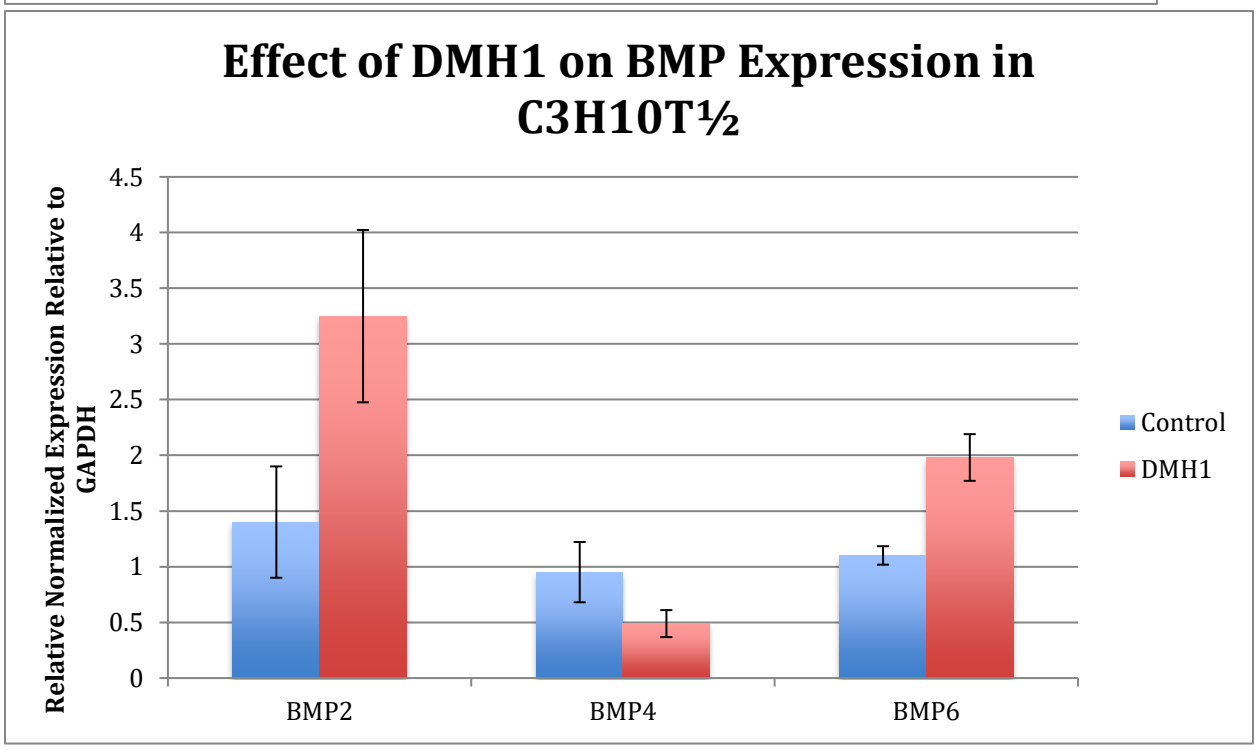
**Effect of DMH1 on Osteoprogenitors**

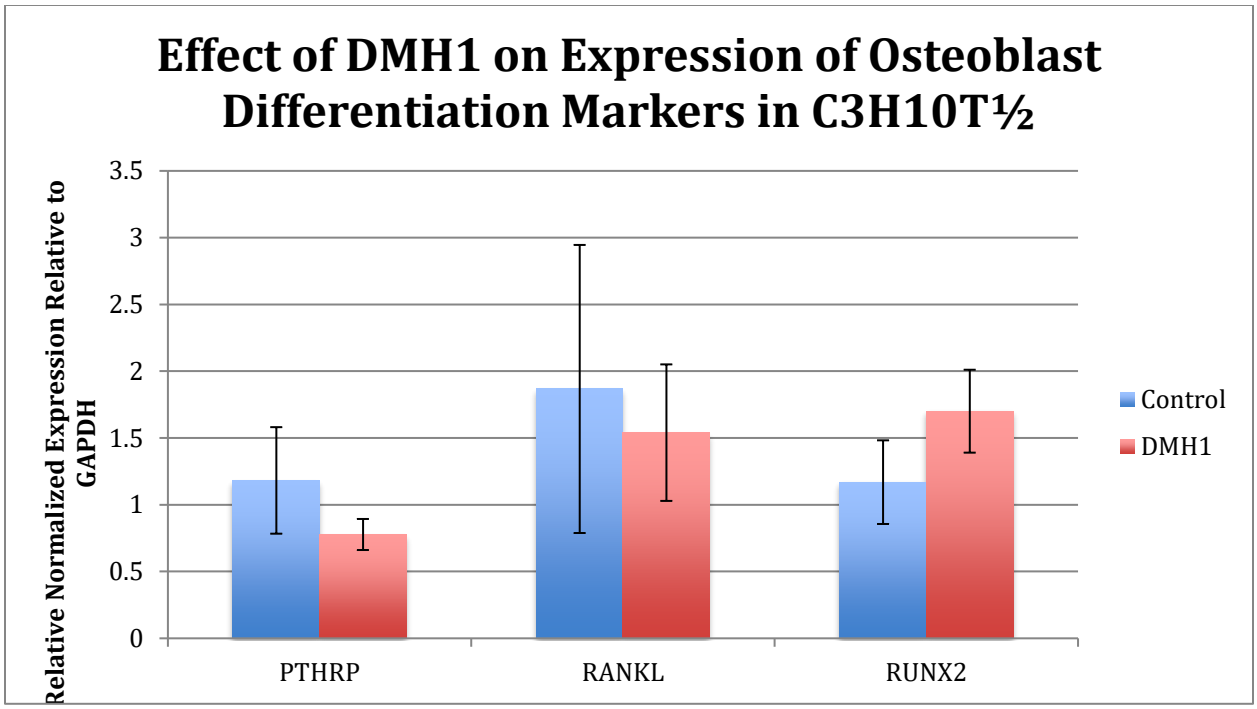
Two separate osteoprogenitor lines, MC3T3.E1 and C3HT10½, were examined in monoculture. MC3T3.E1 exhibited non-significant increases to expression of BMPs 4 and 6 and a non-significant decrease to BMP 2 in response to treatment with DMH1. It also underwent non-significant increases to PTHrP and RUNX2, and a non-significant decrease to RANKL.

