



# KMN-159, a Novel EP<sub>4</sub> Receptor Agonist, Stimulates Osteoblastic Differentiation of Human Bone Marrow-Derived Mesenchymal Stem Cells

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## KEY FINDING KMN-159 induces osteogenesis in human mesenchymal stem cells through EP<sub>4</sub> activation.

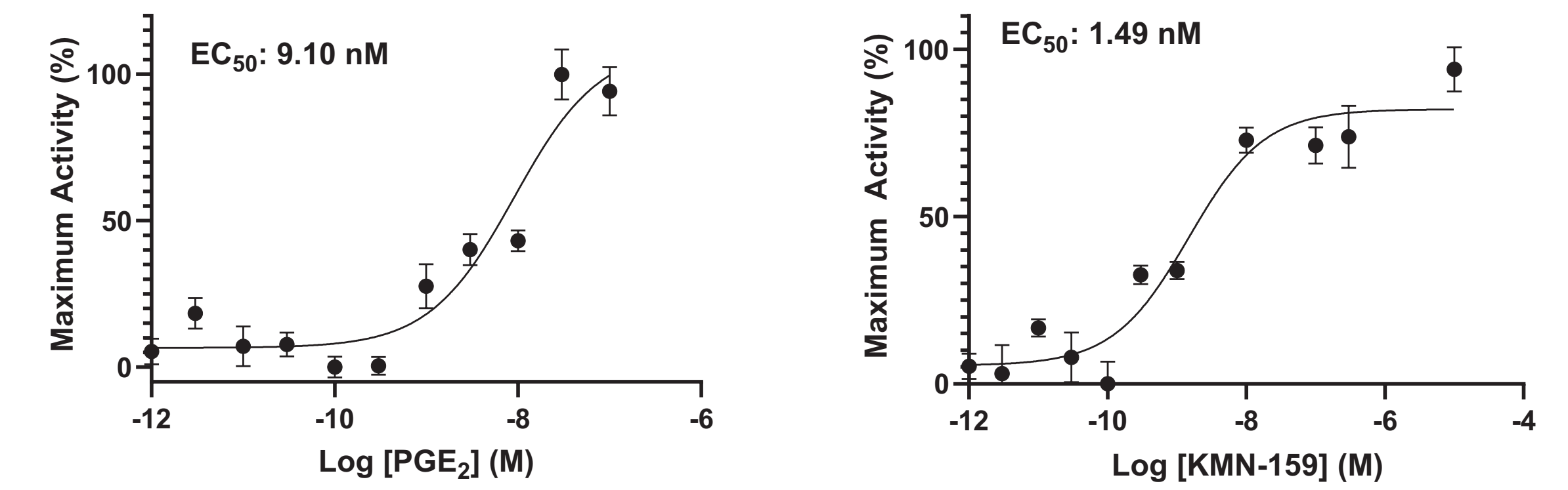
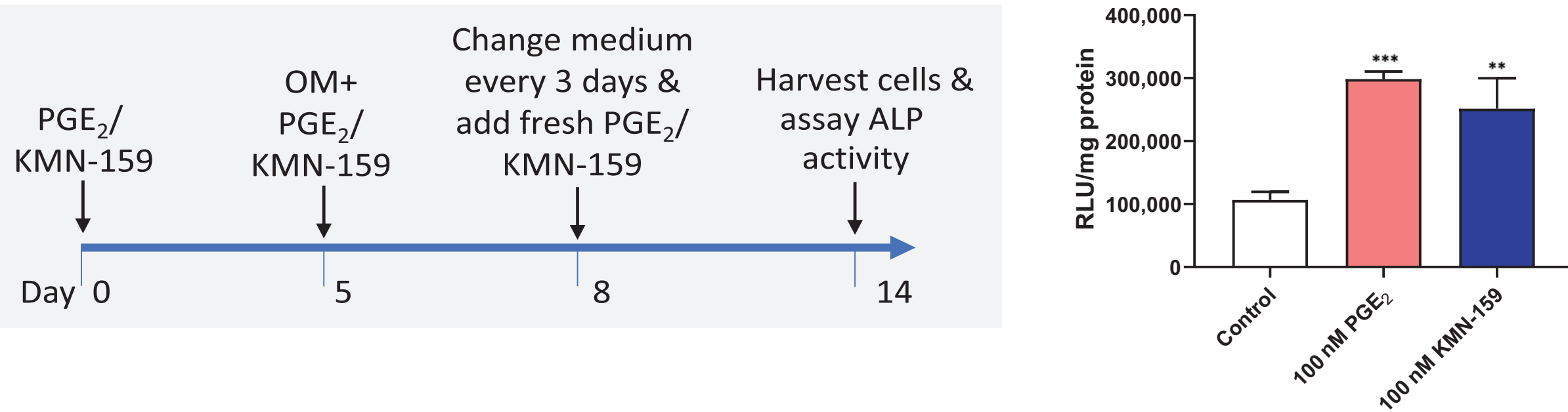
### Introduction

Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) is a potent endogenous molecule that exerts various biological effects by binding to prostaglandin receptors EP<sub>1-4</sub>. Of the four EP receptors, PGE<sub>2</sub> is thought to mediate its bone anabolic effects through the EP<sub>4</sub> receptor. EP<sub>4</sub> has been found to be involved in bone formation by osteoblasts and resorption of existing bone by osteoclasts. However, PGE<sub>2</sub> cannot be used as a therapeutic agent for bone growth/regeneration due to undesired effects mediated through the EP<sub>1</sub> and EP<sub>3</sub> receptors. To circumvent this, we synthesized and developed a series of novel difluorolactam small molecule EP<sub>4</sub> receptor agonists. KMN-159, the lead compound from this series, has a high selectivity and potency for EP<sub>4</sub> receptor binding and activation as well as excellent medicinal chemical properties. In the present work, we demonstrate that KMN-159 stimulates osteoblastic differentiation of human bone marrow-derived mesenchymal stem cells (hBM-MSCs). Cells stimulated with KMN-159 in the presence of osteogenic media (OM) show increased alkaline phosphatase activity and increased mRNA levels of osteoblast phenotype markers. These changes in alkaline phosphatase activity and gene expression do not appear to be related to a simple change in cell number following KMN-159 treatment of hBM-MSCs. KMN-159 also increased the osteoclastic differentiation of RAW 264.7 cells in the presence of RANKL as shown by an increased number of TRAP positive cells.

### Methods and Results

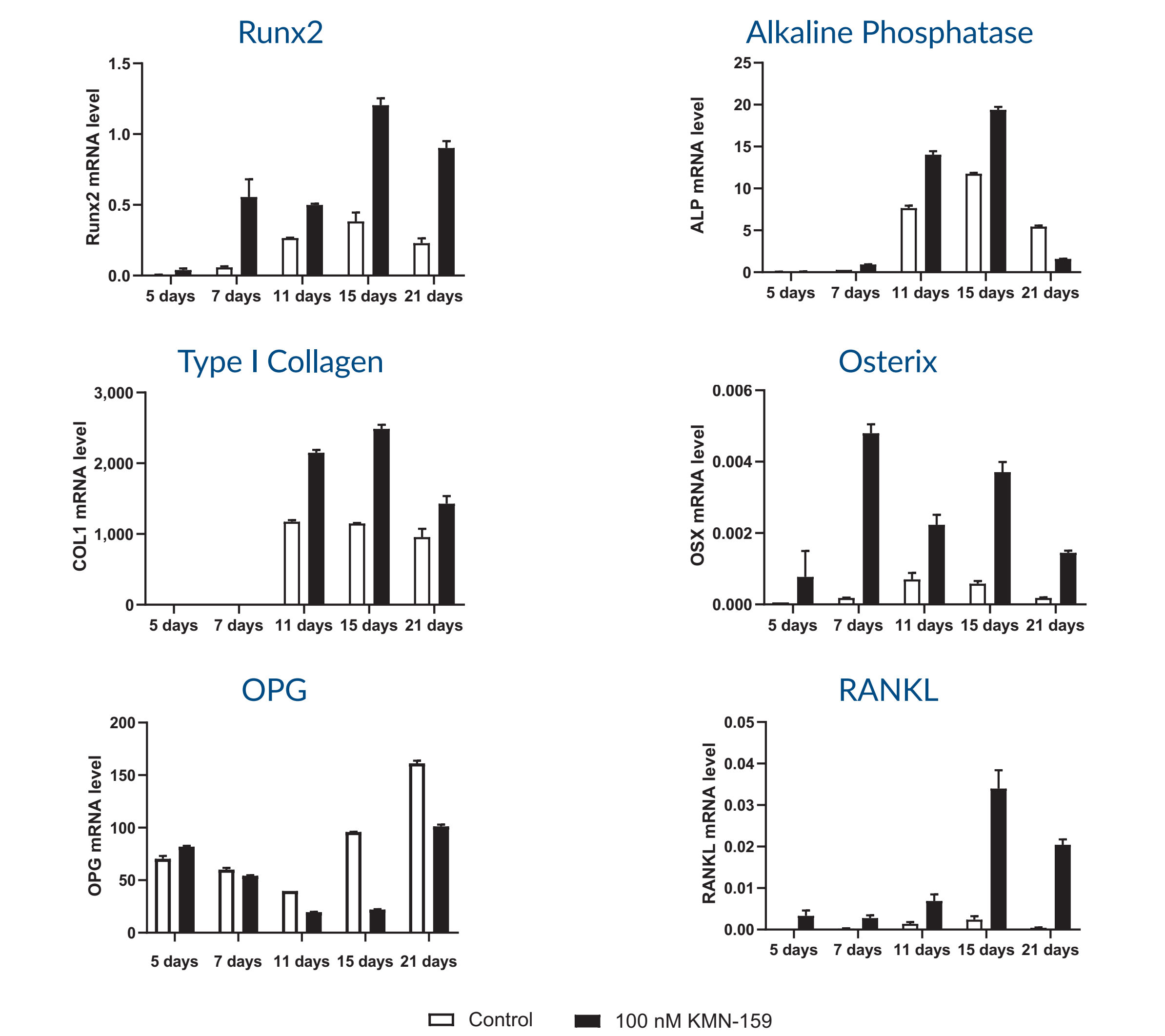
#### KMN-159 Increases Alkaline Phosphatase (ALP) Activity in hBM-MSCs in the Presence of Osteogenic Media

