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

Acevedo, L., Barillas, S., Weis, S., Gothert, J., Cheresh, D. (2008) "Semaphorin 3A Suppresses VEGF-Mediated Angiogenesis Yet Acts as a Vascular Permeability Factor", Blood, 111(5), 2674-2680.

Jin, K., Zhu, Y., Sun, Y., Mao, X., Xie, L., Greenberg, D. (2002) "Vascular endothelial growth factor (VEGF) stimulates neurogenesis in vitro and in vivo", Proceedings of the National Academy of Sciences, 99(18), 11946-11950.

Wang, X., Han, C., Fiscus, R. (1991) "Calcitonin Gene-Related Peptide (CGRP) Causes Endothelium-Dependent Cyclic AMP, Cyclic GMP and Vasorelaxant Responses in Rat Abdominal Aorta", Neuropeptides, 20(2), 115-124.

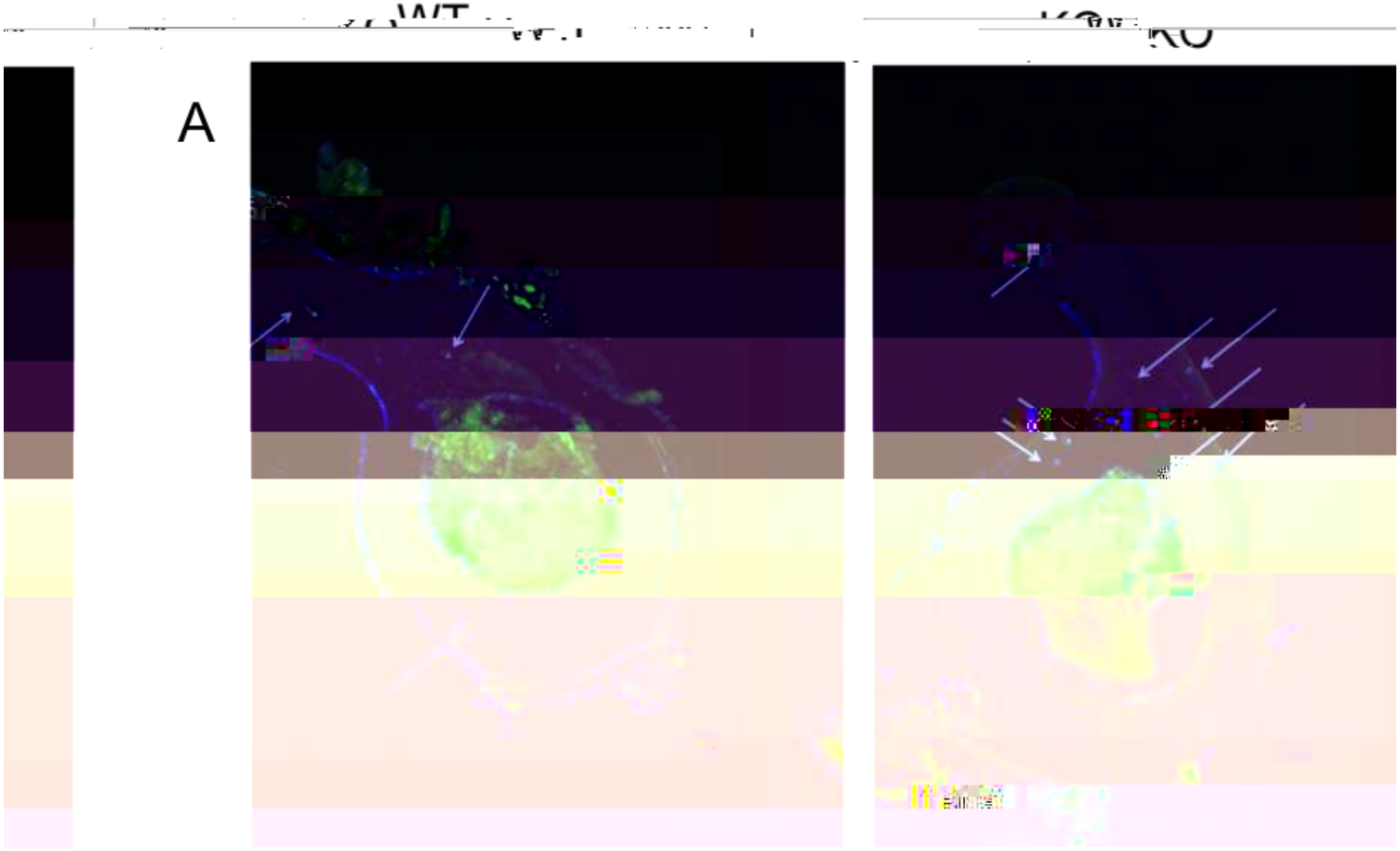
A

Figure 2: Analysis of gene expression in calvaria using qRT-PCR. A) Decrease in CGRP expression in calvaria from OB-