Does the loss of osteoblast-derived Vascular Endothelial Growth Factor (VEGF) affect skeletal innervation?

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Introduction

It is well established that osteoblasts, the cells that form bone, are a predominant source of a blood vessel attracting factor called Vascular Endothelial Growth Factor (VEGF). Recent studies by Dr Claire Clarkin have demonstrated that *in vivo* deletion of VEGF by mature osteocalcin-expressing osteoblasts (OB) from birth (OBVEGFKO) results in highly porous bones in mice which are weaker and more susceptible to fracture. VEGF is essential for angiogenesis and osteogenesis during bone development and repair but it also plays a role in neurogenesis (Jin *et al.,* 2002).
The link between angiogenesis and neurogenesis is also demonstrated by the fact that many neuropeptides are involved in angiogenesis including calcitonin-gene related peptide (CGRP), which promotes endothelial cell proliferation *in vitro* (Seegers *et al.,* 2003). Semaphorin 3A (Sema3A) is an axon inhibitory signalling molecule, which competes with VEGF for the neuropilin 1 receptor (Acevedo *et al.,* 2008), demonstrating a direct regulatory control by this molecule of both angiogenesis and neurogenesis.

Aims and Hypothesis

The main research aim of my studentship is to determine whether the loss of OB-derived VEGF will impact on the density and distribution of nerves in these transgenic mice.
For this, I aimed to study changes in expression of nerve markers in bone sections from wildtype (WT) controls, Vegf $^{fl/+}$ (Het) and Vegf$^{fl/fl}$ (OBVEGFKO) mice. I used CGRP as a marker for sensory neurones and tyrosine hydroxylase (TH) as a marker for sympathetic neurones. I also examined the expression of Sema3A in these sections and used CD31 as a marker for blood vessels.

I hypothesised that mice lacking OB-VEGF will display decreased expression of neuronal markers in bone.

Experimental Plan

<u>Samples</u>
Transverse sections of tibia-fibula junction and calvaria from wildtype (WT), Vegf $^{fl/+}$ (Het) and Vegf$^{fl/fl}$ (KO) 16-week old mice were provided by Alice Goring.

<u>Immunostaining</u>
Sections were blocked for an hour in normal serum of the animal. Primary antibodies (Anti-CD31, Anti-CGRP, Anti-TH and control IgG) were incubated overnight at 4°C and appropriate secondary antibodies were incubated at room temperature for three hours. Slides were mounted on Fluoromount G medium (counterstained with DAPI blue) and imaged on Leica SP5 confocal microscope.

<u>SYBR Green qRT-PCR</u>

Mouse calvaria from WT, HET and KO were pulverised on dry ice, using centrifugation to separate cell lysates, and RNA was extracted through Qiagen RNeasy kits method. cDNA was then synthesised through Qiagen reverse transcriptase kit. Standards for absolute quantification were serially diluted from purified PCR products using primers for CGRP, Sema3A and beta-actin as a housekeeping gene. Brain cDNA was used as a positive control and pure water as a blank. SYBR Green qRT-PCR was run on Bio-Rad Cfx 2.

Results

At the tibia-fibula junction, KO mice have many smaller blood vessels located distally from the tibia cavity rather than fewer larger vessels as shown in the wildtype mice (**Figure 1**). This demonstrates a distinct phenotype caused by the lack of OB-VEGF. Despite this, immunostaining for both nerve markers (TH and CGRP) showed no objective differences between WT and OBVEGF KO mice. Staining was seen mainly in isolated neurones however, some staining around blood vessels was also observed (**Figure 1)**.
qRT-PCR absolute quantification demonstrated that there was a 46% decrease in CGRP expression in the KO mice compared to WT and a small, but insignificant decrease of Sema3A in the KO mice (**Figure 2**).

Discussion

My results indicate that in mice lacking OB-VEGF, there is decreased expression of CGRP mRNA in calvaria, but I couldn't see any difference in CGRP protein expression in WT and KO mice using immunocytochemistry. This could be due to the fact that mice were not perfused with fixator just after euthanasia, leading to some degradation of neurones which may mask possible differences in immunostaining.
The sections used for nerve marker immunostaining were more distal than the sections stained with anti-CD31 with more visible blood vessels. It should be considered that more distal sections contain less nerves. Moreover, the autofluorescence of the bone marrow and cortical bone raised issues in identifying structures of interest.
Several challenges arouse from this project that reduce confidence in the results obtained from the qRT-PCR. The greatest challenge was that much of the mRNA extracted from calvaria was of low quality and degraded in storage or during extraction. Poor nucleic acid integrity may be responsible for the discrepancy of results from the immunostaining and qRT-PCR, although, it could also be due to tissue-specific differences.
Had more time been available, other neuromediators could have been considered such as nerve growth factor or Substance P.

