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Radar/Gdf6a function during zebrafish (Danio rerio) embryogenesis

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A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Molecular Medicine

University of Auckland

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Abstract

In the early vertebrate embryo, the vascular system is one of the first organ systems to form. Recently it has become evident that the development of mature, functional vessels requires not only signals derived from the endothelium itself, but a set of additional molecules that are not necessarily endothelium-specific. In zebrafish and Xenopus embryos two such tissues that are believed to secrete modulators of blood vessel assembly are the hypochord and primitive gut endoderm (PGE). These tissues intimately border the major axial vasculature.

Radar/Growth/differentiation factor 6a (Gdf6a) represents a signalling molecule belonging to the GDF5, 6, 7 subgroup of the transforming growth factor-beta (TGF-β) superfamily. In the zebrafish, transcripts for radar are located in the hypochord, PGE and ventral tail mesenchyme (VTM), all tissues that border the developing axial blood vessels. This prompted an investigation into a potential role for this signalling bone morphogenetic protein (BMP) during the specification and assembly of the closely related vascular and haematopoietic systems in the zebrafish.

Transient forced expression experiments confirmed an early ventralising activity for the Radar signal that resulted in the expansion of the haematopoietic/vascular compartment, known as the intermediate cell mass (ICM). However, a loss-of-function zebrafish model generated using morpholino technology demonstrated a critical requirement for this BMP signal in establishing the integrity of the axial blood vessels. Furthermore, this requirement was independent of the initial establishment of vascular patterning. Zebrafish embryos depleted of the Radar signal initiate a normal primitive circulation. However, soon after this commencement of normal flow, blood cells were observed to extravasate from the axial vasculature. Microangiography confirmed this leakage phenotype.

Such an angiogenic/maturation role for Radar during vascular development was supported by a transgenic zebrafish line carrying an inducible copy of the radar gene.
Homozygous transgenic embryos established a typical early circulation that became progressively restricted until no blood travelled throughout the entire embryonic tissue.

In summary, the work presented in this thesis strongly suggests that Radar is involved in a signalling pathway required for establishing the integrity of the axial vessels during zebrafish development.
Acknowledgements

I would like to thank my supervisors Associate Professor Philip Crosier, Professor Kathy Crosier and Dr. Maria Vega Flores for their much valued help and guidance throughout the course of this thesis. I would also like to thank them for the opportunity to work with a great team of people over the years. In particular I would like to thank Dr. Alan Davidson for introducing me to the world of zebrafish and Dr. Ross Bland for his help and humour at the most unexpected times. Special thanks also goes to Dr. Julia Horsfield for helping to proof-read this thesis and her guidance, Dr. Peter Cattin and Dr. Maggie Kalev for our many varied discussions. I also appreciate the help from Dr. Heather Rooke with the site-directed mutagenesis work. Also, thank you to Latifa Kahn for her excellent lab managerial skills. I am also grateful to past and present members of the Department of Molecular Medicine and Pathology for assistance when required.

This work would not have been possible without Dr. Peter Cattin for his efficient management of the zebrafish facility and sectioning/histology expertise. I would also like to make a special thanks to Dr. Maria Vega Flores who helped keep me calm during the most trying times of this work and who always provided guidance and assistance.

I would also like to take this opportunity to thank my parents for their love, support and a roof over my head (don’t worry, now I can move out). Special thanks also goes to Daniel Vizor (Dan) for his friendship which I will always value. Last, but certainly not least, I would like to say a very special thank you to Katja Reuter who I will always be indebted to because of her love and support.
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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>A</td>
<td>Absorbance</td>
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<td>ACV</td>
<td>Anterior cardinal vein</td>
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<td>ALK</td>
<td>Activin-like kinase</td>
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<td>AMA</td>
<td>Anterior mesenteric artery</td>
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<td>AO</td>
<td>Acridine orange</td>
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<td>Basilar artery</td>
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<td>BCIP</td>
<td>5-Bromo-4-chloro-3-indolyl-phosphate</td>
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<td>bFGF</td>
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<td>bHLH</td>
<td>Basic helix-loop-helix</td>
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<td>BM</td>
<td>Basement membrane</td>
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<td>BMP</td>
<td>Bone morphogenetic protein</td>
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<td>Bone morphogenetic protein receptor</td>
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<td>bp</td>
<td>Base pairs</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>Internal carotid artery</td>
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<td>Days post fertilisation</td>
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<td>E</td>
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<td>Extracellular matrix</td>
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<td>hpf</td>
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<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
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<td>Nano</td>
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<tr>
<td>NBT</td>
<td>4-Nitroblue tetrazolium chloride</td>
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<td>NCI</td>
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<td>OD</td>
<td>Optical density</td>
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<td>O.D.</td>
<td>Outer diameter</td>
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<td>Pico</td>
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<td>P2RAD</td>
<td>Human <em>BMP2</em> pro-domain, <em>radar</em> mature domain fusion</td>
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<tr>
<td>PAV</td>
<td>Parachordal vessel</td>
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<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<td>PC</td>
<td>Peri-endothelial support cell</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PCV</td>
<td>Posterior cardinal vein</td>
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<td>Platelet-derived growth factor</td>
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<td>PDGFR</td>
<td>Platelet-derived growth factor receptor</td>
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<td>PECAM</td>
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<td>PFA</td>
<td>Paraformaldehyde</td>
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<td>Se</td>
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<td>Suprarentestinal artery</td>
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<td>Tris-acetic acid/EDTA</td>
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<tr>
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<td>Tris-HCl/EDTA</td>
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<td>TGF</td>
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<tr>
<td>Tris-HCl</td>
<td>Tris buffer, pH adjusted using hydrochloric acid</td>
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<tr>
<td>tRNA</td>
<td>Transfer ribonucleic acid</td>
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<tr>
<td>UTP</td>
<td>Uridine 5'-triphosphate</td>
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<td>UTR</td>
<td>Untranslated region</td>
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<td>Ultraviolet</td>
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<td>v/v</td>
<td>Volume per volume</td>
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<td>w/v</td>
<td>Weight per volume</td>
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The following one- and three-letter abbreviations were used for amino acid residues in the text and figures.

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<th>Residue</th>
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<td>Isoleucine</td>
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<td>ile</td>
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<td>Valine</td>
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Gene and protein names were written according to standard nomenclature, as described in Wood, 1998. A summary of gene and protein nomenclature for different developmental systems is tabled below.

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<td><strong>Drosophila melanogaster</strong></td>
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<td><em>dpp</em></td>
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<tr>
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<td><strong>Danio rerio and Xenopus laevis</strong></td>
<td>Lower case,</td>
<td><em>radar</em></td>
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<td>Initial letter</td>
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<td>Company Abbreviations</td>
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<td></td>
<td>Eugene, OR 97402, USA</td>
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<td>BDH</td>
<td>British Drug Houses Chemicals NZ Ltd.,</td>
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<td></td>
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<td>Eppendorf</td>
<td>Eppendorf-Netheler-Hinz GmbH, D-22331 Hamberg, Germany</td>
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Falcon
Trademark of:
Becton Dickinson Labware,
Becton Dickinson Co.,
2 Bridgewater Lane,
Lincoln Park, NJ 07035, USA

Gelman Sciences
Gelman Sciences Inc.,
Pall Corporation,
600 S. Wagner Road,
Ann Arbor, MI 48103-9019, USA

Gene Tools, LLC
Gene Tools, LLC,
One Summerton Way,
Philomath, OR 97370, USA

GibcoBRL
Bethesda Research Laboratories,
Life Technologies Inc.,
87/7 Grovemont Circle,
Gaithersburg, MD 20887, USA

Hollywood Fish Farm
Hollywood Fish Farm Aquarium Specialists,
36 Frost Road,
Mt Roskill,
Auckland, New Zealand
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<td>44 Talmadge Road,</td>
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<td>Pharmacia LKB Biotechnology, Björkgata 30, S-751 Uppsala, Sweden</td>
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<td>QIAGEN GmbH, Max-Volmer-sträße 4, 40724 Hilden, Germany</td>
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<td>Riedal-de Haën</td>
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<td>Roche Molecular Biochemicals</td>
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<td>Thermo Hybaid</td>
<td>Thermo Hybaid, Action Court, Ashford Road, Ashford, Middlesex, TW 15 1XB, United Kingdom</td>
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<td>ZM Ltd.</td>
<td>ZM Ltd., 26 Harrow Down, Winchester, Hampshire S022 4LZ, England</td>
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Publications


Manuscripts in Preparation


Presentations

- Hall, C., Crosier, K., Crosier, P. Dept. of Molecular Medicine, Faculty of Medical and Health Sciences, University of Auckland, New Zealand: A potential role for *radar* in early zebrafish haematopoiesis/vasculogenesis. 2000 Zebrafish Development and Genetics meeting, Cold Spring Harbor, NY, USA.
- Hall, C., Flores, M., Crosier, K., Crosier, P. Dept. of Molecular Medicine, Faculty of Medical and Health Sciences, University of Auckland, New Zealand: An essential role for the BMP Radar in establishing vascular integrity in the zebrafish. EMBO World Program Workshop - “Fish as Model Organisms in the Genomic Era”, 2001, Singapore.

- Flores, M., Hall, C., Crosier, K., Crosier, P. Dept. of Molecular Medicine, Faculty of Medical and Health Sciences, University of Auckland, New Zealand: Radar/GDF6a: Potential role in a haematopoietic/vasculogenic signalling cascade. 13th Lorne Cancer Conference, 2001, Victoria, Australia.

- Crosier, P., Horsfield, J., Flores, M., Kalev, M., Hall, C., Cattin, P., Khan, L., Crosier, K. Division of Molecular Medicine, Faculty of Medical and Health Sciences, University of Auckland, New Zealand: Understanding Blood and Vessel Formation Through Zebrafish Genetics. 2002 Lorne Genome Conference, Victoria, Australia.

- Hall, C.J., Flores, M.V., Davidson, A.J., Crosier, K.E. and Crosier, P.S. Division of Molecular Medicine, Faculty of Medical and Health Sciences, University of Auckland, New Zealand: Radar is Required for the Establishment of Vascular Integrity in the Zebrafish. 2002 Zebrafish Development and Genetics meeting, Madison, Wisconsin USA.

- Flores, M., Hall, C., Crosier, K., Crosier, P. Division of Molecular Medicine, Faculty of Medical and Health Sciences, University of Auckland, New Zealand: Radar/GDF6a Plays a Critical Role in the Establishment of Vascular Integrity in Zebrafish. ComBio 2002, Sydney, Australia.
I. Embryonic vascular development

Vascular development occurs via two distinct processes: vasculogenesis and angiogenesis. Early in embryonic development, endothelial precursor cells differentiate, proliferate in situ and migrate to a previously avascular tissue where they coalesce to form a primitive vascular network. This process of initial vascular assembly is termed vasculogenesis (Hanahan, 1997). The primitive vascular system is then further refined and modified to create the stable, integrated circuitry characteristic of the mature vasculature. This remodelling of the de novo vessels is referred to as angiogenesis (Yancopoulos et al., 2000). The mechanisms that govern both vasculogenesis and angiogenesis are similar and employ a number of common molecules throughout early development.

1.1 Vasculogenesis and angiogenesis

1.1.1 Vasculogenesis

During early development, vasculogenesis is responsible for the establishment of the primary vascular system (Figure 1.1). This system carries primitive haematopoietic cells in a restricted path through the major vessels of the embryo, such as the aorta and major veins, as well as the honeycomb-like plexus often interconnecting these major vessels. These earliest initial blood vessels in the developing embryo are derived from
Figure 1.1 Schematic representation of vasculogenesis and some of the key molecules involved in its regulation. Vasculogenesis commences with the bFGF-dependent endothelial commitment of lateral plate mesoderm to the endothelial lineage. The migration, proliferation and assembly of these endothelial cells involves, in part, signalling cascades utilising VEGF and its receptors, VEGFR1/Flt1 and VEGFR2/Flik1 along with TGF-β1 and Ephrin-B2. This results in the establishment of a primitive vascular network that is further remodelled via the recruitment of periendothelial support cells. A process dependent on Ang1 and its receptor Tie2, as well as Ephrin-B2. Graphics adapted from Hanahan, 1997 and Yancopoulos et al., 2000.
angioblasts. Angioblasts differentiate from mesodermal tissue and represent vascular endothelial precursor cells that have yet to form a lumen (Risau, 1997). A proposed intermediate step between the mesodermal precursor cell and the angioblast is the putative haemangioblast. The intimate developmental association between the endothelial and haematopoietic systems initially lead to the concept and hypothesised existence of this bi-potential cell (Sabin, 1920). Recently, this hypothesis has gained substantial evidence, mostly supported by the observation that both the vascular and blood lineages share expression of a number of genes (Choi, 1998; Choi et al., 1998; Gering et al., 1998). The differentiation of lateral plate mesoderm into angioblasts is dependent upon the fibroblast growth factor (FGF) family of molecules, in particular basic-FGF (bFGF) (Amaya et al., 1991; Flamme and Risau, 1992). Interestingly, subtypes of angioblasts are believed to exist within the developing embryo. Intraembryonic angioblasts derived from the splachnopleuric mesoderm have been demonstrated to possess a bi-potential haemangioblastic property, unlike those derived from the somatopleuric mesoderm (Pardanaud et al., 1996). Once angioblasts have migrated to sites of vasculogenesis and differentiated into endothelial cells they begin to interact and coalesce, forming rudimentary vascular tubes. These immature unstable vessels are then further refined, via the recruitment of periendothelial support cells, to create stable vessels capable of supporting primitive blood flow (Figure 1.1).

Although much research has been conducted on the genetics underlying vasculogenesis, the majority of data collected thus far relates to mesoderm-inducing signals. Many unanswered questions remain as to what genes govern the commitment decision made by the haemangioblast to go down the vascular or blood lineage, or what signals control the migration of angioblasts to sites of active vasculogenesis.
1.1.2 Angiogenesis

Following the establishment of the primary vascular plexus, more endothelial cells are recruited to form new vessels by sprouting, extension or splitting of established vessels (Risau, 1997). This angiogenic remodelling also relies on the proliferation, migration and assembly of endothelial cells into vessels capable of supporting blood flow, as well as the recruitment of peri-endothelial support cells such as smooth muscle cells or pericytes for vessel integrity (Hanahan, 1997) (Figure 1.2). Angiogenic sprouting, although not the only mechanism of angiogenic remodelling, is the most extensively characterised. Sprouting angiogenesis occurs in the yolk sac and in the embryo, predominantly during brain development (Risau, 1997). Non-sprouting angiogenesis or intussusception represents another mechanism of angiogenesis involving the splitting of pre-existing vessels by transcapillary pillars of extracellular matrix (ECM) (Risau, 1997). The particular type of angiogenesis that occurs in a particular tissue is thought to depend on how many vessels are present in that tissue when it starts to grow rapidly. Examples of different organs that employ different mechanisms of angiogenesis are the lung and brain which employ non-sprouting and sprouting angiogenesis, respectively (Pardanaud et al., 1989).

