Angiopoietin-like 4 expression during osteogenesis in canine stem cells

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Introduction

In the case of canine fractures, 5-10% result in non-unions[3]. Non-unions rarely heal without surgical intervention which can be very complicated and costly, especially in larger canine breeds. Successful fracture healing requires the migration and differentiation of a variety of **stem** and progenitor cells to support and promote bone formation and angiogenesis[3]. Strategies to enhance bone healing may include application of osteogenic and angiogenic molecules to promote these processes. Angiopoietin-like 4 (Angptl4) is a molecule of particular interest because it has been implicated in both angiogenesis and osteogenesis[1,4]. Furthermore, we have previously identified upregulated expression of *Angptl4* at the fracture site, with high expression in mineralizing osteoblasts[4]. In this study, we chose to investigate the expression of *Angptl4* during osteogenic differentiation of canine mesenchymal stem cells (MSCs). The studies were designed to confirm appropriate osteogenic differentiation of canine MSCs, and to explore the expression of *Angptl4* during the differentiation process. This study is the first step toward identifying a role for Angptl4 in osteogenesis. Future studies will focus on whether Angptl4 promotes osteogenic differentiation, and ultimately whether it may promote bone formation in dogs, *in vivo*.

Overview

Our long term goal is to identify molecules that can be used to promote fracture healing.
Hypothesis: The expression of *Angptl4* is upregulated during osteogenic differentiation of canine adipose-derived MSCs.

Materials and Methods

Cell culture: Canine adipose-derived MSCs from frozen stocks[2], were maintained in alpha-MEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in humidified incubators at 37°C. Osteoblast differentiation media was standard media supplemented with 50µg/mL ascorbic acid-2-phosphate, 5 mM β -glycerol phosphate, and 100 nM dexamethasone. Cells were sub-cultured and plated at a density of 5000 cells/cm^2 and experiments were started the following day.

Quantitative PCR: Samples were collected on days 1, 3, 7 and 14. Cells were washed in PBS and following RNA collection (RNeasy Mini Kit, Qiagen) total RNA was reverse transcribed with simultaneous genomic DNA elimination with Quantitect Reverse Transcriptase Kit (Qiagen). Quantitative PCR and analysis was performed as described in Wilson et al[3]. Expression levels were normalized to Beta2-macroglobulin, a housekeeping gene.

Alizarin Red Stain: Cells were seeded in 6-well plates, at 5,000 cells/cm^2, and grown for 1, 3, 7 and 14 days. After fixation in 5% formaldehyde, the cells were rinsed with deionized water and stained with 0.05% alizarin red. Plates were rinsed with deionized water and digital images were acquired using a flatbed scanner.

Figures

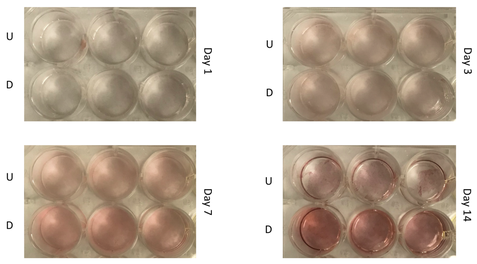

Figure 1: Calcium deposition during osteoblast differentiation using the Alizarin Red Stain (AR). U=Undifferentiated, D=Differentiated

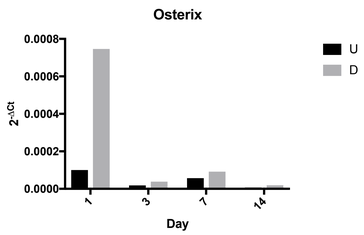

Figure 2: Expression of *Osterix* during osteoblast differentiation. Osterix is a transcription factor required for osteoblastic differentiation. n=2. For all PCR figures, bars represent mean +/-SEM. Black bars (U) indicate cells grown in standard culture media and gray bars (D) represent cells grown in osteogenic media.