To determine the effect of KMN-159 on osteogenesis, hBM-MSCs were plated in mesenchymal stem cell basal media with growth supplements (ATCC) in 24-well plates in the presence of either 100 nM KMN-159 or PGE<sub>2</sub> (or vehicle) with n = 8 per treatment. Beginning on day 5, cells were fed with the compounds in OM (basal growth medium plus 10 mM β-glycerophosphate (βGP), 10 nM dexamethasone, and 100 μg/ml L-ascorbic acid). The cells were harvested on day 14, and ALP activity was assessed. KMN-159 significantly increased ALP activity in hBM-MSCs, with efficacy similar to PGE<sub>2</sub>, and depicted a dose-dependent increase in ALP activity with an EC<sub>50</sub> value of 1 nM, approximately 10 times more potent than that observed following treatment with PGE<sub>2</sub>.



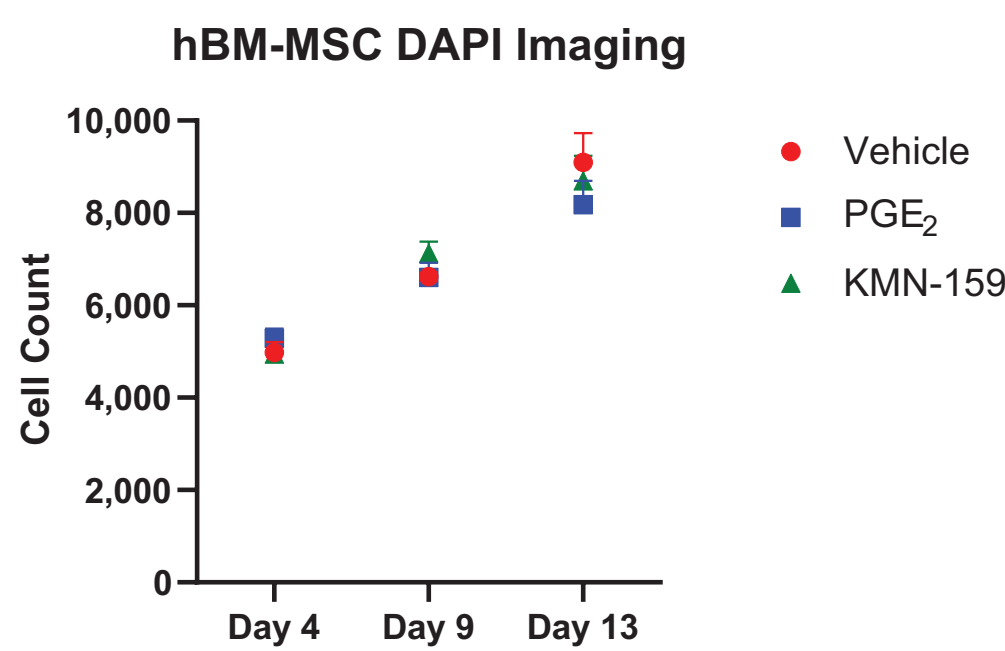
#### KMN-159 Upregulates the mRNA Levels of Osteogenic Marker Genes (ALP, COL1, Runx2, and OSX) in hBM-MSCs