The functions of endothelial cells during angiogenic events can be broadly separated into two phases, activation and resolution (Pepper, 1997). The phase of activation includes: 1) an increase in vascular permeability and extravascular fibrin deposition; 2) basement membrane degradation; 3) cell migration and matrix invasion; 4) cell division; and 5) lumen formation. The resolution phase includes: 1) inhibition of cell proliferation and migration; 2) basement membrane reconstitution; and 3) junctional complex maturation (Pepper, 1997). It should be noted that each of these functions are as equally applicable to vasculogenesis as they are to angiogenesis.
Figure 1.2 Schematic representation of angiogenesis and regression. The initial immature vascular circuitry established via vasculogenesis is further remodelled into a mature system of interconnecting branching vessels. This requires the recruitment of periendothelial support cells such as pericytes and smooth muscle cells and remodelling of the nascent vasculature. Regulators of this process include members of the VEGF, angiopoietin, ephrin and TGF-β families of signalling molecules. An important aspect of this angiogenic remodelling is the ability of vessels to destabilise and even to regress, enabling completely new vascular interconnections to be established. Molecules believed to be involved in these processes include Ang2 and its receptor Tie2 along with VEGF and its receptor VEGFR2/Flok1. Graphics adapted from Hanahan, 1997 and Yancopoulos et al., 2000.
Primary vasculature

VEGF
VEGFR2/Flik1
Ang1
Ephrin-B2
Ang2
Tie2
TGF-βR
TGF-β1

Remodelled vasculature

Immature unstable vessel

Stable mature vessel

Relaxing of matrix contacts and PC cell interactions

BM

PC

Ang2
Tie2
VEGF
VEGFR2/Flik1

Regression

Loss of matrix interactions and structure

Endothelial cell (EC)

Basement membrane (BM)

Periendothelial support cell (PC)
Angiogenesis begins with nitric oxide-dependent vasodilation and increased vascular permeability (Risau, 1997). The control of vascular permeability needs to be tightly regulated, too much permeability can lead to excessive vascular leakage resulting in circulatory collapse, formation of adhesion, metastasis or blindness, depending on the affected tissue. This tight regulation is mediated by the formation of fenestrations, vesiculo-vacuolar organelles and the redistribution of platelet endothelial cell adhesion molecule (PECAM)-1 and vascular endothelial (VE)-cadherin. Vascular permeability is also mediated by the Src kinases (Eliceiri et al., 1999). Increase in vascular permeability allows for the establishment of a temporary scaffold to be used by the migrating endothelial cells. For these endothelial cells to migrate from their resident sites to the site of active angiogenic remodelling, their connections with other neighbouring endothelial cells and periendothelial support cells must be weakened. Once these cell contacts have been relaxed the proliferating endothelial cells can migrate to angiogenic sites. Little is known regarding the spatial cues that guide these endothelial cells to their final destinations. These cues would need to position the endothelial cells within a complex three-dimensional framework, not only during their migration, but also in their positioning within the nascent vascular structure. Once endothelial cells have been integrated into a new vessel, they become quiescent and can survive for years. Their persistence is very important for the survival of blood vessels, since a reduction in survival can lead to vascular regression (Carmeliet et al., 1999a).

Angiogenesis can further be divided into physiological and pathological categories. The main difference between these two forms of angiogenesis lies in the fact that pathological angiogenesis is commonly induced by inflammation, and requires the interaction between angiogenic factors and blood-borne cells such as macrophages, platelets and mast cells. These cells in turn produce angiogenic factors resulting in the recruitment of endothelial cells and smooth muscle cells to the site of inflammation or wound healing (Sunderkotter et al., 1994). A number of mechanisms underlying pathological blood vessel growth seem to resemble those that mediate embryonic vessel
development. However, there is an emerging view that differences may exist. Some examples of molecules crucial for pathological angiogenesis while only minimally required, or not required at all, for embryonic vascular development include proteinases (Heymans et al., 1999), nitric oxide (Murohara et al., 1998) and plasminogen activator inhibitor 1 (Bajou et al., 1998). The requirement for different molecules is not surprising given that during embryonic vascular development endothelial cells are proliferating and loosely connected while in the adult they are quiescent.

1.1.3 Vascular smooth muscle cell recruitment

An essential step in the development of a functional vascular system is the recruitment of smooth muscle cells to provide structural integrity. Smooth muscle cells can originate from a number of different locations and cell types. For example, smooth muscle cells can transdifferentiate from endothelial cells, mesenchymal cells, bone marrow precursors and macrophages (Carmeliet, 2000; Luttun et al., 2002). Platelet-derived growth factor (PDGF)-BB and vascular endothelial growth factor (VEGF) both act as chemoattractants for smooth muscle cells (Lindahl et al., 1998; Carmeliet et al., 1999b) (Figure 1.3). Once these mural cells have been recruited to the nascent endothelial tube, other molecules including Ang1 and its receptor Tie2 are believed to function in stabilising the endothelial-mural cellular interaction (Suri et al., 1996). This endothelial-mural cell interaction is further strengthened by a group of molecules that act in a pleiotropic fashion, inhibiting endothelial proliferation and migration while stimulating ECM production and inducing smooth muscle cell differentiation. These molecules include TGF-β1, TGF-βRII, endoglin and Smad5 (Dickson et al., 1995; Li et al., 1999; Carmeliet, 2000).
Figure 1.3 Schematic representation of arteriogenesis. Once smooth muscle cells (SMC) have been recruited to the nascent vasculature, they provide further muscular support by sprouting or migrating (longitudinal migration) along the existing vasculature, using the vessel as a guidance cue. Many of the molecules involved in vascular myogenesis (PDGF-B, PDGFR-β and VEGF) are also believed to play a role in arteriogenesis. Other potential regulators of arteriogenesis include connexin 43 (Cx43), neuropilin-1 (NP-1), Pax3, Wnt-1, and endothelin-1 (ET-1). Graphics adapted from Hanahan, 1997.
1.1.4 Arteriogenesis

Arteriogenesis describes the further muscularisation of the vasculature via the sprouting or migration (longitudinal migration) of already recruited mural cells alongside pre-existing vessels (Carmeliet, 2000; Luttun et al., 2002). It is believed that the same instructional cues that are involved in the recruitment and growth of smooth muscle cells during vascular myogenesis play a role in arteriogenesis (Figure 1.3).

1.2 Key molecules in vasculogenesis and angiogenesis

Although much remains to be understood concerning the genetic regulators governing embryonic vascular development, a number of key genes have been characterised (summarised in appendix 1, Table A1.1). Experiments in a number of developmental model systems, in particular gene targeting studies in the mouse (summarised in appendix 1, Table A1.2), have helped to dissect the roles that these often pleiotropic genes perform during vasculogenesis and angiogenic remodelling.

1.2.1 Basic fibroblast growth factor

Although it is well known that bFGF acts as a potent stimulator of angiogenesis both in vitro and in vivo, it is unclear whether it functions in the assembly of the early embryonic vasculature. Experiments in Xenopus have shown FGF signalling to be crucial for mesoderm induction. Injection of a dominant-negative form of the FGF receptor into Xenopus embryos resulted in an inhibition of mesoderm induction (Amaya et al., 1991). In addition, FGF-2 has been shown to induce vasculogenesis in quail blastodisc-derived embryoid bodies and dissociated epiblast (Krah et al., 1994). As well as being a potent modulator of mesoderm induction, work in mice carrying a soluble dominant-negative FGF
receptor have demonstrated the requirement of FGF for the induction and patterning of organ systems (Celli et al., 1998).

1.2.2 Vascular endothelial growth factor

Of all the regulators believed to be involved in vasculogenesis and/or angiogenesis, the most compelling evidence is for the potent growth factor VEGF. VEGF is synthesised by a range of cell types including tumour cells, T cells, macrophages, smooth muscle cells, kidney cells, astrocytes and osteoblasts (Klagsbrun and D'Amore, 1996). VEGF was initially discovered and characterised for its ability to induce vascular leak and permeability as well as its proliferative effect on vascular endothelial cells (Ferrara, 1999). VEGF is a member of the PDGF family (Neufeld et al., 1994). Members of the PDGF family include, PDGF and VEGF-A through to VEGF-E. Although VEGF is predominantly active as a homodimer, there is evidence to suggest that functional heterodimers between VEGF isoforms and PDGF do exist (Neufeld et al., 1994). The majority of research conducted on these VEGF related proteins has focussed on VEGF-A. Less is known regarding the potential roles other VEGFs (VEGF-B, -C, -D and -E) play during vascular development.

1.2.2.1 Expression of VEGF during embryogenesis

A significant feature of VEGF is the existence of multiple mRNA species generated through alternate splicing events from a single VEGF gene. These splice variants encode for the VEGF isoforms VEGF<sub>121</sub>, VEGF<sub>165</sub>, VEGF<sub>189</sub> and VEGF<sub>206</sub>. An interesting feature of each of these isoforms is that although each is synthesised with a signal peptide, they are processed differently by the cell. VEGF<sub>121</sub> is secreted into the medium, VEGF<sub>165</sub> is also secreted into the medium as well as being cell-associated, while VEGF<sub>189</sub> is sequestered on cell surfaces and in ECM (Park et al., 1993). Furthermore, the expression of each of these VEGF species appears to be isoform-specific. In the human, isoforms VEGF<sub>121</sub> and
VEGF<sub>164</sub> are the most abundantly expressed while in the mouse VEGF<sub>120</sub> and VEGF<sub>164</sub> but not VEGF<sub>188</sub> is expressed in the developing brain. Mouse VEGF isoforms possess one less amino acid than their human counterparts.

Expression domains for VEGF are found throughout the developing embryo, however, in situ hybridisation studies have demonstrated that the highest levels are present in the endoderm (Breier et al., 1992; Flamme et al., 1995; Cleaver and Krieg, 1998) (Table A1.1). Experiments in Xenopus have demonstrated the expression of a diffusible vegf<sub>122</sub> from the hypochord (an endodermal derivative), a transient structure present in Xenopus and zebrafish that is found immediately dorsal to the dorsal aorta (DA) (Cleaver and Krieg, 1998). Based on tissue transplantation and vital dye-labelling experiments, Cleaver and Krieg believe this VEGF source to provide a chemoattractant signal for angioblasts that migrate to form the DA directly beneath. Expression of VEGF in the mouse by in situ hybridisation detected transcripts from embryonic day E7 in the extraembryonic and embryonic endoderm (Dumont et al., 1995). High levels of expression are then observed (E8) in the myocardium, gut endoderm, embryonic mesenchyme and amniotic ectoderm. By E12.5 transcripts can be detected in the head mesenchyme and neural ectoderm.

An important regulator of VEGF expression is hypoxia, increased levels of VEGF mRNA along with protein and bioactivity have been reported from primary cultures of non-tumorigenic cells when grown under low oxygen conditions (Shima et al., 1995). Promoter-reporter activation studies have been carried out to try and dissect the VEGF promoter and identify which regulatory elements might alter transcription in response to hypoxic conditions (Levy et al., 1995). Using this method a 3' regulatory element that was hypoxia-responsive was identified. This enhancer element showed high homology with a hypoxia-responsive element in the promoter region of the erythropoietin gene, a well-studied oxygen-regulated gene (Minchenko et al., 1994).
1.2.2.2 VEGF function during development

Gene inactivation of VEGF-A in the mouse emphasises the importance of this molecule in the regulation of vascular development. Mice deficient for a single VEGF-A allele do not survive beyond 11 days post coitum (dpc) (Carmeliet et al., 1996) (Table A1.2). Such a result indicates the importance of VEGF dose regulation for normal vascular development. Both heterozygotes and homozygotes failed to form a normal vascular pattern. In the heterozygote this was characterised by a decrease in red blood cells in blood islands, a failure of the vitelline vessels to connect with yolk sac circulation and a lack of vessel invasion into the forebrain (Carmeliet et al., 1996; Ferrara et al., 1996).

The quail embryo has also been used to investigate the role that VEGF performs during vascular development (Drake and Little, 1995). By injecting a recombinant human VEGF₁₆₅ at the onset of vasculogenesis they showed that normal vasculogenesis was profoundly altered. This was characterised by inappropriate neovascularisation of normally avascular areas and unregulated, excessive vascular fusion. Also in the quail, each of the four quail VEGF splice variants (VEGF₁₂₂, VEGF₁₄₆, VEGF₁₆₆ and VEGF₁₉₀) have been ectopically expressed using retroviruses. Overexpression of VEGF₁₂₂ in wing mesenchyme demonstrated its ability to accelerate the progression of vascular development (Flamme et al., 1995). Forced expression of each of the other isoforms revealed a similar potential to induce localised oedema and hypervascularisation of the wing bud (Schmidt et al., 1998). These and other results suggest that the various splice variants of VEGF may act at a number of different stages of vascular development. With diffusible splice variants acting at some distance to attract vascular endothelial cells while non-diffusible forms act locally, perhaps in altering rates of cell coalescence and adhesion during the assembly of the vascular tube.
1.2.2.3 VEGF in the zebrafish

In the zebrafish, genes encoding two Vegf isoforms have been isolated to date vegf<sub>127</sub> and vegf<sub>165</sub> (Liang et al., 1998; Liang et al., 2001). The major domain of expression of these two isoforms, as detected by in situ hybridisation, is the ventro-medial region of the somites. This domain of vegf transcription is closely associated with the developing axial vasculature of the zebrafish. Simultaneous overexpression of these isoforms in the zebrafish resulted in ectopic vasculature and blood cells as well as pericardial oedema (Liang et al., 2001). Expression analysis of vascular and haematopoietic markers within these embryos revealed the premature onset of flk-1, tie1, scl and gata1 expression suggesting a potential role for zebrafish Vegf in patterning the vasculature and haematopoietic lineages (Liang et al., 2001). Further functional evidence relating zebrafish Vegf to vascular development is provided by an antisense morpholino-induced knock-down model. In this model, Vegf-A function was demonstrated to be critical for proper axial vessel formation but not for the initial establishment of axial vessel patterning (Nasevicius et al., 2000).

Recently, another domain of vegf expression has been documented in the zebrafish hypochord, consistent with that displayed in Xenopus (Lawson et al., 2002). In the associated study, this domain of vegf expression, as well as the somitic domain was demonstrated to be dependent upon sonic hedgehog (Shh) signalling, presumably from the notochord. Furthermore, Vegf function was shown to be necessary for the expression of the arterial-specific genes notch5 and ephrin-B2a (Lawson et al., 2002). These experiments highlight another role for this pleiotropic gene in establishing and/or maintaining arterial identity.
1.2.3 VEGF receptors

There are three main receptors to which VEGF binds with varying levels of affinity, VEGFR1/Flt1, VEGFR2/Flk1 and VEGFR3/Flt4 (Figure 1.4). Each of these receptor tyrosine kinases possess a split intracellular tyrosine kinase domain and are characterised by having an extracellular domain containing seven immunoglobulin-like motifs (Klagsbrun and D'Amore, 1996). Of these receptors, it appears that Flk1 represents the main transducer of the VEGF signal in endothelial cells. The third receptor, Flt4 is closely related to Flt1 by sequence homology (Kaipainen et al., 1995). In addition to these main transducers of the VEGF signal there are a number of accessory receptors including neuropilins which are believed to be involved in modulating binding to the main VEGF receptors (Soker et al., 1998).