Conclusion

This project found no relationship between osteoblast-derived VEGF and Sema3A. However, there was a decrease in the mRNA expression of calcitonin-gene related peptide in calvaria from mice where VEGF is lacking in osteoblasts. This has not been confirmed in sections of tibia-fibula junction obtained from the same mice using immunocytochemistry. These results do not however support the conclusion that VEGF has a role in bone innervation from development.

References

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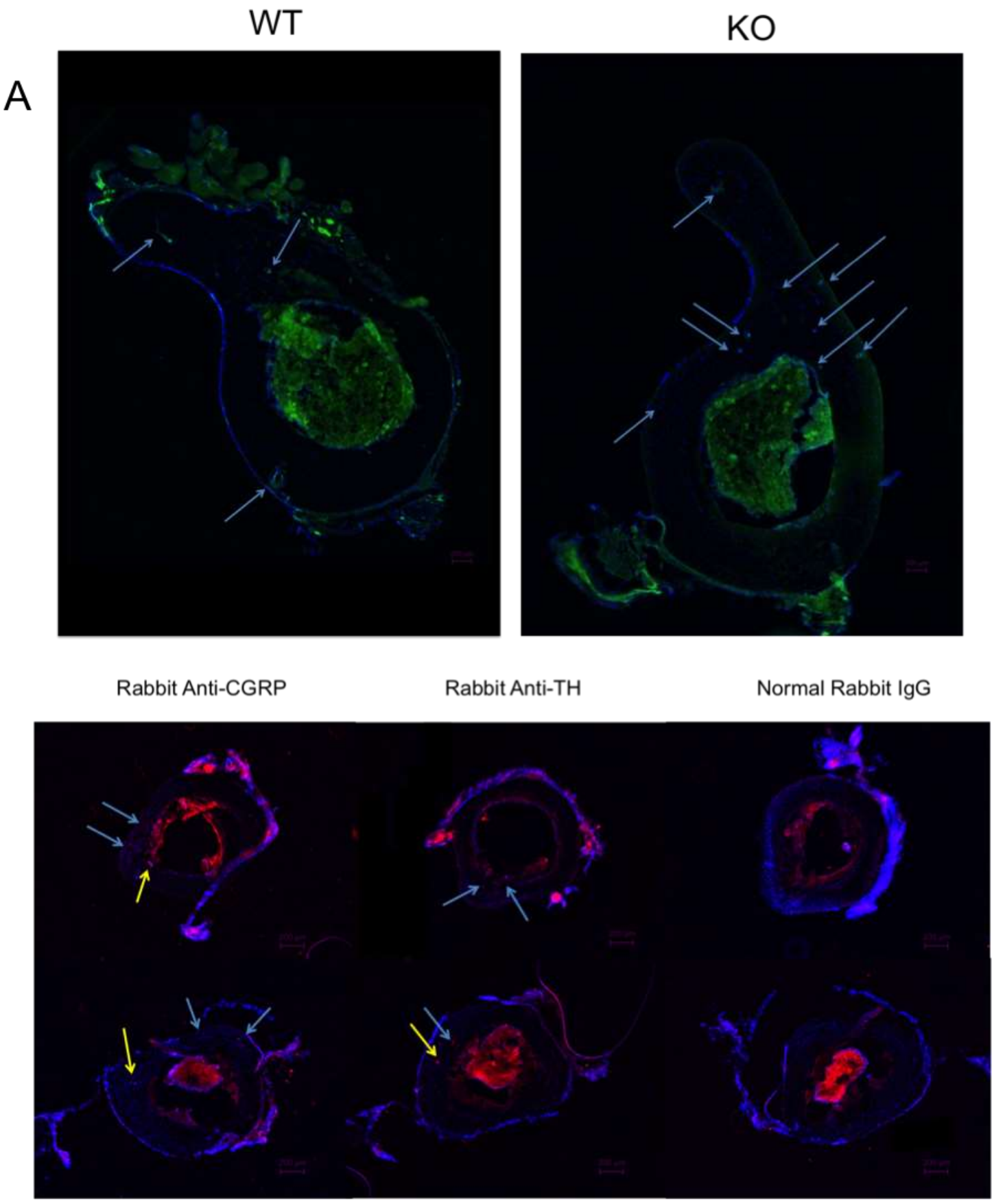

Figure 1: Changes of blood vessels and nerve markers in mouse tibia-fibula junction in absence of osteoblast-derived VEGF.