C3HT10½ treated with DMH1 exhibited higher expression of BMP 2 and lower expression of BMP 4 than the control, but neither result was significant at the $P < 0.05$ level. It also exhibited a significant 1.8-fold increase to BMP6 expression ($p = 0.010$). As for bone differentiation markers, it had non-significant decreases to PTHrP and RANKL and a non-significant increase to RUNX2.




Effect of DMH1 on Expression of Osteoblast Differentiation Markers in MC3T3.E1
Relative Normalized Expression Relative to GAPDH
1.4
1.2
1
0.8
0.6
0.4
0.2
0
PTHRP
RANKL
RUNX2
Control
DMH1

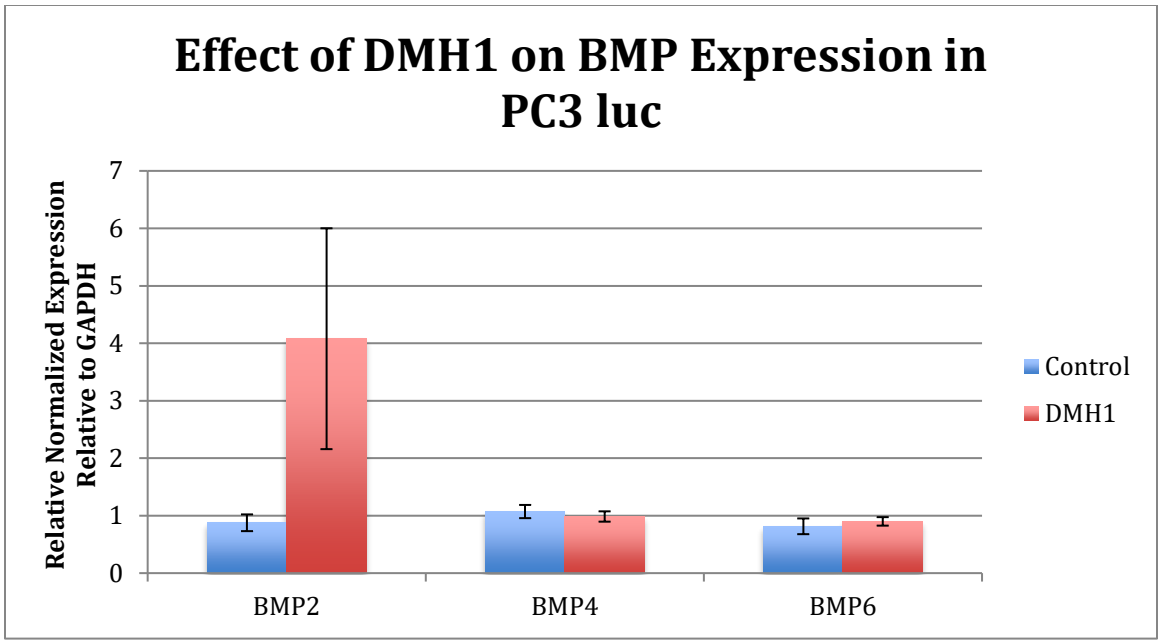

Effect of DMH1 on BMP Expression in C3H10T½
Relative Normalized Expression Relative to GAPDH
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
BMP2
BMP4
BMP6
Control
DMH1