1.2.3.1 Expression of VEGF receptors during embryogenesis

In the developing mouse embryo, transcripts for Flk1 and Flt1 are specifically located in blood vessels and in capillaries of the developing organs. Of particular importance is the complementary expression of these two receptors with that of VEGF throughout phases of vasculogenesis and angiogenesis (Millauer et al., 1993). The onset of Flk1 expression in the mouse coincides with the initiation of vascularisation in the developing embryo, around E7.0. By E8.5 it is expressed throughout the vasculature and remains localised to the blood vessels (Dumont et al., 1995). Flt1 transcripts are found in moderate levels during organogenesis, low levels during fetal growth and highest levels in newborn mice (Peters et al., 1993). In the embryo proper, transcripts for Flt1 are located in blood vessels and capillaries of the developing organs, closely resembling the expression profile of Flk1 (Breier et al., 1995). Expression of Flt4 in the mouse by in situ hybridisation demonstrated the presence of transcripts in angioblasts of the head mesenchyme, the
Figure 1.4 Receptor interactions of three major families of growth factors involved in embryonic vascular development. (A) A simplistic view of the interactions between the VEGFs (VEGF-A, -B, -C and -D) and their receptors (VEGFR1, 2 and 3). (B) A similar view of the specificity between the angiopoietins (Ang1, 2, 3 and 4) and their receptor Tie2, little is known about how the orphan receptor Tie1 participates in these interactions. (C) The ephrins (Ephrin-B1, -B2 and -A1) and their interactions with the receptors (EphB2, B3, B4 and A2). Adapted from Yancopoulos et al., 2000.
cardinal vein and the allantois in E8.5 stage embryos. By E12.5, Flt4 expression was restricted to developing venous and lymphatic endothelia but not arterial endothelia (Kaipainen et al., 1995).

1.2.3.2 VEGF receptor function during development

VEGF receptor gene inactivation in mice has provided additional evidence that Flk1 and Flt1 are essential for normal vascular development. Mice deficient in functional Flk1 die between E8.0 and E9.5 due to an almost complete lack of vascular structures (Table A1.2). Yolk sac blood islands are absent as are any organised blood vessels suggesting a role in the early development of both endothelial cells and haematopoietic precursors, perhaps at the level of the haemangioblast (Shalaby et al., 1995). In contrast, mice deficient in Flt1 possess an excess of endothelial cells in both the embryonic and extraembryonic regions, however these vascular endothelial cells fail to assemble into a normal vascular pattern (Fong et al., 1995) (Table A1.2). This disorganised vasculature is characterised by large fused vessels that contain internally stranded groups of endothelial cells. These results suggest that Flk1 appears to mediate the major growth and permeability activities of VEGF while Flt1 is essential for the organisation of the embryonic vasculature, but not for endothelial cell differentiation. Similar gene inactivation studies of Flt4 in the mouse have demonstrated the requirement for this VEGF receptor in the development of the cardiovascular system (Taipale et al., 1999) (Table A1.2).

1.2.3.3 VEGF receptors in the zebrafish

In the zebrafish, flk-1 transcripts are initially detected by in situ hybridisation between the 5 and 7 somite stages as two bilateral stripes in the lateral plate mesoderm, flanking the embryonic axis (Liao et al., 1997). Throughout early somitogenesis these expression domains expand both rostrally and caudally. The anterior flk-1 expression
presumably lays the foundation for the head vasculature while the trunk lateral plate mesoderm expression domain begins to converge towards the ventral midline axis. Around the 13 somite stage, this *flk-l* expression extends caudally into the developing tail bud. By the 20 somite stage, the anterior and posterior expression domains meet and soon afterwards (24 somite stage) the axial vasculature (the DA and posterior cardinal vein (PCV)) becomes clearly distinguishable. Following 24 hours development, *flk-l*-expressing projections sprout from the DA, these represent the intersegmental vessels. At this time, all endothelial cells lining the vasculature in the zebrafish express *flk-l* (Liao et al., 1997) (Table A1.1). A recently characterised zebrafish mutant that lacks a functional Flk-1 has highlighted a specific angiogenic function for this receptor (Habeck et al., 2002). The embryonic expression of *flt4* in the zebrafish is detectable by the 18 somite stage and resembles the expression pattern of *flk-l* until 48 hours post fertilisation (hpf), when *flt4* is no longer expressed (Thompson et al., 1998). In contrast to *flk-l* expression, which seems to be strongest in the DA, *flt4* expression is highest in the PCV (Thompson et al., 1998). This is consistent with the venous-specific expression of *Flt4* in the mouse (Kaipainen et al., 1995).

### 1.2.3.4 Neuropilins

The accessory receptors neuropilin-1 and neuropilin-2 are also believed to be involved in vascular development. However, their ability to regulate vascular development is indirect via the modulation of VEGF binding to the main VEGF receptors. In the chicken, *neuropilin-1* is preferentially expressed in arterial endothelia while *neuropilin-2* is specific for venous endothelia. This suggests an additional role in determining arterial-venous identity (Herzog et al., 2001). Neuropilin-1 is believed to bind both Flk1 and Flt1 (Fuh et al., 2000; Whitaker et al., 2001). Targeted inactivation of neuropilin-1 in the mouse results in impairment of neural vascularisation and disorganised development of vascular networks in the yolk sac (Kawasaki et al., 1999) (Table A1.2). Neuropilin-2 also forms
complexes with Flt1 (Gluzman-Poltorak et al., 2000), however mice lacking neuropilin-2 function suffer from no serious cardiovascular abnormalities (Giger et al., 2000). Therefore, the role that neuropilin-2 performs, if any, during vascular development remains unclear.

In the zebrafish, neuropilin-1 displays a dynamic expression pattern (Lee et al., 2002). During late somitogenesis (22 somites), transcripts are located in tail angioblasts, gut endoderm, motoneurons, ventro-medial somites and the hypochord. Later in development (24 to 48 hpf) the expression of neuropilin-1 extends to the developing axial vasculature and persists in the gut endoderm throughout the trunk (Lee et al., 2002) (Table A1.1). Targeted knock-down studies in the zebrafish demonstrated that neuropilin-1 regulates angiogenesis through a Vegf-dependent pathway (Lee et al., 2002). Furthermore, zebrafish neuropilin-1 has been shown to be a functional receptor for human VEGF165 (Lee et al., 2002). These results suggest a conservation of function for these molecules during embryonic vascular development.

### 1.2.4 Angiopoietins and their receptors

The angiopoietin family represents an important partner for VEGF during vascular development. Currently, there are four members in the angiopoietin family, Ang1, Ang2, Ang3 and Ang4 (Yancopoulos et al., 2000). These angiopoietins were originally identified as ligands for the Tie receptors, a family of receptor tyrosine kinases that are endothelially-expressed (Figure 1.4). Of the two Tie receptors (Tie1 and Tie2), only Tie2 binds the known angiopoietins. Therefore, the mechanism through which the orphan receptor Tie1 affects vascular development remains unclear.
1.2.4.1 Expression of angiopoietins and their receptors during embryogenesis

Expression of Ang1 is initially detected in the mouse heart myocardium. Later in development, expression is detected in the mesenchyme and smooth muscle cells surrounding the developing vasculature (Suri et al., 1996) (Table A1.1). In contrast, Ang2 is only expressed at sites of active vascular remodelling suggesting a role specific to angiogenesis (Maisonpierre et al., 1997). Transcripts for Tie1 are detected in the mouse by E8.5 in endothelial cells of the head mesenchyme, the DA and yolk sac blood islands (Korhonen et al., 1994) (Table A1.1). This vascular endothelial expression persists throughout development but in the adult becomes restricted to capillaries in the perilveolar septa of the lung. Expression of Tie2 commences in the yolk sac vasculature of the E7.5 stage mouse embryo, and by E8.0, expands to also include the endocardium and DA (Table A1.1).

1.2.4.2 Angiopoietin function during development

Many of the vascular phenotypes observed following the targeted disruption of these genes are highly conserved, as one would expect by inactivating ligand/receptor pairs. Ang1-deficient mice die at E12.5, and although they possess a primary vasculature, the remodelling events leading to a mature vascular system fail to take place (Suri et al., 1996) (Table A1.2). The underlying defect was a failure of endothelial cells to associate with periendothelial support cells (Suri et al., 1996). The researchers used this result to create a model in which Ang1 performs a permissive role, optimising the interaction between the Tie-expressing endothelial cells and the underlying Ang1-expressing support cells (Suri et al., 1996). In support of this, overexpression of Ang1 resulted in vessels that were resistant to vascular leakage induced by VEGF or inflammatory agents (Thurston et al., 1999).

Ang2 is believed to function as a natural antagonist for Ang1 and Tie2 (Maisonpierre et al., 1997). Furthermore, targeted disruption of Ang2 in the mouse results
in disrupted angiogenic and, to a lesser extent, vasculogenic events in the retina (Hackett et al., 2002). These results, together with the fact that Ang2 is only expressed in endothelial cells at sites of active remodelling suggest that Ang2 may provide a key de-stabilising activity at sites of active vascular remodelling (Yancopoulos et al., 2000). Other evidence, however, suggests that Ang2 signalling is much more complex than this. Using an in vitro model of angiogenesis it has been shown that Ang2 may act directly by stimulating the Tie2 receptor and inducing in vitro angiogenesis (Teichert-Kuliszewska et al., 2001). If such an activity exists in vivo, then Ang2 may act alternately to induce or inhibit Tie2 transduced signalling in endothelial cells. The modulators of such a decision are likely to be dependent upon the local microenvironment.

Targeted disruptions to the Tie receptors, Tie1 and Tie2, have revealed important but distinct roles during blood vessel development. Embryos deficient in Tie1 developed oedema and associated localised haemorrhages (Sato et al., 1995) (Table A1.2). This leaky phenotype was demonstrated to be due to a loss of endothelial integrity. In contrast, targeted disruption of the Tie2 locus resulted in a disrupted vascular network and was more reminiscent of the Ang1 knock-out phenotype (Sato et al., 1995) (Table A1.2). These results suggest distinct roles for the Tie receptors during embryonic development. Tie1 appears to function in establishing the integrity of endothelial cells while Tie2 is more involved in the formation of vascular networks (Sato et al., 1995).

1.2.4.3 Angiopoietins in the zebrafish

In the zebrafish, three angiopoietin genes have been isolated to date, ang1, ang2, and angptl3 (angiopoietin-like-3). Expression analysis of these genes by in situ hybridisation has revealed distinct patterns of expression for each (Pham et al., 2001). Transcripts for angl are located in ventral head mesenchyme and mesenchyme surrounding the major trunk vessels (consistent with murine expression) and in ventro-medial regions of the somites in the 24 hpf stage embryo (Table A1.1). This coincides with the development
of the primitive embryonic vasculature. Another site of expression is the hypochord, a single-cell layer immediately dorsal to the DA and ventral to the notochord. Although a similar structure does not exist in mammals, in *Xenopus* and zebrafish the hypochord has been implicated in DA formation (Cleaver and Krieg, 1998; Cleaver et al., 2000). Expression of *ang2* is also detected in the ventral head mesenchyme but its expression domain extends more anteriorly. A domain of *ang2* expression is also located in the pronephric glomeruli (Table A1.1). The expression of *angptl3* is confined to the yolk syncytial layer and has no overlap with the expression of either *angl* or *ang2*. Although the embryonic expression pattern of the zebrafish orthologue of *Angl* does not exactly match that of its mammalian counterpart, it is closely associated with the developing vasculature expressing the Tie receptors. In the zebrafish both *tie1* and *tie2* are predominantly expressed in endothelial cells of the developing vasculature (Lyons et al., 1998) (Table A1.1). This suggests that the central mechanism underlying the angiopoietins role in vascular development is conserved between the zebrafish and its mammalian counterpart.

### 1.2.5 Ephrins

The Eph family of receptor tyrosine kinases represents the largest family of growth factor receptors, with at least 14 members identified to date (Flanagan et al., 1998). There are 8 known ligands that transduce their signals through these receptors (Figure 1.4). The Eph family of receptors and ligands are divided into two subclasses, A and B, based on ligand specificity and structural homologies (Flanagan et al., 1998). An interesting feature of the Eph receptor/ligand interaction is the requirement for the ligand to be tethered to the membrane in order to activate its native Eph receptor (Flanagan et al., 1998). This is in contrast to ligands of other receptor tyrosine kinases that can act as soluble mediators.

The majority of research on this family to date has focussed on its involvement in neural development (Flanagan et al., 1998), but recent evidence suggests that at least a subset of this family functions in development of the early embryonic vascular system.
Targeted gene disruption of both the Eph receptor EphB4 and its ligand Ephrin-B2 provide compelling evidence for their involvement during vascular development (Gerety et al., 1999). Mice deficient in both EphB4 and Ephrin-B2 show defects in early angiogenic remodelling that are similar to those in knock-out models of Tie2 and Ang1 (Table A1.2). An interesting feature of this ligand receptor pair is their expression profiles. Transcripts for ephrin-B2 are found in the endothelium of developing arterial vessels while EphB4 transcripts are found in the endothelium of primordial venous vessels (Wang et al., 1998b) (Table A1.1). Both of the zebrafish orthologues for these genes have been characterised and share the same contrasting expression profiles as their mammalian counterparts (Table A1.1). Such a distribution suggests that these molecules are involved in defining arterial versus venous identity, perhaps in fusing arterial and venous vessels (Figure 1.5) (Yancopoulos et al., 2000). These results suggest that signalling between the tethered ligand Ephrin-B2 and its receptor EphB4 is essential for angiogenic remodelling and morphogenesis.

1.2.6 Platelet-derived growth factor

The PDGF family consists of pairwise assemblies of two highly related PDGF chains, A and B, resulting in homodimers (PDGF-AA or -BB) or heterodimers (PDGF-AB). This arrangement forms three different PDGF isoforms whose action on a target is dependent upon the repertoire of PDGF receptors that the particular cell presents on its surface (Beck and D'Amore, 1997). In a similar fashion to the ligands, the PDGF receptors are also dimers of an α and a β subunit. The receptor subunits confer specificity with the α subunit able to bind both PDGF ligand chains while the β subunit is specific for the PDGF-B chain (Beck and D'Amore, 1997).
Figure 1.5 Schematic representation of the involvement of Ephrin-B2 and EphB4 during angiogenic remodelling. EphB4 and ephrin-B2 are reciprocally expressed by venous and arterial endothelium, respectively. Furthermore, both are presented on the surface of the endothelial cells. Taken together, this supports a model in which they interact at the junction of arterial and venous vessels. Establishing such an arterial/venous identity is crucial for proper angiogenic remodelling of the primary vascular plexus into a mature vascular system. Adapted from Yancopoulos et al., 1998.
The expression pattern of one of these PDGF receptors in the mouse is particularly interesting with respect to angiogenesis due to its close association with the developing vasculature. Transcripts for platelet-derived growth factor receptor β (PDGFR-β) are detected in the mesenchyme surrounding the endothelium of large blood vessels and in the endothelium of small blood vessels (Shinbrot et al., 1994) (Table A1.1). This suggests a role in the growth and/or development of blood vessels. Such a model is supported by the endothelial expression of the gene encoding the PDGF ligand PDGF-B (Lindahl et al., 1998) (Table A1.1). Not surprisingly, targeted inactivation of PDGFR-β and PDGF-B results in similar disruptions to normal vascular development. Mice deficient in PDGFR-β are haemorrhagic, thrombocytopenic, anaemic and die at, or shortly following, birth (Soriano, 1994) (Table A1.2). Mice deficient in PDGF-B also develop severe, fatal haemorrhages prior to birth and suffer from anaemia and thrombocytopenia (Table A1.2). Ultrastructural analysis of the PDGF-B mutant mouse revealed a lack of microvascular pericytes and large numbers of capillary microaneurysms (Lindahl et al., 1997). Endothelial cells that would normally possess a functional PDGF-B were unable to recruit PDGFR-β positive pericytes in the PDGF-B-deficient mutant. Given that these pericytes contribute to the mechanical stability of the vasculature, a lack of such support would result in fragile capillary networks that would be susceptible to ruptures and leaks.