hBM-MSCs were plated and treated with 100 nM KMN-159 as above and beginning on day 5, fed every 3 days with 100 nM KMN-159 in OM. Cells were harvested on day 5, 7, 11, 15, and 21, and mRNA levels of osteogenic marker genes were determined by RT-qPCR using β-actin as a reference gene. All early-stage osteoblastic markers, including runt-related transcription factor 2 (Runx2), ALP, type 1 collagen, and osterix, were found to be upregulated by KMN-159 compared to vehicle control. Runx2 and osterix mRNA were detectable as early as 5 days, and ALP and type I collagen mRNA were detectable by day 7 and 11, respectively. Furthermore, osteoprotegerin (OPG), an osteoclastogenesis inhibitory factor, was down-regulated by KMN-159, whereas receptor activator of NF-κB ligand (RANKL) was significantly upregulated, suggesting that KMN-159 stimulates hBM-MSCs to upregulate genes necessary for crosstalk in promoting osteoclast differentiation.



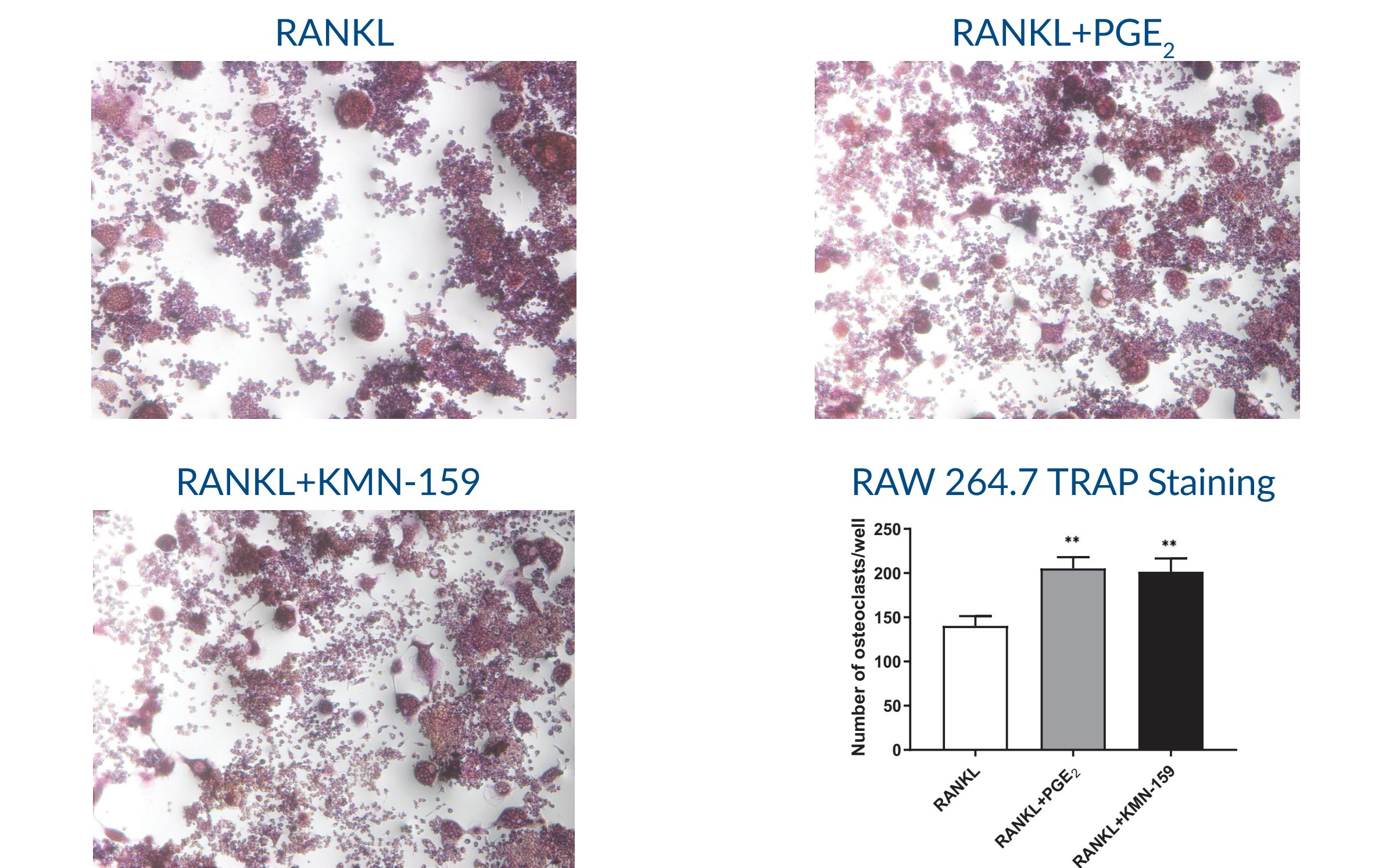
#### KMN-159 Does Not Affect Cell Number