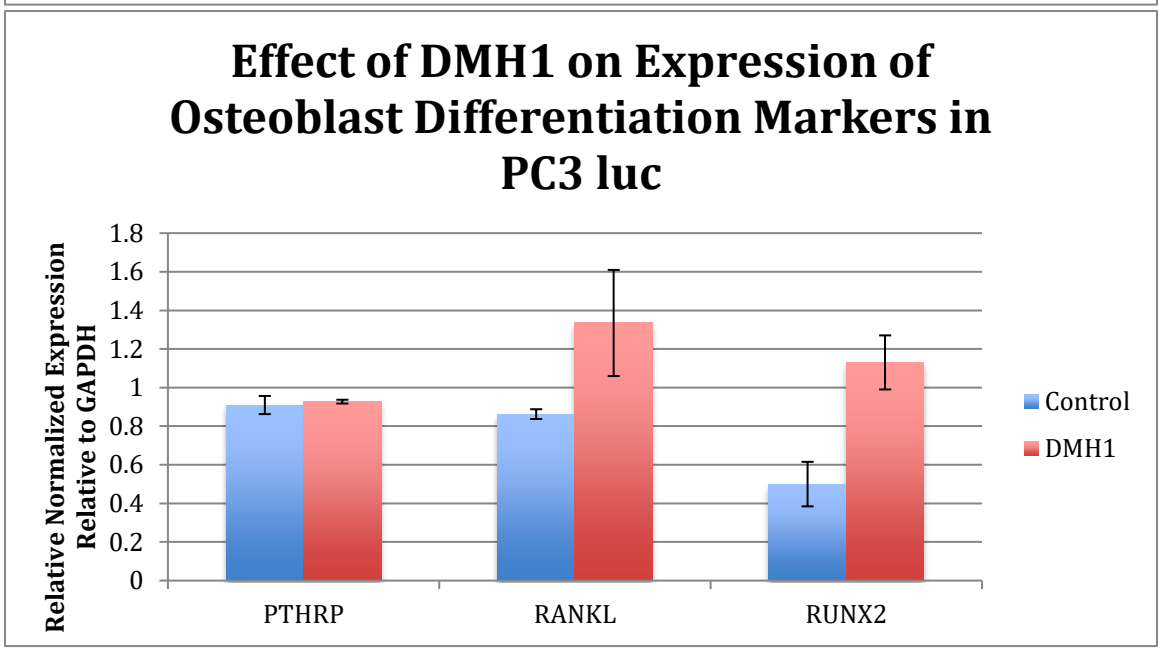


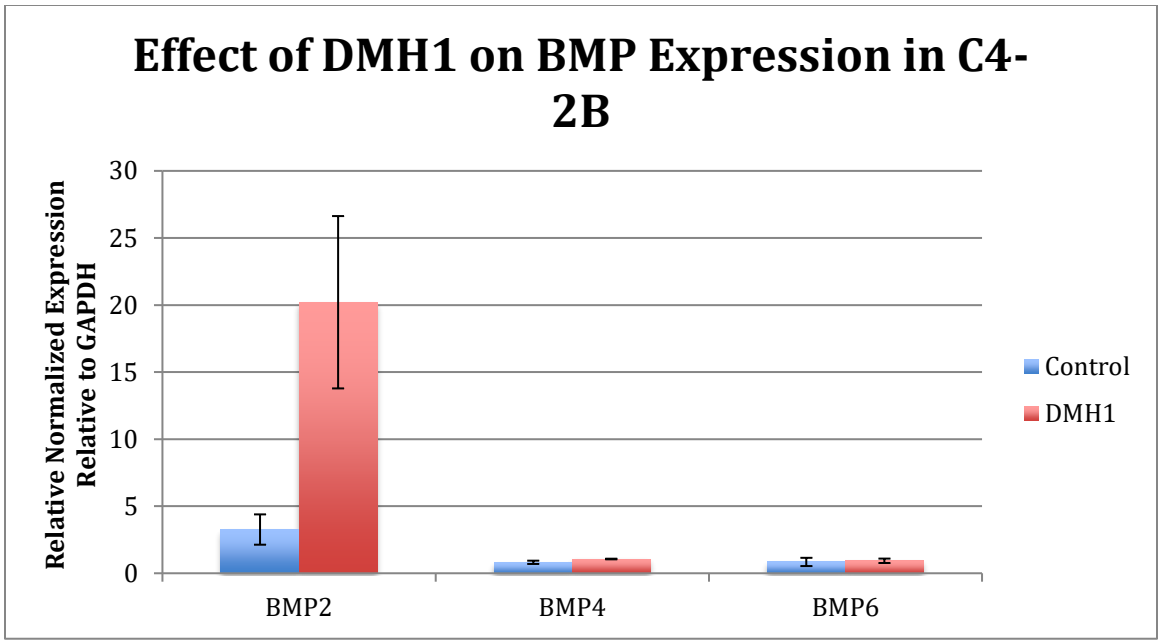

**Effect of DMH1 on Prostate Cancer**

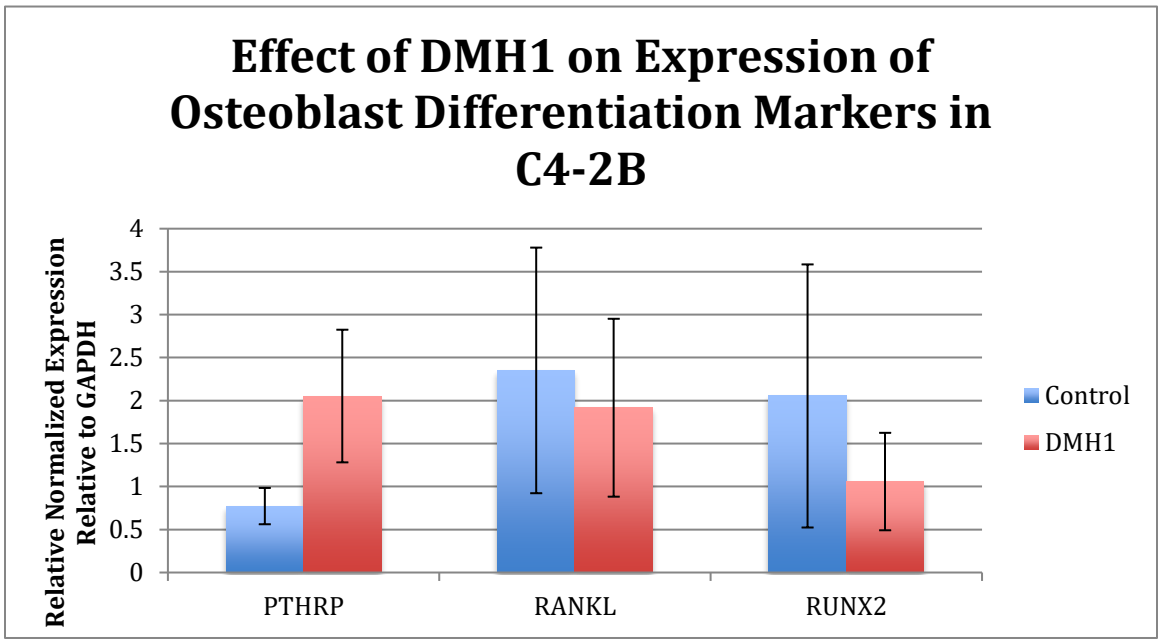
The PCa lines PC3 luc and C4-2B were studied in monoculture. The PCa line PC3 luc exhibited a small, non-significant decrease to BMP4 expression, as well as non-significant to BMP 2 and 6. It also showed small, non-significant increases in PTHrP and RANKL expression, as well as a significant 2.3-fold increase to RUNX2 expression ($p = 0.0008$).