1.2.7 Transforming growth factor-β

TGF-β signalling is transduced through two types of serine/threonine kinase-containing receptors, type I (TGF-βRI) and type II (TGF-βRII), which form a heteromeric complex (Kingsley, 1994). There are three different isoforms of TGF-β in mammals (TGF-β1, 2 and 3). The signal transduction pathway requires ligand binding to the type II receptor which then phosphorylates and activates the type I receptor to signal downstream pathways (Kingsley, 1994).
1.2.7.1 Expression of TGF-β1 and its receptors during embryogenesis

Transcripts for TGF-β1 are first detected in the mouse by in situ hybridisation at around 7.5 dpc in the extraembryonic yolk sac blood islands, mesodermal cells of the allantois and the pro-angioblast progenitors within the cardiogenic mesoderm (Akhurst et al., 1990). The expression in the haemangioblastic cells of the blood islands represents progenitors of both the endothelial and haematopoietic lineages, both of which continue to express TGF-β1 (Akhurst et al., 1990) (Table A1.1). TGF-βRI, also known as activin receptor-like kinase 5 (ALK-5), is a widely expressed type I receptor which can induce the phosphorylation of Smad2 and Smad3 (Larsson et al., 2001). The type II receptor, TGF-βRII, is also widely expressed in vascular smooth muscle cells (VSMCs) and pericytes (Oshima et al., 1996), closely associated with TGF-β1-expressing endothelial cells (Table A1.1).

1.2.7.2 TGF-β1 function during development

Not surprisingly, TGF-β1- and TGF-βRII-deficient mice possess identical defects in vascular development. Both die at midgestation due to defects in yolk sac vasculogenesis and haematopoiesis (Dickson et al., 1995; Oshima et al., 1996) (Table A1.2). The initial differentiation of yolk sac mesoderm to endothelial cells does occur but results in weak vessels with reduced cellular adhesiveness. Furthermore, there is a decrease in erythroid cell number in the yolk sac. This result is interesting in the context that TGF-β1 has been reported as a potent in vitro inhibitor of endothelial and haematopoietic proliferation (Muller et al., 1987; Ottmann and Pelus, 1988). Targeted disruption of the type I receptor TGF-βRI, results in defects to vascular development of the yolk sac, consistent with the defects described for the TGF-β1 and TGF-βRII knock-out mice. However, surprisingly the haematopoietic compartment of the TGF-βRI knock-out mice is not deficient, in contrast there is an increase in erythroid colonies within mutant yolk sacs (Larsson et al., 2001).
The vascular defect in these TGF-βRI-deficient mice is most likely due to impaired fibronectin synthesis and migration displayed by their endothelial cells (Larsson et al., 2001). These differences suggest that TGF-β signalling acts through independent mechanisms to regulate its vascular and haematopoietic functions during embryonic development. Indeed, it seems likely that TGF-β acts to influence vascular development through a variety of different actions, such as inhibition of endothelial cell proliferation and migration, induction of periendothelial cell differentiation, stimulation of matrix accumulation (Beck and D’Amore, 1997) and the maintenance of vessel wall integrity.

1.2.7.3 TGF-β signalling in the zebrafish

A recently characterised zebrafish mutant, violet beauregarde (vbg), has shed light on the role of Alk-1, a TGF-β type I receptor, during vertebrate vascular development (Roman et al., 2002). The expression of this gene is restricted to endothelial cells within the zebrafish embryo with highest levels predominantly in cranial vessels. Zebrafish carrying a disrupted alk-1 locus appear morphologically normal during the first 1.5 days development, with circulation commencing at around 25 hpf. However, following 2 days development, vbg embryos possess dilated cranial vessels, composed of twice the normal number of endothelial cells, that carry the majority of blood flow. Caudal circulation throughout the trunk and tail is almost completely inhibited. Furthermore, these vascular defects do not appear to be due to perturbed vascular patterning (Roman et al., 2002). The authors suggest that the vbg mutant represents a zebrafish model for the human disorder, hereditary haemorrhagic telangiectasia type 2 (HHT2). This condition results from a disruption at the human activin receptor-like kinase I (ACVRLI) locus (Roman et al., 2002).
1.2.8  Endoglin

Endoglin is a TGF-β binding auxiliary protein that is associated with the autosomal dominant disease, hereditary haemorrhagic telangiectasia type 1 (HHT1) in humans, which is characterised by vascular abnormalities (McAllister et al., 1994). Expression of endoglin, analysed by monitoring β-galactosidase expression from a lacZ reporter driven by the endoglin promoter, was first detected by 6.5 dpc in extraembryonic ectoderm. By 8.5 dpc expression was detected in primitive vascular endothelial cells in the yolk sac. This endothelial expression continued during development irrespective of whether the vasculature was derived from vasculogenesis or angiogenesis (Jonker et al., 2002) (Table A1.1). Targeted disruption of this TGF-β binding protein results in lethality by day 11.5 due to defective vascular development (Li et al., 1999) (Table A1.2). Endoglin deficiency resulted in angiogenic remodelling defects characterised by poor VSMC development and arrested endothelial remodelling (Li et al., 1999). This suggests an important role for endoglin during embryonic angiogenesis.

1.2.9  Notch

There is a growing amount of evidence that Notch signalling possesses a role during embryonic vascular development (Gridley et al., 2001). The Notch intracellular signalling mechanism is a highly evolutionary conserved process in which both the ligands and receptors are transmembrane bound. This restricts the Notch signalling cascade to regulating interactions between neighbouring cells. To date four mammalian Notch receptors (Notch1, 2, 3 and 4) and five Notch ligands (Delta1, 3 and 4; Jagged1 and 2) have been identified (Krebs et al., 2000; Kortschak et al., 2001; Villa et al., 2001).
1.2.9.1 Expression of Notch receptors and ligands during embryogenesis

In the mouse, transcripts for Notch1 are detected in the descending aorta while Notch3 expression is restricted to arterial smooth muscle cells (Villa et al., 2001) (Table A1.1). Also specific for arteries is Notch4 expression, although this Notch receptor is selective for the endothelium (Villa et al., 2001). Notch2 expression has not been reported in either arteries or veins of the developing vasculature. In situ hybridisation has also revealed transcripts for the Notch ligands Delta4 in arterial endothelium, Jagged1 in arterial smooth muscle cells (Table A1.1) and Jagged2 in arteries (Gridley et al., 2001). So, in summary, Notch1, Notch3, Notch4, Delta4, Jagged1 and Jagged2 are all specifically expressed by arteries, and not veins.

1.2.9.2 Function of Notch signalling

Targeted inactivation of members of the Notch signalling pathway has provided compelling evidence for their involvement in angiogenic remodelling of the primitive embryonic vasculature. Mice deficient for Jagged1 die early during embryogenesis due to severe haemorrhages and exhibit defects in angiogenic remodelling of the yolk sac vasculature (Xue et al., 1999) (Table A1.2). Vascular defects are also present in mice deficient for Notch1 and in Notch1/Notch4 double mutant embryos (Krebs et al., 2000) (Table A1.2). These defects were present in the placenta, yolk sac and embryo proper. In brief, vasculogenesis appeared normal in the yolk sac as evident by a normal primary vascular plexus. However, there was no subsequent angiogenic remodelling of this plexus into large vitelline blood vessels. In the placenta, defective angiogenesis prevented the normal invasion of blood vessels into the placental labyrinth. Angiogenesis defects in the embryo proper resulted in malformed vasculature, such as the DA, anterior cardinal veins and the intersomitic vasculature (Krebs et al., 2000).
1.2.9.3 Notch signalling in the zebrafish

In the zebrafish, four Notch receptors have been identified, Notch 1a, 1b, 5 (also known as Notch 3) and 6 (also known as Notch 2) (Bierkamp and Campos-Ortega, 1993; Kortschak et al., 2001; Lawson et al., 2001). Zebrafish notch3 is expressed specifically by arterial endothelial cells of the DA and not by venous endothelial cells that constitute the PCV (Lawson et al., 2001). Zebrafish embryos lacking Notch activity have been shown to possess defects in arterial-venous specification (Lawson et al., 2001). Such embryos fail to induce arterial-specific expression of ephrin-B2 and also demonstrate misexpression of venous-specific markers in the DA. Furthermore, these defects in specification correlate with defective remodelling of the major trunk vessels, presumably through a lack of arterial-venous identity (Lawson et al., 2001). More recent studies have demonstrated that the arterial-specific expression of notch5 in the zebrafish DA is dependent upon functional Vegf activity, which in turn is mediated by notochord-derived Shh signalling (Lawson et al., 2002).

1.3 Adhesion events during angiogenesis

Angiogenic remodelling requires the establishment of intimate cell-to-cell interactions between endothelial cells and supporting cells in the local microenvironment. The angiogenic process is highly dynamic, requiring the loosening of existing contacts between endothelial cells and the establishment of new ones. The formation and maintenance of these cell contacts requires a complex relationship between plasma membrane proteins, cytoskeletal components and associated signalling molecules (Vestweber, 2000). The greatest amount of research conducted on endothelial cell contacts has focussed on two different types of junctional complexes, adherens junctions and tight junctions. Adherens junctions are characterised by a cytoplasmic electron-dense structure on the plasma membrane of neighbouring cells (Gumbiner, 1996). They are ubiquitously
expressed by the endothelial cells of all vascular beds. Tight junctions are defined by the apparent partial fusion of closely juxtaposed plasma membranes (Anderson et al., 1995) and act to seal the endothelial cell layer. The adherens junction protein VE-cadherin has been demonstrated to be critical for normal angiogenesis. VE-cadherin can mediate angiogenesis, endothelial barrier function and support cross-talk with VEGF. Blocking VE-cadherin function, using antibodies, inhibits angiogenesis while targeted gene disruption results in embryonic lethality due to abnormal organisation and remodelling of the vasculature (Carmeliet et al., 1999a; Dejana et al., 1999; Liao et al., 2000a; Corada et al., 2001) (Table A1.2).

In addition to endothelial cell-cell contacts, contacts between endothelial cells and components of the ECM are of critical importance to normal angiogenic remodelling. Cell adhesion to the ECM is mediated by integrins, one such endothelial cell-specific integrin, $\alpha_\beta_3$, is believed to be required for the formation and/or maintenance of de novo vessels (Beck and D'Amore, 1997). Inhibition of $\alpha_\beta_3$ function during chick angiogenesis results in the inhibition of neovascularisation (Brooks et al., 1994). The integrin $\alpha_\beta_3$ also appears to possess a vasculogenic role. In quail embryos with compromised $\alpha_\beta_3$ activity dorsal aortae develop in their normal positions but lack patent lumens and are fragmented (Drake et al., 1995). Furthermore, targeted deletion of the $\alpha_\beta$ gene in mice is lethal due to severe haemorrhaging in a subset of organs (Eliceiri et al., 2001).

In summary, the relationship between neighbouring endothelial cells and between endothelial cells and the ECM is essential for key aspects of vascular development. Not only do the contacts provide a physical connection between cells, but also in co-ordinating the response to inputs, they regulate cell migration, proliferation and differentiation.

1.4 Shear stress-induced vascular remodelling

The assembly of the primitive vascular system takes place in the absence of any blood flow. However, further angiogenic remodelling of this rudimentary system occurs in
the presence of circulating blood. This early observation resulted in the hypothesis that haemodynamic forces such as shear stress influence vascular remodelling. Many early studies on such haemodynamic-dependent maturation focussed on ultrastructural analysis of ECM development and VSMC differentiation during vascular development (Gonzalez-Crucci et al., 1971; Murphy and Carlson, 1978). These early structural studies correlated ECM development and VSMC differentiation with an increase in blood pressure during embryonic development in the chick. This was primarily based on the observation that the initial circulation flowed through primitive blood vessels that lacked any presumptive VSMCs. It was not until an increase in blood pressure was experienced that VSMCs were recruited around the embryonic vasculature. More recent studies have focussed on the genetic regulatory networks involved (Wilson et al., 1993; Ohno et al., 1995; Resnick and Gimbrone, 1995; Wilson et al., 1995; Reusch et al., 1996; Resnick et al., 1997).

Following the application of mechanical strain, rat neonatal VSMCs have been demonstrated to elevate their production and secretion of PDGF (Wilson et al., 1993). This increase in PDGF activity resulted in a mitogenic response in the VSMCs, as detected by increased DNA synthesis. This activity was found to be ECM-dependent (Wilson et al., 1995). Mechanical strain was reported to increase DNA synthesis in VSMCs cultured on collagen and fibronectin, but not on elastin or laminin. Furthermore, this response to cyclic mechanical stress was abrogated in the presence of antibodies to specific integrin isoforms. This suggests that interactions between specific integrins and matrix proteins are responsible for sensing and/or transducing mechanical stress (Wilson et al., 1995). Further evidence for shear stress-induced vascular remodelling is provided by similar experiments that demonstrate an elevation in TGF-β production from endothelial cells subjected to mechanical strain (Ohno et al., 1995).

In summary, both endothelial and periendothelial cells possess the capacity to respond to haemodynamic forces. This has led to a model by which flow-induced vascular remodelling and maturation is mediated by shear-stress induced expression of autocrine and paracrine molecules.
1.5 Vascular anatomy of the zebrafish

Recently, the advent of confocal microscopy, along with sophisticated image processing software and micromanipulation, has led to the complete characterisation of the zebrafish embryonic vasculature in very high detail (Isogai et al., 2001) (Figure 1.6). In addition, it has led to the establishment of a standard reference nomenclature (summarised in appendix 2, Table A2.1). Having a standard reference such as this is extremely valuable, enabling very small perturbations in vascular development to be characterised. For example, microangiography proved useful in detecting disrupted vascular modelling in zebrafish embryos exposed to cadmium (Cheng et al., 2001). Microangiography employs the use of small fluorescent microspheres that can penetrate even the smallest of blood vessels. Furthermore, unlike more traditional methods used to delineate vasculature that use coloured dyes or plastic resins, these fluorescent spheres do not significantly harm the specimen, enabling live, real-time image capture. In vivo imaging of vascular development in fli1:EGFP transgenic zebrafish has demonstrated that blood vessels undergoing angiogenic modelling display filopodial and pathfinding behaviour reminiscent of neuronal growth cones (Lawson and Weinstein, 2002).

