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BMP4 and noggin control embryonic blood vessel formation by antagonistic regulation of VEGFR-2 (*Quek1*) expression

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Abstract

Regulation of VEGFR-2 (*Quek1*) is an important mechanism during blood vessel formation. In the paraxial mesoderm, *Quek1* expression is restricted to the lateral portion of the somite and later to sclerotomal cells surrounding the neural tube. By grafting of either intermediate mesoderm or BMP4 beads into the paraxial mesoderm, we show that BMP4 is a positive regulator of VEGFR-2 (*Quek1*) expression in the quail embryo. Separation of somites from intermediate mesoderm leads to down-regulation of *Quek1* expression. The expression of *Quek1* in the medial somite half is normally repressed by the notochord and becomes up-regulated and lateromedially expanded after separation of the notochord. Our results show that up-regulation of BMP4 leads to an increase of the number of blood vessels, whereas inhibition of BMP4 by noggin results in a reduction of blood vessels.

Keywords: *Quek1*; VEGFR-2; Bone morphogenetic proteins; Noggin; Endothelial cells; Quail embryo

Introduction

The growth and maintenance of the blood vascular system is to a large extent mediated by members of the vascular endothelial growth factor (VEGF) family via their tyrosine kinase receptors (VEGFRs) that are expressed in angioblastic and endothelial cells (Ferrara, 2000). One member of the VEGF receptor (VEGFR) family is VEGFR-2 (Flk-1/KDR). In the quail, the homologue of VEGFR-2 has been cloned and named as *Quek1* by Eichmann et al. (1993, 1996). *Quek1* possesses 69% and 71% identity to murine *Flk-1* and human KDR. The mesoderm of the posterior two-thirds of the gastrulation-stage chick embryo expresses VEGFR-2 (Eichmann et al., 2002; Kubo and Alitalo, 2003).

The functional aspects of VEGFR-2 have been studied in detail. For instance, VEGFR-2 expressing cells isolated from chick blastoderm and cultured in vivo can give rise to both endothelial and hematopoietic cells (Eichmann et al., 1997). VEGFR-2 deficient mice failed to form yolk-sac blood islands and organized blood vessel formation in the embryo proper (Shalaby et al., 1995). Thus, embryonic blood vessel formation depends on this receptor. However, the mechanisms of the regulation of VEGFR-2 expression are far from being understood. In the paraxial mesoderm of the quail embryo, expression of *Quek1* can be observed in the lateral portion of both the segmental plate mesoderm and the epithelial somite. Initial expression in the somite is restricted to its dorsolateral portion (Eichmann et al., 1993; Nimmagadda et al., 2004; Wilting et al., 1997). Later, a medial expression domain is established in the part of sclerotome surrounding the neural tube. In the avascular notochord, *Quek1* is being expressed from day 4 onwards (Eichmann et al., 1993; Nimmagadda et al., 2004; Wilting et al., 1997).

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Studying the regulation of these expression patterns could give us a key to understand the mechanisms controlling VEGFR-2 expression. The lateral plate is subdivided into somatic and splanchnic mesoderm by the coelom. By means of quail/chick transplantation experiments, it has been shown that somitic and splanchnic mesoderm have the potential to give rise to endothelial progenitors (Pardanaud and Dieterlen-Lièvre, 1999; Pardanaud et al., 1987). Since angioblasts do not cross the embryonic midline (Klessinger and Christ, 1996), it has been suggested that notochord-derived signals inhibit midline crossing and negatively regulate blood vessel formation. The antagonistic activities of BMP4 and noggin control mediolateral differentiation of the paraxial mesoderm with BMP4 promoting lateral plate formation (Tonegawa et al., 1997). It is still unknown, if the BMP4 and noggin pathway is also involved in the regulation of VEGFR-2 expression.

In the present study, we examined the role of BMP4 in the regulation of *Quek1* expression in the somite. We show that the notochord exerts an inhibiting effect on *Quek1* expression. We also show that over-expression of BMP4 induces the formation of additional vasculature whereas noggin inhibits *Quek1* expression and leads to a reduction of blood vessels. These results demonstrate that the regulation of *Quek1* expression in somites is mediated by the BMP4 signaling pathway.

Materials and methods

Preparation of chick and quail embryos

Fertilized chick (*Gallus gallus*) and quail eggs (*Coturnix coturnix japonica*) were incubated at 38°C under 80% humidity and the embryos were staged according to Hamburger and Hamilton (1951). Experiments were performed on embryos at stages 11–14.

Grafting experiments

Grafting methods used in this study have been described previously (Wilting et al., 1995, 2000; Zhi et al., 1996).

Grafting of medial somite

The lateral portion of a epithelial brachial somite (somite 18–20) was extirpated from a day-2 quail embryo and replaced by a medial half of a somite from another quail embryo of the same stage (Fig. 1A). The embryos were reincubated for 1 day, fixed and processed for in situ hybridization.

Grafting of intermediate mesoderm

Intermediate mesoderm from HH-stage 13–14 chick embryos was grafted into a somite (18–20) of quail embryos of the same stage (Fig. 1B). Embryos were reincubated for 24 h, fixed, and processed for in situ hybridization.

Separation of somite from intermediate mesoderm

A longitudinal slit through all the three germ layers was made between the segmental plate mesoderm and intermediate mesoderm of HH-stage 12–14 quail embryo (Fig. 1C). An aluminum foil barrier was inserted into the slit. The embryos were reincubated for 24 h and then processed for in situ hybridization.

Separation of somite from axial organs

A longitudinal slit was made between neural tube and adjacent segmental plate mesoderm on one side of HH-stage 12–14 quail embryo (Fig. 1D). An aluminum foil barrier was inserted into the slit and the embryo reincubated for 24 h and then processed for in situ hybridization.

Separation of somite from notochord

After opening the vitelline membrane, neural tube and overlying ectoderm were cut open along the dorsal midline with a tungsten needle (Fig. 4A). A longitudinal cut was made on one side of the ventrolateral neural tube, isolating the notochord from segmental plate mesoderm on one side of HH-stage 12–14 embryo, leaving the neural tube in contact. Then, an aluminum foil barrier was inserted into the slit. The embryos were reincubated for 24 h, processed for whole mount in situ hybridization. Embryos reincubated for 60 h were used to analyze blood vessel pattern after ink injection.

Implantation of BMP4 beads

BMP4 was obtained from Genetics Institute, Cambridge MA. For application of BMP4, Affigel beads of approximately 80–120 μm in diameter (BioRad Laboratories) were rinsed in PBS and incubated in 25 μg/ml BMP4 protein solution for 1 day at 4°C. For bead implantation, paraxial mesoderm (somite 18–20 of HH-stage 13–14 embryo) was punctured with a electrolytically sharpened tungsten needle, and a bead was inserted into the mesenchyme using a blunt glass needle. Embryos were reincubated for 24 h, processed for whole mount in situ hybridization and for 60 h, for ink injection and blood vessel pattern analysis. Beads soaked in PBS were used as control beads. None of the controls had an effect on *Quek1* expression.

Noggin cell injection

CHO B3 cells expressing noggin protein and DHFR control CHO cells were kindly provided by Dr. Richard Harland, University of California at Berkeley. Cell lines were cultured as described elsewhere (Lamb et al., 1993). Confluent cultures were harvested, cells were washed in phosphate-buffered saline (PBS), pelleted and resuspended in a minimal volume of medium. For cell injection, the ectoderm (somite 18–21 of HH-stage 14 embryo) was punctured with a