C4-2B demonstrated increases to BMPs -2, -4 and -6, but none of those increases were significant. It showed non-significant increases to PTHrP, and a non-significant decrease to RANKL and RUNX2.


Effect of DMH1 on BMP Expression in PC3 luc
Relative Normalized Expression Relative to GAPDH
7
6
5
4
3
2
1
0
BMP2
BMP4
BMP6
Control
DMH1


Effect of DMH1 on Expression of Osteoblast Differentiation Markers in PC3 luc
Relative Normalized Expression Relative to GAPDH
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
PTHRP
RANKL
RUNX2
Control
DMH1



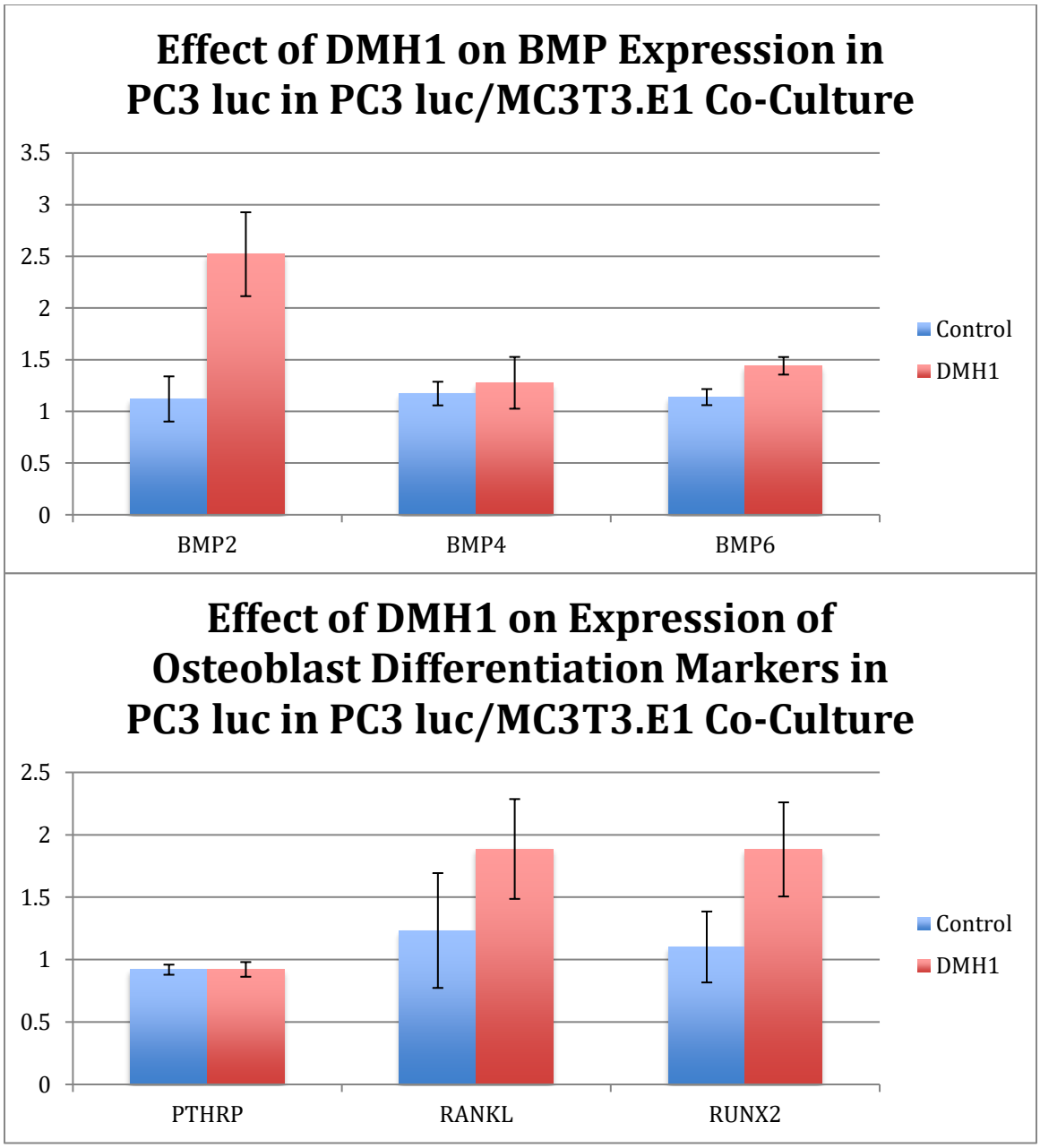



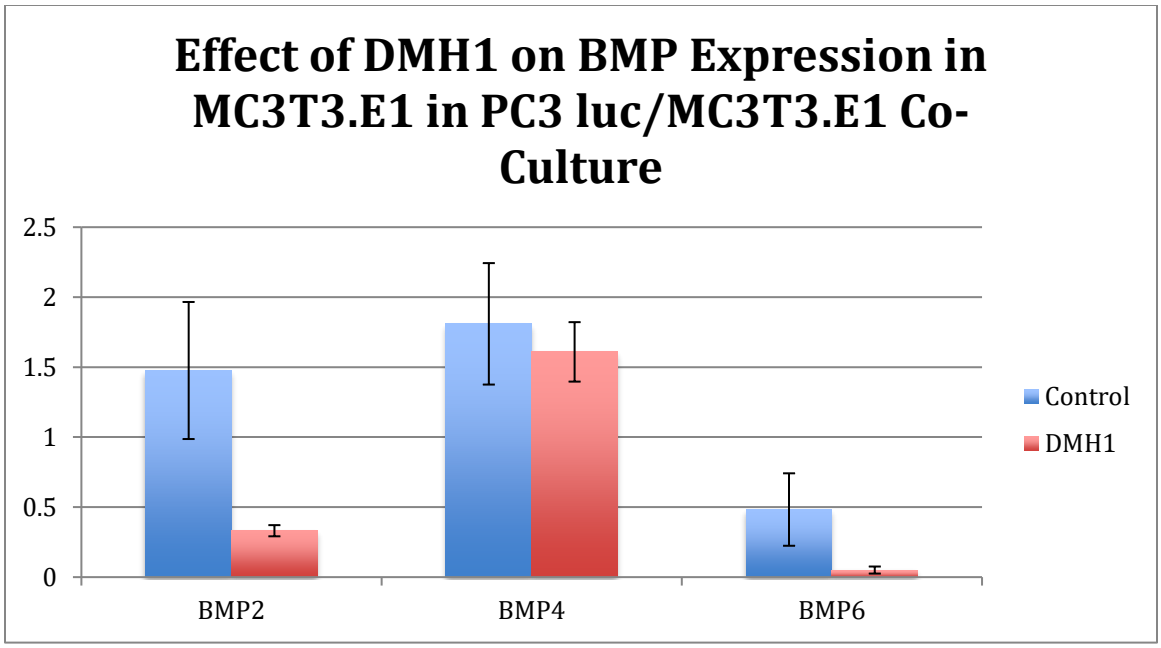

**Effect of DMH1 on Osteoprogenitor/Prostate Cancer Co-Culture**

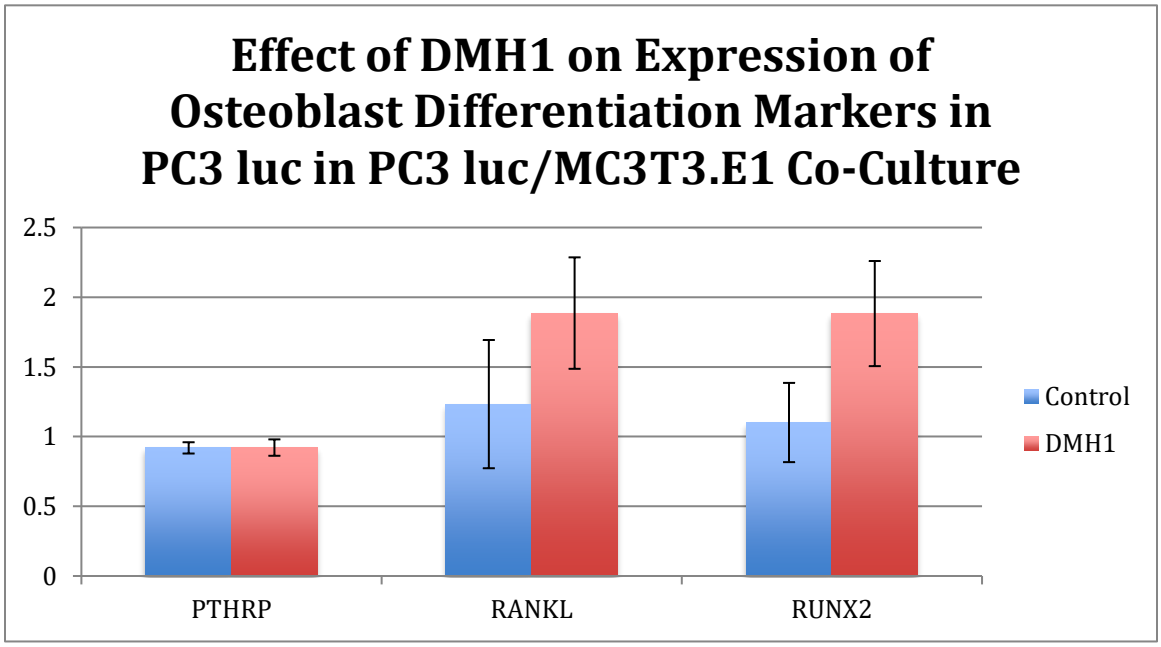
PC3 luc and MC3T3.E1 were co-cultured to study the effect of BMP inhibition on prostate cancer and bone cells together. I found that prostate cancer upregulated two classes of BMP in response to DMH1 treatment; PC3 luc exhibited a significant 2.25-fold increase in BMP2 expression and a significant 1.27-fold increase in BMP-6 expression (with

p-values of 0.042 and 0.006, respectively), and a 1.09-fold increase to BMP-4, but this was not significant ($p = 0.307$). PC3 luc also showed a significant 1.71-fold increase to expression of RUNX2, as well as non-significant increases to PTHrP and RANKL. Interestingly, MC3T3.E1 showed decreased expression of all classes of BMP (2, 4 and 6), as well as decreases in PTHrP and RANKL and an increase in RUNX2, but none of these changes were significant at the $p < 0.05$ level. A full summary of all statistical results may be found in the appendix.