1.5.1 Initiation of circulation

Blood can first be observed circulating at around 24-26 hpf. This initial blood flow exists as a simple rudimentary circuit (Figure 1.6A). Starting from the heart, blood moves into the mandibular aortic arches through the bulbus arteriosus and the ventral aorta, where it empties into the paired left- and right-lateral dorsal aortas (LDA). These two vessels run caudally, progressively approaching one another until they bisect and fuse into a single DA that then carries the blood caudally through the developing trunk into the developing tail.
Figure 1.6 The vascular anatomy of the developing zebrafish. Microangiography of the developing vascular system in 1.5 dpf (A), 2.0 dpf (B), 2.5 dpf (C), 3.0 dpf (D) and 3.5 dpf (E) stage zebrafish. Red arrows denote direction of blood flow in the developing trunk and tail. The dorsal aorta (DA), posterior cardinal vein (PCV), caudal artery (CA), caudal vein (CV), dorsal longitudinal anastomotic vessel (DLAV) and intersegmental vessels (Se) provide circulation to the developing trunk and tail regions (highlighted in red). For a translation of the abbreviated vessels, see Table A2.1. Adapted from Isogai et al., 2001.
The posterior-most region of the DA, that extends beyond the anal pore, represents the caudal artery (CA). This is the vessel that carries the primitive circulation into the tail where it turns 180° and begins its rostral passage back to the heart, beginning at the caudal vein (CV). The CV initially exists as an undefined plexus of very small vessels. Eventually this labyrinth of small channels becomes remodelled into a single defined vessel, more similar to the CA that lies immediately dorsal. The CV then empties into the PCV which carries blood anteriorly through the trunk from the anal pore to the cranial trunk. At this point, the PCV splits into a pair of vessels that terminate just rostral to the LDA/DA fusion point (Figure 1.7A). Each posterior cardinal then empties into the duct of Cuvier (DC)/future common cardinal vein (CCV) which fans the blood out over both sides of the yolk. These flows then merge cranioventrally at the sinus venosus where it re-enters the heart. Soon after the initiation of this crude circulatory network, a cranial circuit starts. Where blood exiting the first aortic arch only travelled caudally into the paired LDA, now, it also flows rostrally and empties into the primitive internal carotid arteries (PICA). From here blood flows into the caudal division of the internal carotid artery (CaDI) where it eventually joins with the equivalent branch from the other side of the embryo through the basal communicating artery (BCA) that runs along the cranial midline. The other branch of the PICA, named the cranial division of the internal carotid artery (CrDI), carries blood to the optic capsule where is turns caudally into the primordial midbrain channel (PMBC) which then fuses with the primordial hindbrain channel (PHBC). At this stage, rudimentary vessels that sprout from the PMBC/PHBC junction represent primitive midcerebral veins (MCeV). These vessels have yet to fuse at the midline to complete a circuit.

1.5.2 Circulation at 1.5 dpf

Following 1.5 days post fertilisation (dpf), throughout the developing trunk vessels can be seen sprouting and elongating from the DA and PCV (Figure 1.6A). Although the
**Figure 1.7** Vasculogenesis and angiogenic remodelling of vessels in the cranio-trunk region of the developing zebrafish embryo. (A) Dorsal view (anterior to left) of a zebrafish embryo with a superimposed view of the main axial vessels in the cranial trunk region as they appear at around 1.2-1.5 dpf. Blue and red lines represent venous and arterial vessels, respectively. (B) Whole mount *in situ* hybridisation of a 1.2 dpf embryo (lateral view, anterior down), displaying β-globin-expressing blood cells travelling over the yolk sac through the common cardinal vein (CCV). (C) Diagram illustrating the extensive angiogenic remodelling and regression that occurs during the interconnection of the minor trunk vessels (DLAV and Se) with the cranial trunk/head vasculature throughout development stages 1.5 to 4.5 dpf. BA = Basilar artery, PHBC = Primordial hindbrain channel, DLAV = Dorsal longitudinal anastomotic vessel, Se = Intersegmental vessel, LDA = Lateral dorsal aorta, ACV = Anterior cardinal vein. Adapted from Isogai et al., 2001.
majority of these vessels do not support blood flow at this time, the fluorescent spheres highlight their genesis as they elongate dorsally away from the main axial vessels. These intersegmental vessels (Se) anastamose with other neighbouring Se vessels initially along the longitudinal axis to form a pair of dorsal longitudinal anastomotic vessels (DLAVs). However, at this stage of development, there are no connections between the left and right DLAVs that run the length of the trunk. The development of the Se and DLAV occurs in a caudal to rostral progression, with the caudal vessels functional before their more rostral equivalents. The DA and PCV remain relatively unchanged.

1.5.3 Circulation at 2.0 dpf

After 2 days development, the Se throughout the trunk and tail are lumenised and functional, carrying blood dorsally into the developing somitic and dorsal neural tissues. Also at this stage of development, a number of anastomotic vessels can be seen connecting the caudal regions of the left and right DLAVs (Figure 1.6B). This change, however, is not mirrored by the rostral DLAVs, which remain as separate paired parallel structures. The DA and PCV are again relatively unchanged from their appearance at 1.5 dpf. One change that is noticeable in the anterior trunk is the vascularisation of the pronephric glomus which occurs just caudal to the CCV and ventral to the DA. Another change in the cranio-trunk region is the commencement of regression of the left-paired PCV. The right PCV will progressively carry the majority of venous blood flow from the trunk with the left PCV carrying flow only from the anterior-most SeV. By 2 dpf, the circuitry of the developing brain becomes more complex with many new projections sprouting from the main cranial arterial passage. An important cranial-to-trunk connection occurs around this time point, linking the dorsal DLAV to the more medial paired PHBC. Eventually this DLAV/PHBC connection regresses and is replaced solely by the BA (Figure 1.7C). It is interesting to note
that the Se vessels do not follow a pre-designated arterial-venous identity, except for the first four sets of Se which do follow an inherent pattern.

1.5.4 Circulation at 2.5 dpf

By 2.5 dpf a small extension of the CA sprouts caudally into the base of the caudal fin (Figure 1.6C). This projection then splits into a pair of vessels to allow a simple circulatory loop to penetrate into the developing caudal fin. Also noticeable is the increased separation between the DA and PCV along the dorsal-ventral axis, and the continued remodelling of the CV plexus into a more defined single vessel. Despite an obvious increase in the complexity of the wiring within the developing head of the zebrafish, the overall framework of the major vessels remains unaltered after 2.5 dpf.

1.5.5 Circulation at 3.0 to 3.5 dpf

Following 3-3.5 days development, the paired DLAVs in the tail and caudal trunk are remodelled into a single vessel, and two completely new types of vessel sprout from the Se throughout the trunk (Figure 1.6D and E). These sprouts extend from a number of Se in both directions along the anterior-posterior axis. The ventral sprouts represent the parachordal vessels (PAV) while the more dorsal vessels are the primordial vertebral arteries (VTA). The PAVs form as a pair of vessels, lateral to the notochord at the horizontal myoseptum while the VTAs are located on each side of the base of the neural tube, immediately adjacent to the myotome. These vessels eventually elongate to span the somites at around the 5 dpf stage of development. New vessels also span the increasing dorsal-ventral gap between the DA and PCV. These vessels exist transiently and regress by
4.5-5 dpf. The CV continues its condensation and more resembles its final state as a single vessel in the ventral region of the tail.

1.5.6 Genetic cues control vascular development

While the zebrafish vascular anatomy is similar to that of other vertebrates, some differences in vascular circuitry do exist. These differences are most likely the result of adaptations to unique blood flow requirements in the developing zebrafish. A possible explanation could also be found in the rapid development the zebrafish experiences, with certain non-essential aspects of vascular development eliminated in the interests of speed (Isogai et al., 2001). Vascular modelling in the zebrafish is a highly dynamic process with new vessels and connections appearing and then disappearing, often to be replaced by completely new vascular tracts. However, despite this complex and seemingly chaotic process, a conserved and reproducible vascular plan does exist. This would suggest that the cues governing vessel modelling are genetically programmed. An example of such a cue is disrupted in the out of bounds (obd) zebrafish mutant (Childs et al., 2002). The genetic loci underlying this mutation is required for the proper sprouting of endothelial cells along the DA that constitute the intersegmental vasculature. Furthermore, these mutants display inappropriate and disorganised growth of these minor trunk blood vessels (Childs et al., 2002). A number of other loci have been found to be essential for various aspects of vascular development through mutagenesis screens in the zebrafish (Stainier et al., 1996), providing further evidence for underlying genetic programs that control vessel formation. These include vascular integrity mutants such as bubble head (BBH), leaky heart (leh), mush for brains (mfb) (Stainier et al., 1996) and gridlock (gdl) (Weinstein et al., 1995; Zhong et al., 2001).

In summary, this staged 3-dimensional atlas of the zebrafish has greatly increased our understanding of the dynamic steps involved in vertebrate vascular development. This
knowledge will prove invaluable in the interpretation of abnormal vascular patterns. Placing the zebrafish in a position to make a substantial impact on the level of knowledge regarding the regulators involved in embryonic vasculogenesis and angiogenesis.

1.6 Signals from the hypochord and gut endoderm are involved in vertebrate vascular development

There is an increasing amount of data to suggest that molecules involved in the assembly and maturation of the main axial vessels in the zebrafish are produced by bordering tissues like the notochord, the hypochord and the endoderm (Figure 1.8). The notochord has long been known to play a role in patterning midline and paraxial tissues such as the floor plate and somites (Halpern et al., 1995; Talbot et al., 1995). Characterisation of the zebrafish notochord mutants floating head (flh) and no tail (ntl) suggest that the notochord, that lies dorsal to the developing vasculature, is a source of signals that directs the formation of the DA (Fouquet et al., 1997; Sumoy et al., 1997; Brown et al., 2000). Both flh and ntl possess defects in the formation of the notochord, flh embryos completely lack a notochord while ntl embryos lack its differentiation. Vascular endothelial cells, highlighted by in situ hybridisation using the endothelial-specific marker flk-1, failed to organise into a DA in both mutants. However, the PCV was less effected (Fouquet et al., 1997). Further investigation into a role for the notochord in DA formation looked at flii expression, a vascular-specific transcription factor, in the mutants, flh, one-eyed-pinhead (oep), squint (sqt) and sonic-you (syu) (Brown et al., 2000).

The oep gene encodes an extracellular EGF-CFC family protein that appears to be essential for signalling by nodal-related proteins (Gritsman et al., 1999). In contrast to the notochord mutants, oep embryos do possess a notochord but lack anterior axial mesendoderm, the prechordal plate and ventral neuroectoderm as well as gut and other endodermal derivatives (Hammerschmidt et al., 1996). Consistent with the presence of a
**Figure 1.8** Diagrammatic representation of the sources of signals involved in zebrafish vascular development. (A) Lateral view of a 22 somite stage embryo with the hypochord, intermediate cell mass (ICM) and primitive gut endoderm (PGE) highlighted in blue, red and black, respectively. (B) Formation of the zebrafish ICM. Diagrammatic representation of a transverse section through the trunk of a developing zebrafish embryo. Vascular endothelial/haematopoietic precursors migrate from the lateral plate mesoderm (LPM) to the midline, beneath the developing somites (S), and coalesce to form the ICM. The ICM is sandwiched between the hypochord (H), that lies immediately dorsal, and the PGE, which is immediately ventral. (C) Schematic representation of possible mechanisms by which zebrafish Shh and Vegf signal to the developing vasculature. Three potential mechanisms are: (1) Shh signals from the notochord (N) to induce Vegf signalling in the ventro-medial somites and/or hypochord which, in turn, signals to the developing vasculature; (2) Shh signals from the notochord to induce the secretion of another signalling molecule from the hypochord, that acts on the developing vasculature; (3) Shh signalling from the notochord acts directly on the migrating angioblasts. Signal(s) from the underlying PGE are also believed to mediate the organisation of the axial vasculature. NT = Neural tube, DA = Dorsal aorta and PCV = Posterior cardinal vein. Adapted from Roman and Weinstein, 2000.
Hypochord
Intermediate cell mass (ICM)
Primitive gut endoderm (PGE)

22 somites
notochord, oep mutants possess a normal DA, however instead of the typical PCV that normally forms, there is a disorganised collection of flil-expressing endothelial precursor cells (Brown et al., 2000). The PCV is situated immediately dorsal to the gut endoderm, which is predominantly absent in oep mutants. This implies that signalling from the underlying endoderm is required for proper formation of the PCV. Such a role for the endoderm is supported by tissue ablation experiments in Xenopus. Although Xenopus embryos lacking endoderm possess aggregates of angioblasts, these disorganised structures fail to assemble into endothelial tubes (Vokes and Krieg, 2002). This suggests that endoderm signalling is not required for angioblast specification, but is essential for endothelial tube formation.

The sqt gene encodes a nodal-related signalling molecule (Feldman et al., 1998). The sqt mutant lacks ventral central nervous system (CNS) tissue and has a reduced prechordal plate (Heisenberg and Nusslein-Volhard, 1997). Another interesting phenotype of this mutant is the absence of the anterior notochord. Examining flil expression within this background revealed that no DA formed below the region of the ablated notochord, consistent with a role for the notochord in DA assembly (Brown et al., 2000).

The syu mutant results from a mutation at the sonic hedgehog (shh) locus (Schauerte et al., 1998). Transcripts for this gene are found in a number of zebrafish tissues, including both the notochord and endoderm (Krauss et al., 1993). Neither the DA nor the PCV form normal endothelial tubes in this mutant. This result is intriguing in that the signal that emanates from the notochord and endoderm to organise the axial vasculature may be Shh, or another signal whose expression is dependent on Shh (Figure 1.8C). Indeed, the Shh signal, presumably from the notochord, has been demonstrated to be necessary for vegf expression in the zebrafish (Lawson et al., 2002). Support for this comes from another study in which vegf expression was shown to be dependent upon a functional notochord (Liang et al., 2001). These results provide compelling evidence implicating the notochord and endoderm in the formation of the DA and PCV, respectively.
Several groups have hypothesised that the formation of the DA is actually mediated not by the notochord, but by signals secreted from the hypochord, which lies between the notochord and the DA (Lofberg and Collazo, 1997; Cleaver et al., 2000; Eriksson and Lofberg, 2000; Pham et al., 2001). The hypochord is a transient single-cell layer of endodermal origin that exists in fishes and amphibians (Lofberg and Collazo, 1997). In the zebrafish, it separates from a subset of endodermal cells at around the 14 somite stage (Eriksson and Lofberg, 2000). This specification of hypochord cell fate is believed to be dependent on Delta-Notch signalling (Latimer et al., 2002). While maintaining tight association with the notochord, the hypochord moves dorsally away from the underlying endoderm, creating a gap in which precursors of the future DA and PCV converge (Figure 1.8B). A number of studies have found that the hypochord expresses a wide variety of genes. These include transcription factors such as twist (Halpern et al., 1995; Schauerte et al., 1998), fkd1, 2, 4 and 7 (Odenthal and Nusslein-Volhard, 1998), adhesion molecules such as f-spondin (Ruiz i Altaba et al., 1993) and ECM components such as type II collagen (Yan et al., 1995). Several secreted growth factors are also expressed in the hypochord including shh (Ruiz i Altaba, 1998) and radar (Rissi et al., 1995). Moreover, Vegf (in Xenopus and zebrafish) and Angl (in zebrafish), two potent modulators of axial vascular development are synthesised by the hypochord (Cleaver and Krieg, 1998; Eriksson and Lofberg, 2000; Pham et al., 2001; Lawson et al., 2002).