A.) Increased number of blood vessels formed in male osteoblast-derived VEGF specific knockout (KO) compared to wildtype (WT). Blue arrows denote CD31 staining of endothelial cells. B) Patterns of CGRP and TH nerve staining observed in male WT and KO mice compared to normal IgG control. Blue arrows denote staining around blood vessels and yellow arrows denote staining naked nerve fibres.

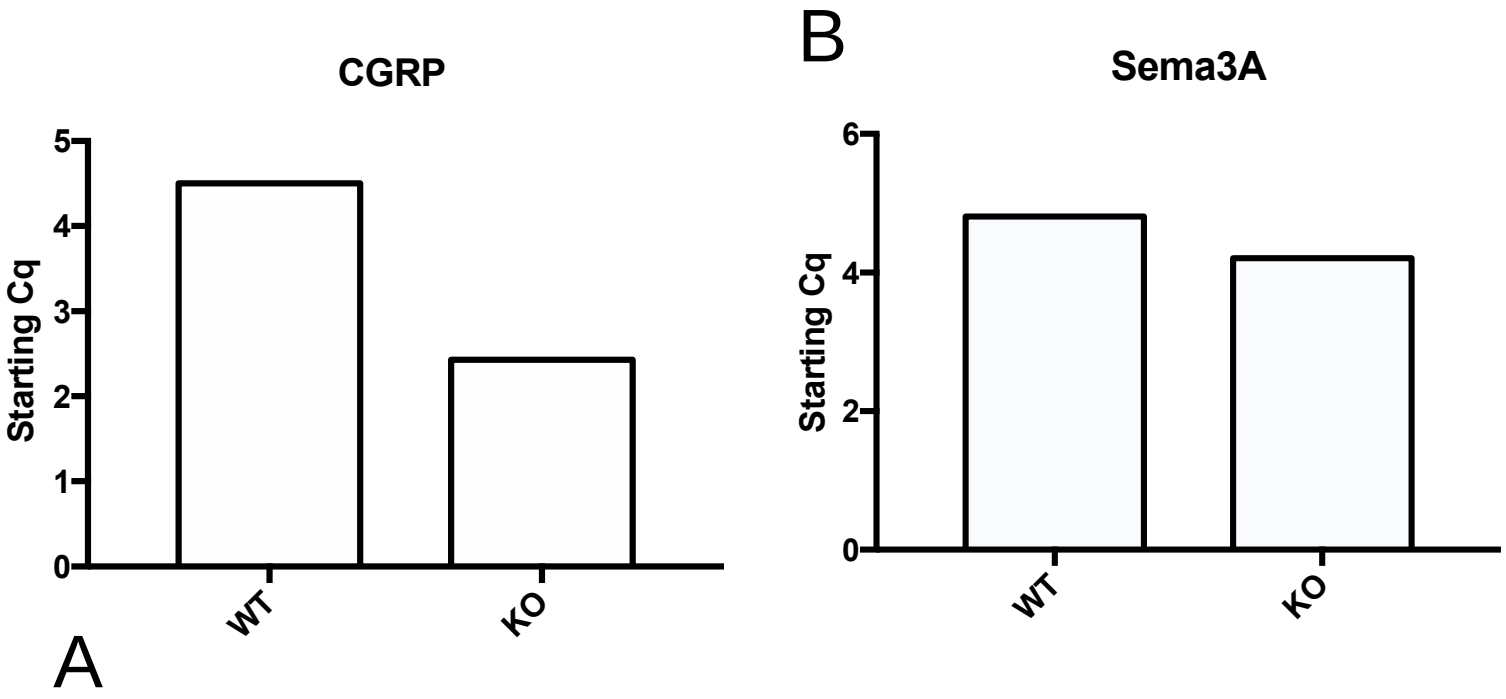

Figure 2: Analysis of gene expression in calvaria using qRT-PCR. A) Decrease in CGRP expression in calvaria from OB-KO compared to WT. B) No significant change in semaphorin 3A expression between OB-KO and WT. Starting Cq indicates mean number of cycles to cross an arbitrary threshold of gene of interest in the ratio of the number of cycles required for a housekeeping gene (Beta-actin) to cross the same threshold. This result gives a ratio of the expression of the gene of interest in wildtype (WT) and knock-out (KO) samples.