Visually, the untreated PC3 luc cells of the co-culture grew more densely than the treated PC3 cells, and were more clustered around bone cells. When treated with DMH1, PC3 luc in co-culture grew less densely and with less clustering around bone cells.


Effect of DMH1 on BMP Expression in PC3 luc in PC3 luc/MC3T3.E1 Co-Culture
3.5
3
2.5
2
1.5
1
0.5
0
BMP2
BMP4
BMP6
Control
DMH1
Effect of DMH1 on Expression of Osteoblast Differentiation Markers in PC3 luc in PC3 luc/MC3T3.E1 Co-Culture
2.5
2
1.5
1
0.5
0
PTHRP
RANKL
RUNX2
Control
DMH1


Effect of DMH1 on BMP Expression in MC3T3.E1 in PC3 luc/MC3T3.E1 Co-Culture
2.5
2
1.5
1
0.5
0
BMP2
BMP4
BMP6
Control
DMH1


Effect of DMH1 on Expression of Osteoblast Differentiation Markers in PC3 luc in PC3 luc/MC3T3.E1 Co-Culture
2.5
2
1.5
1
0.5
0
PTHRP
RANKL
RUNX2
Control
DMH1

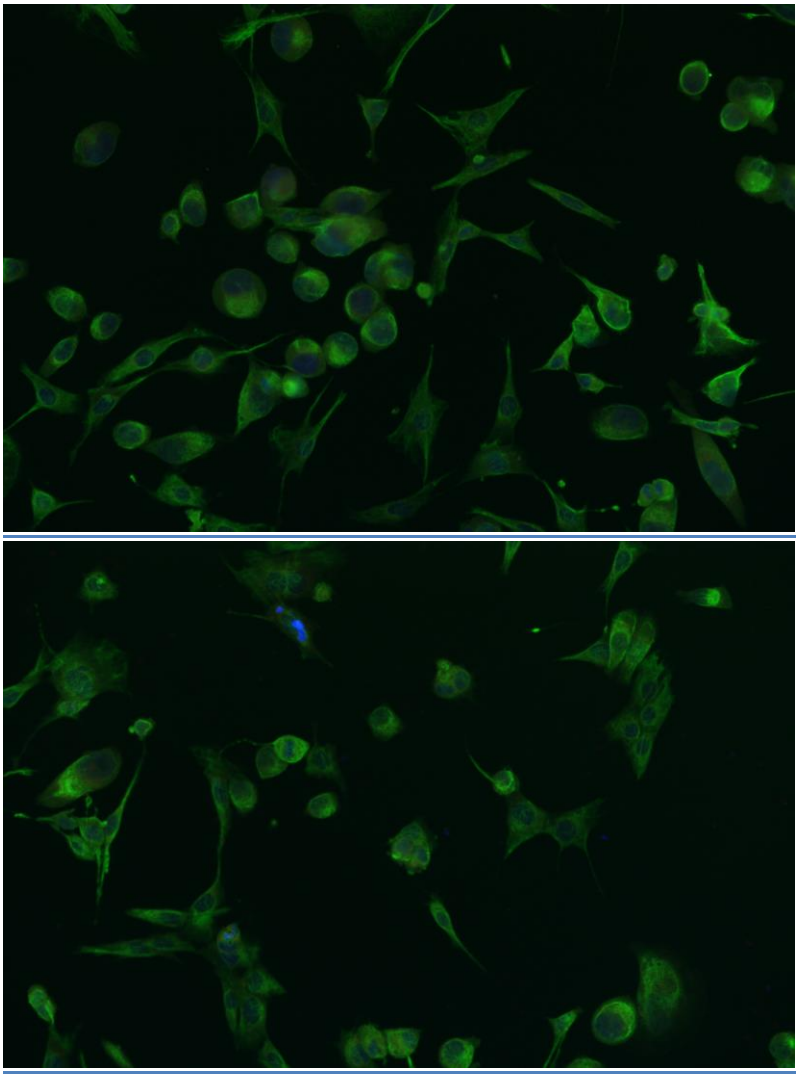


**Figure 4 – top: PC3 luc/MC3T3.E1 co-culture, untreated (20x magnification); bottom: PC3 luc/MC3T3.E1 co-culture, DMH1 (20x magnification)**

## Discussion

Given the tumorigenesis and metastasis-promoting properties of BMPs, as well as the observation that BMP inhibition blocks these processes, BMP signaling is a potential target for cancer therapeutics. However, the effects of BMP-signaling are often dependent on context; in general, BMPs appear to slow the progression of cancer in isolation and accelerate it in the presence of stromal cells. To properly target BMPs, it is necessary to understand the interactions between cancer and stroma that influence the effect of BMPs.

The present study characterized the effects of BMP inhibition on both prostate cancer and osteoprogenitors in isolation and together, and to determine if the interactions between the two are analogous to parasitic manipulation.

**Osteoprogenitor monoculture**

BMPs are both a cause and consequence of osteoblast differentiation. BMPs induce osteoprogenitors to become osteoblasts; in turn, osteoblasts produce more BMPs than their undifferentiated precursors did. An additional consequence of osteoblast differentiation is the increased expression of the bone differentiation markers PTHrP, RANKL, and RUNX2.

If DHM1 inhibits the action of BMP, which in turn inhibits osteoblast differentiation, then the DMH1-treated group is expected to show lower differentiation than the untreated group. I therefore hypothesized that MC3T3.E1 and C3H10T½ treated with DMH1 would show lower levels of BMP and bone differentiation marker expression than the untreated group.