Experiments conducted by Cleaver and Krieg in Xenopus have demonstrated that when the notochord is removed during early neuralation there is a complete inhibition of hypochord development. In reciprocal experiments in which ectopic notochord is transplanted, there is an expansion of the adjacent hypochord region (Cleaver et al., 2000). These results suggest that signals from the notochord instruct cells in the dorsal most aspect of the underlying endoderm to adopt a hypochord fate during early neural stages. The early nature of this inductive signal, from the notochord, is supported by normal collagen 2a expression, in morphologically normal hypochords, in the zebrafish notochord maturation mutants grumpy (gup), bashful (bal), dopey (dop), sneezy (sny) and gno (gno) (Stemple et
In these mutants, only the maturation of the notochord is affected (notochord cells fail to vacuolate). However, in the zebrafish notochord mutants \textit{flh} and \textit{ntl}, that fail to produce a functional notochord, the hypochord would be ablated.

The hypochord is a source of a diffusible isoform of Vegf in \textit{Xenopus} and zebrafish (Cleaver and Krieg, 1998; Lawson \textit{et al.}, 2002) and Ang1 in the zebrafish (Pham \textit{et al.}, 2001). In \textit{Xenopus}, ectopic expression experiments have demonstrated that this diffusible Vegf can act as a chemoattractant for migrating angioblasts that form the future DA and PCV (Cleaver and Krieg, 1998). In the zebrafish, \textit{vegf} is expressed by the ventro-medial region of the somites and the hypochord (Liang \textit{et al.}, 2001; Lawson \textit{et al.}, 2002). Both domains are closely associated with the developing axial vasculature. In addition, morpholino-mediated knock-down of the Vegf signal in the zebrafish has revealed an angiogenic role for this signal in axial vessel assembly (Nasevicius \textit{et al.}, 2000). Furthermore, signalling from the notochord, or indeed the hypochord, is required for \textit{vegf} expression in the somites, as revealed by a lack of somitic \textit{vegf} expression in the \textit{ntl} and \textit{flh} notochord mutants (Liang \textit{et al.}, 2001). This signal is most likely Shh (Lawson \textit{et al.}, 2002). Lawson believes that the somitic domain of \textit{vegf} expression, which is dependent upon Shh activity, is required for arterial identity within the DA. However, this function may also be mediated via Vegf in the hypochord, which is also dependent upon functional Shh and is immediately juxtaposed to the DA.

In summary, signals from the hypochord and underlying gut endoderm, two structures that sandwich the developing DA and PCV, respectively, are believed to provide a source of signals that participate in axial blood vessel assembly.
1.7 Relevance of the zebrafish in vascular development, disease and therapy

The processes of physiological and pathological angiogenesis are tightly regulated by a range of pro- and anti-angiogenic factors (discussed earlier). A number of diseases can result from imbalances in these processes. As reviewed earlier, these processes include the migration, differentiation and proliferation of endothelial cells, reorganisation of ECM and the mechanical process of vessel tube assembly. Some angiogenesis-dependent diseases can be controlled in animal models through the induction or inhibition of new vessel formation. Such conditions include wound healing, inflammatory diseases, ischemic heart and peripheral vascular disease, myocardial infarction, diabetic retinopathy and cancer (Battegay, 1995). Rapid advances in molecular genetics, molecular probes and imaging technology has provided a framework in which to study physiological and pathological angiogenic processes in detail never seen before, providing new and exciting therapeutic possibilities (Battegay, 1995; Ferrara and Alitalo, 1999; Carmeliet and Jain, 2000). A number of angiogenic factors have been tested in animal model systems, such as bFGF, FGF-5, VEGF and the angiopoietins (Ferrara and Alitalo, 1999). Of these molecules, VEGF has been the most extensively tested angiogenic regulator. Not surprisingly, the mouse and rat have been the model systems of choice to date. However, recently, the characterisation of a number of zebrafish mutant models of known human disorders has provided support for the use of this developmental model in unravelling the genetics behind human disorders (Table 1.1). One such example is represented by the zebrafish mutant *violet beauregarde* that lacks a functional Alk-1 receptor. These mutant embryos display defects in vascular development and may represent a zebrafish model of the human autosomal dominant disorder, HHT2, that results from the disruption of the human homologue, *ACVRL1*. These zebrafish were the result of chemical mutagenesis screens.
Table 1.1 Zebrafish mutants which represent models of human diseases.

A recently conducted Tubingen 2000 ENU mutagenesis screen has revealed approximately 750 mutants, representing 150 different genes that are required for normal vascular development in the zebrafish (Gerritsen et al., 2001). This forward genetics approach is in contrast to the traditional reverse genetics used in the mouse to inactivate a gene by gene targeting via homologous recombination.

The well documented advantages that the zebrafish offers as a model system makes it an ideal candidate in which to test potential therapeutic agents in zebrafish models of
human disorders (Vogel and Weinstein, 2000). New mutagenesis screens in the fish are being designed to target specific developmental events. These new generation screens can employ the use of promoter/enhancer-reporter transgenic lines. For example, transgenic zebrafish lines have been generated using an endothelial-specific tie2 promoter/enhancer-green fluorescent protein (GFP) reporter, allowing the visualisation of the vasculature in embryos and adults at the single-cell level (Motoike et al., 2000). A similar endothelial-specific fli1:enhanced GFP (EGFP) line has been established (Roman et al., 2002). A recent pilot haploid mutagenesis screen in this line (of approximately 500 genomes) generated 11 potential vascular mutants (B. Weinstein, personal communication). In brief, haploid embryos from F1 females were scored under fluorescent illumination at 1.5 dpf for vessel defects. Using this approach, defects in both vasculogenic and angiogenic events have been detected. Such promoter-reporter transgenic lines have also been used to characterise mutants generated from traditional random morphology mutagenesis screens. To highlight the effectiveness of such an approach, the vbg mutant (generated from such a traditional screen), when crossed with zebrafish harbouring a fli1:EGFP transgene, revealed that a disruption to the alk-l locus, the gene underlying the mutation, resulted in elevated numbers of endothelial cells in the cranial vasculature (Roman et al., 2002). In another similar example, the mosaic nature of transient transgene expression was utilised to highlight individual endothelial cells by injecting obd mutant embryos with a tie2:EGFP expression cassette (Childs et al., 2002). The obd zebrafish mutant is characterised by defective intersegmental vessel formation (Childs et al., 2002). Employing this technique, individual angioblasts destined to contribute to the intersegmental vasculature were tracked throughout their migration from the lateral plate mesoderm. The obd gene product was demonstrated to be critical for the normal migration of endothelial cells that are fated to the intersegmental blood vessels (Childs et al., 2002). The use of such transgenic lines enables both patent and forming blood vessels to be observed during development, as well as migrating angioblasts. This is an advantage not shared by conventional microangiography
which only highlights patent endothelial tubes connected to a functional circulatory network.

Other techniques that the zebrafish offers as a developmental system include the transient inactivation of selected genetic targets using morpholino antisense technology (Ekker, 2000; Nasevicius et al., 2000; Nasevicius and Ekker, 2000; Ekker and Larson, 2001). In addition, the use of Gal4-UAS binary transgenesis has been demonstrated in the zebrafish (Scheer and Campos-Ortega, 1999; Scheer et al., 2001), enabling a greater control of tissue-specific misexpression of genes of interest. Furthermore, transgenic zebrafish incorporating an inducible hsp70 promoter allows for precise temporal control of forced gene expression (Halloran et al., 2000; Scheer et al., 2001). Indeed, by utilising hsp70:Gal4 and UAS: notch1a transgenic lines, temporal ectopic activation of the Notch pathway was demonstrated to repress venous cell fate. This was detected by analysing expression of the endothelial marker flt4, which becomes restricted to venous blood vessels by the 30 somite stage of development (Lawson et al., 2001).

A relatively new technique that holds great potential for the zebrafish is the implementation of chemical genetic approaches. The advantages that the zebrafish offers as a model system positions it as an ideal candidate system in which to use this technique, making the zebrafish a potential model for drug target identification and a prescreen model for drug evaluation. This approach enables the precise temporal and dosage control of gene activity allowing for accurate functional blockage initiation and termination. Such a chemical genetic approach has already been used to explore angiogenic signalling in the zebrafish embryo, in particular signalling through the VEGF receptors (Chan et al., 2002).

In summary, the biology and genetics of the zebrafish make this system extremely attractive, both for the further investigation of vascular development, and for the discovery of novel therapeutic targets to treat cardiovascular disorders.
II. Radar and the TGF-β superfamily

1.8 Radar is a member of the GDF5, 6, 7 subgroup of the TGF-β superfamily

The TGF-β superfamily of secreted growth factors can be loosely divided into three groups: the TGF-βs, the activins and the BMPs (Kingsley, 1994; Hild et al., 2000; von Bubnoff and Cho, 2001). Members of the TGF-β superfamily share a conserved structure. Initially, a pre-pro-protein is synthesised containing an amino-terminal signal sequence and a carboxy-terminal pro-domain of variable size. A mature carboxy-terminal domain is then cleaved at a dibasic RXXR recognition site. Hetero- or homodimers comprising these mature-domains then signal to activate downstream signalling cascades (Kingsley, 1994). The pro-domain is poorly conserved between different family members but often well conserved for a particular member isolated from divergent organisms. This amino-terminal domain can vary greatly in both length and sequence but appears to be necessary for normal synthesis and secretion of TGF-β family members (Gray and Mason, 1990; Hammonds et al., 1991). In contrast, the mature domain is more highly conserved, containing seven, almost invariantly conserved, cysteine residues. Members of the TGF-β family of signalling molecules, of which there are more than 30 in mammals (Miyazono et al., 2001), have been found in a wide variety of multicellular animals and have been characterised as being involved in many essential processes throughout development (Kingsley, 1994; Hild et al., 2000; Massague et al., 2000; Miyazono et al., 2001; Padgett et al., 2001).

TGF-β signals are transduced across the cell membrane by two serine/threonine kinase receptors, known as type I and type II receptors (Kingsley, 1994). Both these types of receptors contain serine/threonine kinase domains in their intracellular portions and reside on the cell surface in a number of oligomeric arrangements (Miyazono et al., 2001). For example, type II homodimers, type I homodimers and type I/II heterodimers. A
summary of the phenotypes following the targeted inactivation of some of these receptors are shown in Table 1.2. The general model for TGF-β signalling involves the binding of a hetero- or homodimer to the type I and type II receptor chains, followed by a possible receptor conformational change. The type I receptors are then phosphorylated and subsequently activated by type II receptor kinases. Type I receptor kinases can then transmit the signal through the cytoplasm by phosphorylating Smad proteins (Miyazono et al., 2001). These Smad proteins then transmit the signal into the nucleus where they assemble complexes that directly regulate gene expression.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALK-1</td>
<td>Vascular abnormalities characterised by severe fusion of capillary plexes and hyperdilation of large vessels.</td>
<td>(Oh et al., 2000)</td>
</tr>
<tr>
<td>ALK-2/ActRI</td>
<td>Homozygous mutants were smaller than controls. Mutants formed an abnormally thickened primitive streak and were arrested around the late streak stage.</td>
<td>(Mishina et al., 1999)</td>
</tr>
<tr>
<td>ALK-3/BMPRIA</td>
<td>Homozygote embryos smaller than wild type siblings and lacked mesoderm formation.</td>
<td>(Mishina et al., 1995)</td>
</tr>
<tr>
<td>ALK-5/TGF-βRI</td>
<td>Die due to vascular defects in the yolk sac.</td>
<td>(Larsson et al., 2001)</td>
</tr>
<tr>
<td>Type II:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGF-βRII</td>
<td>Die at midgestation due to defects in yolk sac vasculogenesis and haematopoiesis.</td>
<td>(Oshima et al., 1996)</td>
</tr>
<tr>
<td>ActRII</td>
<td>Mutant mice developed into adults with a reduced reproductive performance and a reduction of their follicle-stimulating hormone.</td>
<td>(Matzuk et al., 1995)</td>
</tr>
<tr>
<td>BMPRII</td>
<td>Failed to form an organised structure and lacked mesoderm.</td>
<td>(Beppu et al., 2000)</td>
</tr>
</tbody>
</table>

Table 1.2 Mouse phenotypes following targeted inactivation of genes encoding type I and type II TGF-β receptors.

On the basis of amino acid similarity within the mature domain, a number of subgroups exist within the TGF-β superfamily, one of these groups comprises the Gdf5, 6
and 7 genes. Homologous genes for this group have been identified in the zebrafish, as well as in other organisms (Chang et al., 1994; Rissi et al., 1995; Bruneau et al., 1997; Bruneau and Rosa, 1997; Wolfman et al., 1997; Morotome et al., 1998; Chang and Hemmati-Brivanlou, 1999) (Figure 1.9). In the zebrafish, four genes have been isolated that belong to the Gdf5, 6, 7 subgroup. They have been named contact, radar, dynamo and gdf7 (Rissi et al., 1995; Bruneau et al., 1997; Bruneau and Rosa, 1997; Davidson et al., 1999). Syntenic relationships between the human, mouse and zebrafish Gdf5, 6, 7 subgroup of genes suggest that contact is the zebrafish orthologue of mammalian GDF5/Gdf5, radar and dynamo are orthologues of mammalian GDF6/Gdf6 and zebrafish gdf7 is the orthologue of mammalian GDF7/Gdf7 (Davidson et al., 1999). The Gdf6 orthologues, radar and dynamo, are believed to have been generated following a genome duplication event in teleosts, from a single ancestral gene (Postlethwait et al., 1998; Davidson et al., 1999). These zebrafish orthologues display restricted expression patterns during embryonic development (summarised in Table 1.3). Transcripts for contact are detected in the pharyngeal arches and the pectoral fin buds of the zebrafish embryo (Bruneau et al., 1997) while dynamo is expressed in the posterior neural plate and the ventral region of the neural tube (Bruneau and Rosa, 1997). Zebrafish gdf7 expression includes dorsal and ventral regions of the developing head, pronephric ducts and the DA. Of all the members of the zebrafish Gdf5, 6, 7 subgroup, radar exhibits the most dynamic expression pattern, both spatially and temporally, throughout zebrafish embryonic development.
Figure 1.9 Phylogenetic relationship between members of the Gdf5, 6, 7 subgroup of the TGF-β superfamily of signalling molecules. The genes encoding members of the mammalian and zebrafish Gdf5, 6, 7 subgroup can be grouped into three orthology groups: Gdf5, Gdf6 and Gdf7. (Hs) Homo sapiens (black), (Bt) Bos taurus (dark blue), (Mm) Mus musculus (green), (Dr) Danio rerio (red), (Dm) Drosophila melanogaster (light blue). Adapted from Davidson et al., 1999.
Table 1.3  Expression domains of the Gdf5, 6, 7 subgroup of genes during mammalian and zebrafish development.