hBM-MSCs were plated in three 24-well plates and treated with 100 nM PGE<sub>2</sub> or KMN-159 (n = 8 wells per treatment) as described previously and beginning on day 4, fed every 3 days with 100 nM PGE<sub>2</sub> or KMN-159 in OM. Cell plates were fixed with 100% ice-cold methanol on day 4, 9, or 13, incubated with 1 μg/ml DAPI in 1X PBS for 10 minutes, and rinsed with water twice. The number of nuclei per well was then quantified using a BioTek Cytation™ imaging system. No significant change in cell number was observed between cells treated with vehicle, PGE<sub>2</sub>, or KMN-159.



#### Effect of KMN-159 on Osteoclastogenesis

RAW 264.7 cells were treated with 100 nM PGE<sub>2</sub> or 100 nM KMN-159 together with 50 ng/ml RANKL for 5 days to induce the osteoclastic differentiation of the cells. After 5 days, the cells were stained for TRAP activity using the Acid Phosphatase, Leukocyte (TRAP) Kit (Sigma-Aldrich) to detect osteoclasts. As expected, osteoclastic differentiation of RAW 264.7 cells was not stimulated by PGE<sub>2</sub> or KMN-159 in the absence of RANKL (data not shown). In the presence of RANKL, the cells underwent osteoclastogenesis, and both PGE<sub>2</sub> and KMN-159 treatment showed increased number of TRAP-positive cells as compared to RANKL alone.



### Conclusions

- KMN-159 significantly enhances the osteoblastic differentiation of hBM-MSCs under osteogenic culture conditions as measured by induction of ALP activity.
- Osteoblast phenotype marker genes including Runx2, ALP, type I collagen, and osterix are expressed at higher levels in hBM-MSCs treated with KMN-159 as compared to the vehicle-treated controls.
- KMN-159 has no effect on the cell number of hBM-MSCs.
- KMN-159 increases RANKL and decreases OPG gene expression in hBM-MSCs, demonstrating that it stimulates hBM-MSCs to secrete the factors necessary for regulating osteoclastogenesis.
- KMN-159 significantly enhances osteoclast formation in RAW 264.7 cells in the presence of RANKL as measured by the increase in TRAP-positive multinucleated cells.

#### Future Directions

- Compare the effects of a single exposure of hBM-MSCs to KMN-159 to those following the chronic treatment with the compound reported here.
- Treat whole human bone marrow with KMN-159 to determine if cells in addition to the MSCs are involved in the osteogenic response to EP<sub>4</sub> agonists.
- Co-culture human macrophages with hBM-MSCs to study the role of macrophages in osteogenesis.
- Determine the mechanism of action of KMN-159 by studying the signaling pathways downstream of EP<sub>4</sub>.

#### Acknowledgments

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