The results were not at all consistent with what I hypothesized. MC3T3.E1 exhibited increases in the expression of BMP-4, BMP-6, PTHrP, and RUNX2, and decreases in BMP-2 and RANKL. Of these, the only significant result was the increase in RUNX2, which was a 1.27-fold increase. C3H10T½ saw increases in BMP-2, BMP-6, and RUNX2, and decreases in BMP-4, PTHrP. Of these, the only significant result was the 1.8-fold increase to BMP-6. I had hypothesized that both BMP and bone differentiation marker levels would decrease across the board, but instead found an inconsistent pattern of increase and decrease, with very few significant results, and thus concluded that DMH1 had no discernible effect on osteoblast differentiation in co-culture.

Apart from the possibility of human error during experimentation, one possible explanation is that DMH1 treatment failed to inhibit osteoblast differentiation, and consequently, there was no difference between the control and DMH1 groups because both had differentiated. A second possibility is that neither the DMH1-treated cells nor the control cells differentiated, and thus both groups were indistinguishable. Given that osteoblasts require about a month to differentiate while the timeframe of the experiment was only 48 hours, the second explanation is more likely. If I were to repeat the study, I would treat the cells for a longer period of time.

**Prostate cancer monoculture**

In isolation, PCa is expected to exhibit slower proliferation when exposed to BMP-4. Furthermore, the PCa lines LNCaP and CWR22 (Nishimori et al. 2012) do not exhibit high baseline expression of BMP-4. If PCa in monoculture doesn't require BMP to proliferate, and in fact proliferates more efficiently without BMP, this suggests that PCa would be largely indifferent to BMP inhibition. Based on this, I hypothesized that PC3 luc and C4-2B treated with DMH1 would not demonstrate significant changes in BMP expression relative to the untreated group.

The results were generally consistent with my hypothesis. In the case of both PC3 luc and C4-2B, the expression of BMP4 and 6 were nearly unaffected by DMH1 (less than a 1.5-fold increase or decrease in all cases), and none of the results were significant. Slightly less consistent with my hypothesis were the results for BMP2. Both PC3 luc and C4-2b exhibited greatly increased (4 to 6-fold) expression of BMP2, but these results were nonsignificant ($p = 0.130$ and $p = 0.076$, respectively). Though not significant, the magnitude of the increases was much larger than any other increase or decrease in gene

expression in the study, and both PCa cell lines exhibited upregulation in the same class of BMP. This could indicate that PCa cells in isolation *do* respond to BMP inhibition after all; if so, it could indicate that under the conditions the PCa cells were grown, BMP was more important to the function of those cells than I had thought. Further research is needed to clarify this question.

The observation that PCa is indifferent to DMH1 in monoculture, coupled with prior research on BMP's tumor suppressor effect on prostate cancer in isolation (Nishimori et al. 2012, Ye et al. 2008), shows that DMH1 may be more effective as a treatment for metastatic prostate cancer. Indeed, if BMP slows the progress of non-metastatic PCa, DMH1 may even accelerate the development of non-metastatic PCa.

**Inhibition of prostate cancer and osteoprogenitor interactions**

When the two halves of the PCa/bone cell loop are put together, BMP exhibits different effects than on either one separately. Instead of inhibiting PCa growth, BMP-4 encourages PCa proliferation, perhaps by inducing bone cells to produce additional growth factors. Furthermore, osteoprogenitors became more sensitive to BMP signaling in the presence of PCa cells, perhaps because BMP-4 induced PCa to upregulate SHH, which in turn enhanced BMP signaling by upregulating other molecules in the BMP signaling pathway (Nishimori et al.). The common element is BMP signaling; by inhibiting it, DMH1 has the potential to disrupt both sides of this loop, preventing excessive osteoblast differentiation and prostate cancer proliferation. I therefore hypothesized that treatment of PCa/bone cell co-culture with DMH1 would lead to lower expression of bone differentiation markers by bone cells and less proliferation of PCa.

I found no evidence that BMP inhibition interfered with osteoblast differentiation in co-culture. DHM1-treated MC3T3.E1 cells did not express significantly reduced levels of osteoblast differentiation markers relative to the control; furthermore, the non-significant changes were not all decreases- while DMH1 lowered expression of PTHRP and RANKL, it increased expression of RUNX2. Again, this was likely due to the short timeframe of the experiment; if I were to repeat the study, I would treat the cells over a longer period.

PCa proliferation appeared to be inhibited by DMH1 treatment. Microscopy revealed thinner, less dense clustering of PCa cells treated with DMH1; furthermore, PCa cells were less clustered around bone cells. However, this evidence is fairly weak as it is not quantitative; if I were to repeat the study, I would count the number of cells.

**Prostate cancer as a behavior-manipulating parasite**

There appear to be parallels between PCa and behavior-manipulating parasites. The behavior of bone changes when PCa metastasizes to that site; instead of dynamic equilibrium between bone formation and resorption, PCa induces excess bone formation via osteoblasts. The abnormal behavior of hosts benefits their parasites' reproduction and dispersal, and analogously, the excess activity of osteoblasts creates an environment suitable for the formation of PCa metastases. Parasites tend to be host-specific, preferentially parasitizing certain host types (Roberts et al. 2009); similarly, PCa preferentially metastasizes to the bone and the brain over other potential sites in the body. For the parasitism analogy to hold, the behavior modification would have to be driven by PCa, not by BSCs. I hypothesized that DMH1 in co-culture would lead to increased BMP expression in PCa but not in BSCs.