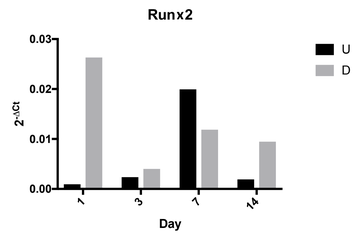

Figure 3: Expression of Runx2 during osteoblast differentiation. Runx2 is a transcription factor required for osteoblastic differentiation . n=2

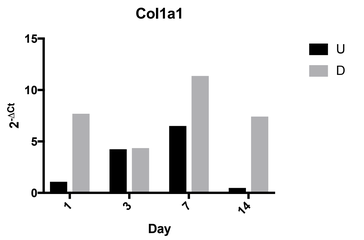

Figure 4: Expression of *Collagen 1* during osteoblast differentiation. Collagen 1 is a major structural protein in the bone matrix. n=2

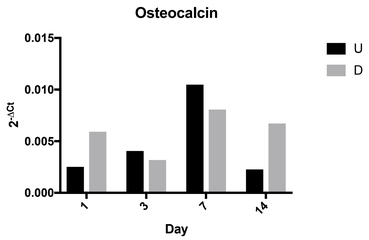

Figure 5: Expression of *Osteocalcin* during osteoblast differentiation. Osteocalcin is a non-collagenous protein hormone found in bone and dentin). n=2

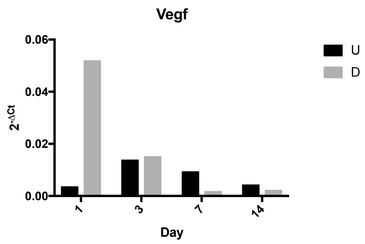

Figure 6: Expression of *Vegf* during osteoblast differentiation. Vegf stimulates the formation of blood vessels. n=2

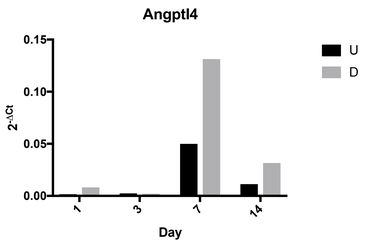

Figure 7: Expression of *Angptl4* during osteoblast differentiation. Angptl4 (gene of interest) is induced under hypoxia and regulates LPL. n=2

Results

Alizarin Red staining, indicating calcium deposition, was increased in cells grown in osteogenic media, Fig 1. This confirmed that canine MSCs were undergoing osteogenic differentiation under our culture conditions. As expected, expression of *Runx2* and *Osterix,* early osteogenic transcription factors, was upregulated early, at day 1, Figs 2 and 3. *Col1a* and *Osteocalcin* showed variable expression during differentiation but were elevated compared to undifferentiated cells at the later time points, Figs 4 and 5. *Vegf* expression, which is critical for vascularization of newly forming bone was upregulated early in osteoblastic differentiation, Fig 6. *Angptl4*, showed a dramatic increase in expression at day 7, Fig 7.

Discussion

Canine MSCs underwent normal osteogenic differentiation as indicated by abundant calcium deposition, and expression of a variety of osteogenic genes in a temporally appropriate manner. *Angptl4* expression was increased one week after initiation of osteogenic differentiation. This is similar to what we observed in murine pre-osteoblasts undergoing osteogenic differentiation where *Angptl4* was significantly upregulated at day 14[4]. Upregulation at this later timepoint in murine cells, most likely reflects differences in the speed of osteogenic differentiation in these two cells types.

Conclusion

Angptl4 has been shown to be highly expressed in regenerating bone in the fracture callus[4], in differentiating murine pre-osteoblasts, and in the current study, in differentiating canine MSCs. Further studies are required to determine whether *Angptl4* plays a role in osteogenic differentiation and bone formation. It is also possible that *Angptl4* is secreted by bone cells to affect other cell processes that are required for bone development and bone healing such as angiogenesis. As such *Angptl4* represents an interesting therapeutic target for future studies aimed at accelerating and improving bone healing.

References

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2. Chung, D.-J., Hayashi, K., Toupadakis, C. A., Wong, A., & Yellowley, C. E. (2012, February). Osteogenic proliferation and differentiation of canine bone marrow and adipose tissue derived mesenchymal stromal cells and the influence of hypoxia. *Research in Veterinary Science, 92*(1), 66-75.
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4. Wilson, S. S., Wong, A., Toupadakis, C. A., & Yellowley, C. E. (2015, September). Expression of Angiopoietin-like protein 4 at the fracture fite: regulation by hypoxia and osteoblastic differentiation. *Journal of Orthopedic Research, 33*(9), 1364-73.

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