1.9 Expression of radar during zebrafish embryonic development

During early embryogenesis, transcripts for radar are detected in a temporally and spatially dynamic pattern (Figure 1.10). Initially, a maternal radar transcript is detected in a ubiquitous pattern throughout the early cleavage stage embryo that persists until the blastula stage. At this point, radar expression is no longer detected until the end of gastrulation when zygotically derived transcripts are detected in two rows of cells flanking the neural plate (Rissi et al., 1995; Goutel et al., 2000). This zygotic expression is first detected at 90% epiboly (9.5 hpf, late gastrula stage) in the lateral lining of the neural plate region. This expression domain then expands both caudally and rostrally, lining the entire
Figure 1.10 Embryonic expression of radar detected by in situ hybridisation. (A) Dorsal posterior view of double in situ hybridisation staining for radar (purple) and krox-20 (blue) in a 12 hpf embryo. Krox-20 staining landmarks the developing rhombomeres (small arrow in A). Expression of radar is detected as bilateral stripes in the lateral plate mesoderm (LPM) and in the ectoderm above the neural keel which splits into lateral stripes caudally (arrow heads in A). (B) Dorsal anterior view of the same embryo displaying radar expression in putative neural crest cells (arrowheads) and krox-20-expressing rhombomeres (small arrow). (C) Lateral view of a 24 hpf embryo displaying the location of radar transcripts throughout the entire embryo. (D) Lateral view of the trunk of a 24 hpf stage embryo (anterior to left) displaying radar expression in the hypochord (H) and primitive gut endoderm (PGE). (E) Lateral view of the tail of a 24 hpf stage embryo (anterior to left) showing radar expression in the posterior hypochord and the ventral tail mesenchyme (VTM) (F) Transverse section through the trunk of a 24 hpf stage embryo stained for radar expression. Transcripts are detected in the developing dorsal fin (DF), roof plate of the neural tube (RP), hypochord and the PGE. Small arrow and arrowhead denotes the DA and PCV, respectively. NT = Neural tube, N = Notochord. Adapted from Davidson, 1998.
neural region of the 11 hpf stage embryo (Rissi et al., 1995). By the 6 somite stage (12 hpf) an entirely new domain of radar expression is activated in the lateral plate mesoderm (Davidson, 1998) (Figure 1.10A). Interestingly, this region of expression was not reported in the initial paper characterising radar expression (Rissi et al., 1995). In 12 somite (15 hpf) stage embryos, radar-expressing cells are detected in putative neural crest cells surrounding the forebrain and midbrain and as bilateral stripes in the trunk (Davidson, 1998). Transcripts are also detected at this stage in the posterior endoderm as well as a narrow band of ectodermal cells along the neural keel. In 18 somite (18 hpf) stage embryos, additional sites of radar expression include the posterior aspect of the developing eye and caudal neural tube. Furthermore, radar-expressing cells of the hypochord can be seen detaching from the underlying radar-expressing posterior endoderm (Davidson, 1998). By 24 hpf, strong expression is present in the posterior retina, the neuroepithelium of the developing brain, while expression in the trunk and tail include the developing dorsal fin, the roof plate of the neural tube, the hypochord, the PGE and the VTM (Figure 1.10C, D, E and F).

This dynamic pattern of expression suggests the potential involvement of the radar gene product in a number of developmental events throughout zebrafish development, including haematopoiesis, vasculogenesis/angiogenesis as well as neural, hypochord and dorsal fin development.

1.10 Radar functions in early dorsal/ventral specification and as a maintenance factor for dorsal neuroectodermal cells

Characterisation of a zebrafish deletion mutant spanning the radar locus revealed a function for the radar gene product as a maintenance factor for dorsal neuroectoderm cells (Delot et al., 1999). In brief, these mutants lacked the dorsal expression domain of a dorsal neural tube marker, msxC, and displayed an associated loss of identity and death of cells in
the dorsal neural tube, a domain where radar transcripts are found. In this deletion mutant, a 27.4 cM region encompassing the radar locus was deleted from the short arm of linkage group 16 (LG16). In reciprocal experiments, forced expression of a full-length radar transcript resulted in an expansion of msxC expression in its typical expression domains. Therefore, no ectopic msxC expression was observed. This would suggest that Radar may regulate the expression of msxC, but requires the presence of other spatially-restricted regulators. Alternatively, Radar may be involved in the maintenance of cells that normally express msxC. Given that the deletion mutant displayed cell death in this region, the later explanation appears most likely (Delot et al., 1999). Other phenotypes exhibited by this radar-spanning deletion mutant included a reduced anterior-posterior axis and a reduction of head structures. It was postulated that this reduced axis was due to apparent cell death in the dorsal part of the brain, most notably posterior to the developing eyes, and in the tail region (Delot et al., 1999). Mutant embryos failed to develop beyond 48 hpf and their phenotypes were tightly linked to the deletion. The large nature of this deletion and the inability of the researchers to effect a rescue, by injection of full-length radar into mutant embryos, made the interpretation of this cell death phenotype in the brain and tail difficult (Delot et al., 1999). Further insight into functional roles for Radar during embryonic development have focussed on a maternal radar transcript that is believed to be involved in early ventral specification through an Alk-6-related signalling mechanism. Furthermore, this ventralising activity is thought to be dependent on a functional Bmp2/4 pathway (Goutel et al., 2000).

All of the functional work on Radar to date has focussed on its early or neural expression domains. However, nothing is known regarding the function of the Radar signal from the sites that intimately flank the developing axial vasculature and haematopoietic compartments of the zebrafish embryo. Functional analysis of radar's orthologues in other developmental systems is limited. In the rat, ectopic GDF6 signalling can induce tendon and ligament formation (Wolfman et al., 1997), while in other related studies, GDF6 has been demonstrated to enhance tendon healing (Aspenberg and Forslund, 1999).
Assembly of the embryonic vasculature remains poorly understood. There is an increasing amount of data emerging that supports roles for non-endothelial tissues, that border vascular events, in the formation of patent endothelial tubes. Two such tissues include the hypochord and PGE. At the commencement of the research presented in this thesis, the embryonic expression pattern for the BMP, radar, had been characterised by \textit{in situ} hybridisation analysis (Rissi \textit{et al.}, 1995; Davidson, 1998). However, no function had been ascribed to the signalling protein encoded by these transcripts. The spatio-temporal expression pattern of \textit{radar} suggested that the encoded signalling molecule may participate in vascular and/or haematopoietic events for the following reasons: 1) transcripts were located in an unidentified population of lateral mesodermal cells, an embryonic domain where early progenitors of the vascular and haematopoietic lineages first arise; 2) during later segmentation stages of vertebrate development, \textit{radar} was strongly expressed by the hypochord and PGE; and 3) in the developing tail bud, transcripts for \textit{radar} were located in the VTM, where a number of genes implicated in vascular and blood development are expressed.

To functionally characterise a potential vascular/haematopoietic role for the Radar signal, we took advantage of the benefits that the zebrafish embryo offers as a developmental model system. In particular, the ability to regulate the expression of \textit{radar} using a transgenesis approach and a reciprocal loss-of-function approach.

Chapter Three describes the use of double whole mount \textit{in situ} hybridisation in an attempt to further characterise \textit{radar} expression relative to vascular and haematopoietic compartments. Chapter Four explores the use of transient forced expression of \textit{radar} during embryonic development as a means to provide functional insight into Radar signalling. Chapter Five describes efforts to generate a zebrafish transgenic line carrying an inducible copy of the \textit{radar} gene to facilitate the temporal control of ectopic \textit{radar} delivery. Finally,
Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Bacterial growth media

2.1.1.1 Liquid media

Luria-Bertani (LB) medium was prepared according to Sambrook et al., 1989. LB medium contained 1% (w/v) bacto-tryptone (Difco), 0.5% (w/v) bacto-yeast extract (Difco) and 85.5 mM sodium chloride, pH adjusted to 7.0.

SOB medium contained 2% (w/v) bacto-tryptone (Difco), 0.5% (w/v) bacto-yeast (Difco), 10 mM magnesium chloride 6-hydrate, 2.5 mM potassium chloride and 8.5 mM sodium chloride.

2.1.1.2 Solid media

LB agar plates were composed of LB medium containing 1.5% (w/v) bacto-agar (Difco) and supplemented with the appropriate selective antibiotic when required. Plates were stored at 4°C until required.
2.1.1.3 Antibiotics

Stock solutions of ampicillin (Roche Molecular Biochemicals) and kanamycin (Sigma) were prepared at concentrations of 25 and 10 mg/ml, respectively. Antibiotic stock solutions were filter sterilised (0.22 μm, Millipore) and stored at -20°C. Working concentrations for liquid and solid media were 100 and 30 μg/ml for ampicillin and kanamycin, respectively.

2.1.2 Bacterial strains

DH5α (Clonetech) was stored in LB with 15% (v/v) glycerol at -70°C. The DH5α strain was employed to propagate DNA plasmid vectors.

2.1.3 Buffers and solutions

All buffers and solutions used, and their contents, are listed in Table 2.1. Unless otherwise stated, buffers and solutions were sterilised either by autoclaving or filtration and prepared according to manufacturer’s instructions or as described in Sambrook et al., 1989.

<table>
<thead>
<tr>
<th>Buffer/Solution</th>
<th>Components/Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acridine orange (AO) staining solution</td>
<td>5 μg/ml (w/v) acridine orange dissolved in 1X E3 medium</td>
</tr>
<tr>
<td>Aniline blue solution B</td>
<td>1% (w/v) aniline blue, 0.5% (w/v) orange G and 8% (v/v) glacial acetic acid in sterile distilled water</td>
</tr>
<tr>
<td>Antibody Solution</td>
<td>1:5000/1:2000 dilution of anti-DIG/anti-FLU Fab fragments (Boehringer Mannheim) in MABT buffer containing 10% (v/v) lamb serum and 2% (w/v) Boehringer Mannheim</td>
</tr>
<tr>
<td><strong>Table:</strong></td>
<td><strong>Description</strong></td>
</tr>
<tr>
<td>------------------</td>
<td>----------------</td>
</tr>
<tr>
<td><strong>Azocarmine solution A</strong></td>
<td>1% (w/v) azocarmine B 0.25 and 1% (v/v) glacial acetic acid in sterile distilled water</td>
</tr>
<tr>
<td><strong>Blocking stock</strong></td>
<td>10% (w/v) Boehringer Mannheim blocking reagent in MAB buffer (autoclaved and stored at −20°C)</td>
</tr>
<tr>
<td><strong>Danieau buffer</strong></td>
<td>58 mM sodium chloride, 0.7 mM potassium chloride, 0.4 mM magnesium sulphate, 0.6 mM calcium nitrate and 0.5 mM HEPES, adjusted to pH 7.6</td>
</tr>
<tr>
<td><strong>Deoxynucleotide (dNTP) stock (10 mM)</strong></td>
<td>10 mM deoxyadenosine 5'-triphosphate (dATP), 10 mM deoxycytidine 5'-triphosphate (dCTP), 10 mM deoxyguanosine 5'-triphosphate (dGTP), 10 mM deoxythymidine 5'-triphosphate (dTTP) and 10 mM Tris-HCl (pH 7.5)</td>
</tr>
<tr>
<td><strong>DNase buffer (10X)</strong></td>
<td>500 mM potassium chloride, 25 mM magnesium chloride and 200 mM Tris-HCl (pH 8.3)</td>
</tr>
<tr>
<td><strong>DNA extraction buffer</strong></td>
<td>10 mM Tris-HCl (pH 8.2), 10 mM EDTA, 200 mM sodium chloride, 0.5% (w/v) SDS and 200 μg/ml pronase (Roche Molecular Biochemicals)</td>
</tr>
<tr>
<td><strong>DNA loading buffer (6X)</strong></td>
<td>0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF and 30% (v/v) glycerol</td>
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<tr>
<td><strong>E3 medium (50X)</strong></td>
<td>16.5 mM calcium chloride, 16.5 mM magnesium sulphate, 8.5 mM potassium chloride and 250 mM sodium chloride</td>
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<tr>
<td><strong>Fast Red staining solution</strong></td>
<td>2 ml Fast Red staining buffer supplemented with 1 Fast Red tablet (Boehringer Mannheim); undissolved particles removed by filtration (0.45 μm, Gelman Sciences)</td>
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<tr>
<td><strong>Injection dye (10X)</strong></td>
<td>1.25% (w/v) tetramethyl-rhodamine-dextran and 1 M potassium chloride</td>
</tr>
<tr>
<td><strong>MABT buffer</strong></td>
<td>MAB buffer supplemented with 0.1% (v/v) Tween-20</td>
</tr>
<tr>
<td>-----------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>Maleic acid buffer (MAB)</td>
<td>100 mM maleic acid and 150 mM sodium chloride, adjusted to pH 7.5 with 5 N sodium hydroxide</td>
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<tr>
<td>NBT/BCIP staining solution</td>
<td>NBT/BCIP staining buffer supplemented with 225 µg/ml NBT (Boehringer Mannheim) and 175 µg/ml BCIP (Boehringer Mannheim)</td>
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<td>Paraformaldehyde solution (PFA)</td>
<td>4% (w/v) paraformaldehyde in PBS buffer, warmed into solution and stored at -20°C</td>
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<td>PBST</td>
<td>PBS buffer supplemented with 0.1% (v/v) Tween-20</td>
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<tr>
<td>PCR buffer (10X)</td>
<td>100 mM Tris-HCl (pH 8.3), 500 mM potassium chloride and 15 mM magnesium chloride 6-hydrate</td>
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<tr>
<td>Phosphate buffered saline (PBS) (10X)</td>
<td>1.5 M sodium chloride, 100 mM sodium hydrogen orthophosphate and 40 mM potassium dihydrogen orthophosphate, adjusted to pH 7.2</td>
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<tr>
<td>PTU stock (100X)</td>
<td>0.3% (w/v) 1-phenyl-2-thiourea (PTU) dissolved in 1X E3 medium and stored at 4°C</td>
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<td>RNA extraction buffer</td>
<td>0.1 M β-mercaptoethanol, 4 M guanidinium isothiocyanate, 0.5% (w/v) N-lauroylsarcosine and 25 mM sodium citrate, pH 7.0</td>
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<td>SSC buffer (20X)</td>
<td>3 M sodium chloride and 300 mM sodium citrate, adjusted to pH 7.0</td>
</tr>
<tr>
<td>SSCT buffers (2X and 0.2X)</td>
<td>20X SSC buffer diluted to 2X and 0.2X and supplemented with 0.1% (v/v) Tween-20</td>
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<td>Staining buffer (for Fast Red)</td>
<td>100 mM Tris-HCl (pH 8.2)</td>
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</tbody>
</table>
| Staining buffer (for NBT/BCIP) | 50 mM magnesium chloride 6-hydrate, 100 mM sodium chloride, 100 mM Tris-HCl (pH 9.5) and 0.1% (v/v) Tween-
<table>
<thead>
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<tr>
<td>T4 DNA ligase buffer (10X)</td>
<td>10 mM dATP, 0.02% (w/v) BSA, 100 mM DTT, 100 mM magnesium chloride 6-hydrate and 500 mM Tris-HCl (pH 7.5)</td>
</tr>
<tr>
<td>TA buffer (10X)</td>
<td>5 mM DTT, 100 mM magnesium acetate, 750 mM potassium acetate, 330 mM Tris-acetate (pH 7.8) and 40 mM spermidine-HCl</td>
</tr>
<tr>
<td>TAE buffer (50X)</td>
<td>2 M Tris-acetic acid (pH 8.0) and 50 mM EDTA</td>
</tr>
<tr>
<td>TB buffer</td>
<td>15 mM calcium chloride, 55 mM manganese (II) chloride, 10 mM PIPES and 250 mM potassium chloride. Prior to the addition of manganese chloride the pH was adjusted to 6.7. The manganese chloride was then added and the buffer filter sterilised (0.45 μm, Gelman Sciences)</td>
</tr>
<tr>
<td>TE buffer</td>
<td>10 mM Tris-HCl (pH 8.0) and 1 mM EDTA</td>
</tr>
<tr>
<td>Whole mount <em>in situ</em> blocking solution</td>
<td>2% (w/v) Boehringer Mannheim blocking reagent, 10% (v/v) lamb serum and 0.1% (v/v) Tween-20 in MAB buffer</td>
</tr>
<tr>
<td>Whole mount <em>in situ</em> hybridisation buffer</td>
<td>50% (v/v) formamide, 5X SSC buffer, 0.1% (v/v) Tween-20, 0.5 mg/ml yeast tRNA (Sigma) and 50 μg/ml heparin (Sigma)</td>
</tr>
<tr>
<td>Whole mount <em>in situ</em> pre-hybridisation buffer</td>
<td>50% (v/v) formamide, 5X SSC buffer, 0.1% (v/v) Tween-20</td>
</tr>
</tbody>
</table>

Table 2.1 Buffers and solutions and their respective contents.
2.1.4 Chemicals

All chemicals used in this work were analytical grade and supplied by Roche Molecular Biochemicals, BDH Laboratory Supplies, Sigma, GibcoBRL, Riedal-de Haën, Boehringer Mannheim or Aldrich.