This hypothesis was supported. PC3 luc cells in co-culture expressed significantly higher levels of BMP-2 and BMP-6, as well as a non-significant increase to BMP-4. MC3T3.E1 cells in co-culture did not undergo significant changes to BMP expression. Since PCa (the parasite) but not BSCs (the host) resists efforts to disrupt the loop, this shows that the loop is being driven by PCa, not by BSCs or as an incidental consequence of the two cell types interacting. This result is consistent with the parasitic manipulation analogy.

To further investigate if PCa's effect on bone is analogous to parasitism, I would investigate the effects of metastases to bone from cancer types that don't typically locate to bone. If colon cancer, for example, failed to change the behavior of bone cells, this would eliminate the possibility that the altered bone cell behavior is merely a byproduct of metastasis by any cancer type, strengthening the parasitic analogy of PCa. Another relevant question is whether PCa upregulate BMPs when exposed to BSCs. BMPs inhibit PCa growth in isolation, and stimulate it in the presence of BSCs; if PCa were analogous to a parasite, it would begin producing chemical signals to manipulate its "host" when exposed to the "host."

## Conclusion

*'The haft of the arrow had been feathered with one of the eagle's own plumes. We often give our enemies the means of our own destruction.'*

*-Aesop, The Eagle and the Arrow*

The response of PCa cells to BMP inhibition by DMH1 depends on the context. Consistent with literature, I found that PC3 luc and C4-2B were largely indifferent to DMH1 when treated in monoculture. In co-culture with MC3T3.E1 osteoprogenitors, PC3 luc resisted the efforts to disrupt its interactions with MC3T3.E1 by upregulating BMP

expression. Whether that disruption was successful or not is unclear; prostate cancer proliferation appeared inhibited, but osteoblast differentiation did not.

In either case, the observation that PC3 luc resisted interference in its interactions with bone cells fits into a larger pattern. Prostate cancer disproportionately metastasizes to bone, and depends on bone stromal cells to become established. By turning BMPs from a liability into an asset, bone cells create a more hospitable environment for prostate cancer growth, perhaps even more hospitable than the native site of the prostate.

Through an ecological lens, metastasis can be seen as analogous to dispersal. By metastasizing to bone, PCa cells avoid stressors in their native site and become established in bone through interactions with bone cells. These interactions are themselves analogous to parasitic manipulation, in which a parasite alters the behavior of the host for its own benefit, often destroying the host in the process.

This opens the door to an alternative way to treat cancer. In addition to directly killing cancer cells, we can halt the progress of cancer by blocking its interactions with other cells, denying them a chance to become established as metastases. By understanding the factors that lead to metastasis, perhaps one day we can even prevent cancer from leaving the primary tumor in the first place.

## Appendix

"mRNA" is the gene whose expression is being tested. "Ratio" is the relative normalized expression relative to GAPDH of the DMH1 group divided by that of the control group. The "P-value" is for a t-test of whether the difference described in "ratio" is significant. Significant results are color-coded red, non-significant results are in black.

**PC3 luc monoculture**

| mRNA | Ratio | P-value |
|---|---|---|
| BMP2 | 4.65 ↑ | 0.130 |
| BMP4 | 0.920 ↓ | 0.245 |
| BMP6 | 1.105 ↑ | 0.362 |
| PTHRP | 1.019 ↑ | 0.392 |
| RANKL | 1.548 ↑ | 0.125 |
| RUNX2 | 2.261 ↑ | 0.001 |

**C4-2B monoculture**

| mRNA | Ratio | P-value |
|---|---|---|
| BMP2 | 6.200 ↑ | 0.076 |
| BMP4 | 1.303 ↑ | 0.098 |
| BMP6 | 1.101 ↑ | 0.315 |
| PTHRP | 2.658 ↑ | 0.075 |
| RANKL | 0.815 ↓ | 0.264 |
| RUNX2 | 0.515 ↓ | 0.281 |

**MC3T3.E1 monoculture**

| mRNA | Ratio | P-value |
|---|---|---|
| BMP2 | 0.766 ↓ | 0.255 |
| BMP4 | 1.402 ↑ | 0.329 |
| BMP6 | Undefined ↑ | 0.097 |
| PTHRP | 1.882 ↑ | 0.155 |
| RANKL | 0.496 ↓ | 0.182 |
| RUNX2 | 1.273 ↑ | 0.007 |

**C3H10T½ monoculture**

| mRNA | Ratio | P-value |
|---|---|---|
| BMP2 | 2.321 ↑ | 0.079 |
| BMP4 | 0.514 ↓ | 0.061 |
| BMP6 | 1.799 ↑ | 0.010 |
| PTHRP | 0.658 ↓ | 0.186 |
| RANKL | 0.825 ↓ | 0.339 |
| RUNX2 | 1.454 ↑ | 0.095 |

**PC3 luc in PC3 luc/MC3T3.E1 co-culture**

| mRNA | Ratio | P-value |
|---|---|---|
| BMP2 | 2.250 ↑ | 0.042 |
| BMP4 | 1.089 ↑ | 0.307 |
| BMP6 | 1.266 ↑ | 0.006 |
| PTHRP | 1.002 ↑ | 0.492 |
| RANKL | 1.530 ↑ | 0.247 |
| RUNX2 | 1.710 ↑ | 0.038 |

**MC3T3.E1 in PC3 luc/MC3T3.E1 co-culture**

| mRNA | Ratio | P-value |
|---|---|---|
| BMP2 | 0.225 ↓ | 0.074 |
| BMP4 | 0.889 ↓ | 0.273 |
| BMP6 | 0.105 ↓ | 0.110 |
| PTHRP | 0.875 ↓ | 0.092 |
| RANKL | 0.235 ↓ | 0.123 |
| RUNX2 | 1.478 ↑ | 0.065 |

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