2.1.5 Enzymes

2.1.5.1 Restriction endonucleases

Restriction endonucleases were obtained from GiboBRL, New England Biolabs or Roche Molecular Biochemicals.

2.1.5.2 Other enzymes

Other enzymes used in this work are listed in Table 2.2.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calf intestinal alkaline phosphatase (CIP)</td>
<td>Roche Molecular Biochemicals</td>
</tr>
<tr>
<td>DNase I (RNase-free)</td>
<td>Roche Molecular Biochemicals</td>
</tr>
<tr>
<td>E. coli DNA polymerase I, large (Klenow) fragment</td>
<td>Boehringer Mannheim</td>
</tr>
<tr>
<td>Expand High Fidelity PCR enzyme mix</td>
<td>Roche Molecular Biochemicals</td>
</tr>
<tr>
<td>Pronase</td>
<td>Roche Molecular Biochemicals</td>
</tr>
<tr>
<td>Proteinase K</td>
<td>Boehringer Mannheim</td>
</tr>
<tr>
<td>RNase H</td>
<td>GibcoBRL</td>
</tr>
<tr>
<td>SP6 RNA polymerase (DIG-/FLU-labelling)</td>
<td>Roche Molecular Biochemicals</td>
</tr>
<tr>
<td>SP6 RNA polymerase (synthetic transcripts)</td>
<td>Ambion</td>
</tr>
<tr>
<td>Superscript II RNase H- reverse transcriptase</td>
<td>GibcoBRL</td>
</tr>
<tr>
<td>T3 RNA polymerase</td>
<td>GibcoBRL</td>
</tr>
<tr>
<td>MO</td>
<td>Target gene</td>
</tr>
<tr>
<td>---------------</td>
<td>-------------</td>
</tr>
<tr>
<td>vegf-A MO</td>
<td>vegf_165</td>
</tr>
<tr>
<td>Negative control MO</td>
<td>No target</td>
</tr>
<tr>
<td>oep MO</td>
<td>oep</td>
</tr>
<tr>
<td>rad-MO1</td>
<td>radar</td>
</tr>
<tr>
<td>rad-MO2</td>
<td>radar</td>
</tr>
<tr>
<td>rad-MO1-mm</td>
<td>radar</td>
</tr>
</tbody>
</table>

Table 2.3 Summary of MO sequences and the genes they target. Nucleotides in lowercase denote mispaired bases with respect to target sequence.
2.1.7 **Nucleic acid molecular size markers**

The DNA molecular size standards used to estimate the size of DNA separated by gel electrophoresis are summarised in Table 2.4.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Size range (bp)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 bp DNA ladder</td>
<td>100 to 1,500</td>
<td>GibcoBRL</td>
</tr>
<tr>
<td>1 kb DNA ladder</td>
<td>500 to 12,000</td>
<td>GibcoBRL</td>
</tr>
<tr>
<td>1 kb plus DNA ladder</td>
<td>100 to 12,000</td>
<td>GibcoBRL</td>
</tr>
</tbody>
</table>

**Table 2.4** DNA molecular size standards, their size ranges and sources.

2.1.8 **Oligonucleotides and primers**

Desalted oligonucleotides, obtained from Life Technologies, were resuspended in sterile distilled water to a concentration of 500 pmole/μl, according to the manufacturer’s instructions, and stored at -20°C. A summary of the oligonucleotides used in this work is provided in Table 2.5.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Gene</th>
<th>Position</th>
<th>Sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>EF1α3DO</td>
<td>eflα</td>
<td>361-380</td>
<td>ATACCCAGCCTCAAATCTCACCC</td>
</tr>
<tr>
<td>EF1α3UP</td>
<td>eflα</td>
<td>82-101</td>
<td>ATCTACAATGCCGCGAAT</td>
</tr>
<tr>
<td>EGFP3’BglII</td>
<td>EGFP</td>
<td>705-715</td>
<td>CCCGCTAGATCTGACGCTCG</td>
</tr>
<tr>
<td>EGFP5’KpnI</td>
<td>EGFP</td>
<td>1-18</td>
<td>ACCATGGTACCAAAGGCCGAG</td>
</tr>
<tr>
<td>EGFPDO</td>
<td>EGFP</td>
<td>123-146</td>
<td>CAGATGAAACTTCAGGTCAGGTTG</td>
</tr>
<tr>
<td>GFP:Gly-Asp</td>
<td>EGFP-radar</td>
<td>767-791</td>
<td>CTTGATGGTACCAATGCGAGAG</td>
</tr>
<tr>
<td>GFP:Pro-Ser</td>
<td>EGFP-radar</td>
<td>69-94</td>
<td>CTCGCGCTTGATACCGCAGCTGAC</td>
</tr>
<tr>
<td>Primer</td>
<td>Region</td>
<td>Sequence</td>
<td></td>
</tr>
<tr>
<td>------------------</td>
<td>--------------</td>
<td>---------------------------</td>
<td></td>
</tr>
<tr>
<td>PHSP70UP</td>
<td>hsp70 promoter</td>
<td>CCGAGAGAAGCGACTTGACAAAG</td>
<td></td>
</tr>
<tr>
<td>RADBlgIII</td>
<td>radar</td>
<td>GCGCTAAAGATCTCAGAAAAAGG</td>
<td></td>
</tr>
<tr>
<td>RADKpnI</td>
<td>radar</td>
<td>CGATGGTACCCACGCTGACTG</td>
<td></td>
</tr>
<tr>
<td>RADSmalI</td>
<td>radar</td>
<td>GACATCCCCGGGCGATTTGCG</td>
<td></td>
</tr>
<tr>
<td>SP6RAD5'</td>
<td>SP6 primer</td>
<td>CCCAAGCTTGATTTAGTGAC</td>
<td></td>
</tr>
<tr>
<td>Trans:XmnI-EcoRV</td>
<td>PBS-KS</td>
<td>GCTCATCATTGGATATCGTTCTTCCG</td>
<td></td>
</tr>
<tr>
<td>TRCHECKUP</td>
<td>radar</td>
<td>GGAGTTACCGGTGGCAGCTC</td>
<td></td>
</tr>
<tr>
<td>TRCHECKDO</td>
<td>EGFP</td>
<td>GAAAGTACCCTTGGATGCGGTTCC</td>
<td></td>
</tr>
<tr>
<td>Wnt5aDO</td>
<td>wnt-5α</td>
<td>ACTTCCGCGTGGTGGAGAATTC</td>
<td></td>
</tr>
<tr>
<td>Wnt5aUP</td>
<td>wnt-5α</td>
<td>CAGTTTCTACCTGCTTCTCGCA</td>
<td></td>
</tr>
<tr>
<td>ZKALK6L1</td>
<td>alk-6</td>
<td>AAATGGTTTCTGCTGAGCGGTCTC</td>
<td></td>
</tr>
<tr>
<td>ZFALK6U1</td>
<td>alk-6</td>
<td>CTGCTCATGGTCTCTGCGGAC</td>
<td></td>
</tr>
<tr>
<td>ZFANG1L</td>
<td>angl</td>
<td>CACCTTTTTGCTCTTGTGAGG</td>
<td></td>
</tr>
<tr>
<td>ZFANG1U</td>
<td>angl</td>
<td>TGGGGTTGGTTTCTTGCC</td>
<td></td>
</tr>
</tbody>
</table>

- Primer sequences to detect the zebrafish wnt-5α gene were obtained from Meng et al., 1999.

**Table 2.5** A summary of the oligonucleotide primers used, together with the region of the cDNA or genomic clone from which the sequence was derived. Underlined sequence for primer EGFP3’BglIII denotes pIRE-EGFP sequence (Clontech).

### 2.1.9 Plasmid vectors

Plasmid DNA vectors used for subcloning, synthetic transcript synthesis, overexpression analysis and riboprobe synthesis are summarised in Table 2.6. These DNA vectors were stored either in sterile distilled water or TE buffer at −20°C.
Forced expression, in vitro synthetic transcript synthesis (Turner and Weintraub, 1994)

PCR subcloning

Subcloning

Subcloning

Forced expression

Subcloning

Forced expression (Halloran et al., 2000)

PCR subcloning

Table 2.6 Summary of plasmid DNA vectors used, together with their respective functions.

2.1.10 Whole mount in situ hybridisation templates

Riboprobe templates, the restriction endonucleases used to linearise them and the RNA polymerases used to transcribe the antisense RNA digoxigenin (DIG)-/fluorescein (FLU)-labelled probes are summarised in Table 2.7.
<table>
<thead>
<tr>
<th>Gene/Marker</th>
<th>Restriction endonuclease</th>
<th>RNA polymerase</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>radar</td>
<td>EcoRI</td>
<td>T3</td>
<td>Crosier Laboratory</td>
</tr>
<tr>
<td>scl</td>
<td>Sall</td>
<td>T7</td>
<td>L. Zon</td>
</tr>
<tr>
<td>flk-1</td>
<td>SmaI</td>
<td>T7</td>
<td>(Liao et al., 1997)</td>
</tr>
<tr>
<td>fliI</td>
<td>EcoRI</td>
<td>T7</td>
<td>A. Sharrocks</td>
</tr>
<tr>
<td>angl</td>
<td>NolI</td>
<td>SP6</td>
<td>Synthesised in-house using primers ZFANG1U and ZKANG1L</td>
</tr>
<tr>
<td>tie2</td>
<td>EcoRI</td>
<td>T7</td>
<td>L. Zon</td>
</tr>
<tr>
<td>βEγ-globin</td>
<td>KpnI and XmaI</td>
<td>T7</td>
<td>L. Zon</td>
</tr>
<tr>
<td>alk-6</td>
<td>Apal</td>
<td>SP6</td>
<td>Synthesised in-house using primers ZFALK6U1 and ZKALK6L1</td>
</tr>
</tbody>
</table>

Table 2.7 Whole mount *in situ* hybridisation markers used, shown together with the restriction endonuclease used to linearise the template and the RNA polymerase used to transcribe the antisense riboprobe.

2.1.11 Zebrafish stocks

Wild-type zebrafish (*Danio rerio*) strains used in this work were supplied by Hollywood Fish Farm (Auckland, New Zealand) and maintained within the in-house designed Zebrafish Facility in the Faculty of Medical and Health Sciences, The University of Auckland, Auckland, New Zealand.
2.1.12 Computer analysis

Nucleotide sequences were analysed and assembled on Apple Macintosh computers using MacVector 7.0 (Oxford Molecular) and AssemblyLIGN 1.0 (Oxford Molecular) software applications.

2.2 Methods

2.2.1 Preparation of transformation-competent DH5α cells

Approximately 10 to 12 DH5α colonies, grown on LB agar plates, were used to inoculate 250 ml of SOB medium in a 2 L flask which was grown at room temperature in a rotary incubator (Innova 4230, New Brunswick Scientific) at 150 rpm until an A₆₀₀ of 0.6 was reached. The liquid culture was then chilled on ice for 10 minutes before the cells were harvested by centrifugation at 2,500 x g (4,000 rpm in a Sorvall RC26 Plus, SLA-1500 rotor) for 10 minutes at 4°C. The cell pellet was then resuspended in 80 ml of ice-cold TB buffer and chilled on ice for 10 minutes before being harvested again by centrifugation as above. The cell pellet was then resuspended in 20 ml of ice-cold TB buffer and dimethylsulphoxide (DMSO) added to a concentration of 7%. Following a final incubation on ice for 10 minutes, the cells were dispersed into 200 µl aliquots in Nunc CryoTube vials (Nunc), snap frozen in liquid nitrogen and stored at −70°C.

2.2.2 Transformation of competent DH5α cells

A 100 µl aliquot of competent DH5α cells was gently thawed on ice then transferred to an ice-cold 15 ml Falcon tube. β-mercaptoethanol was then added to give a final concentration of 25 mM and the solution gently mixed and incubated on ice for 10
minutes. Typically, 1 to 5 µl of DNA solution containing between 1 to 50 ng of DNA was added to the competent cells and incubated on ice for 30 minutes. Following this incubation the cells were heat-shocked for 30 seconds at 42°C then incubated on ice for a further 2 minutes. These transformed cells were then used to inoculate approximately 900 µl LB medium (1 ml final volume) and incubated at 37°C for 1 hour with gentle agitation on a rotary shaker (Innova 4230, New Brunswick Scientific) at 225 rpm. Cells were then plated onto LB agar plates supplemented with the appropriate selective antibiotic (100 µg/ml ampicillin and 30 µg/ml kanamycin) and incubated overnight (12 to 16 hours) at 37°C.

### 2.2.3 Glycerol stocks

Bacterial cells and recombinant strains were stored as glycerol stocks at −70°C. Overnight bacterial cultures grown with the appropriate selective antibiotic (100 µg/ml ampicillin and 30 µg/ml kanamycin) were supplemented with 15% (v/v) glycerol and stored at −70°C.

### 2.2.4 Isolation and purification of DNA

#### 2.2.4.1 Small scale preparation of plasmid DNA from bacteria

Plasmid DNA was isolated from small scale (5 ml) bacterial cultures using the QIAprep spin miniprep kit (QIAGEN) according to the manufacturer’s instructions. In brief, 5 ml of LB medium supplemented with the appropriate antibiotic (100 µg/ml ampicillin or 30 µg/ml kanamycin) was inoculated with a single recombinant bacterial colony and incubated at 37°C on a rotary shaker (Innova 4230, New Brunswick Scientific) overnight (12 to 16 hours). The bacterial cells were then harvested by centrifugation at 5,000 rpm for 5 minutes (Eppendorf 5417C), resuspended and then lysed under alkaline conditions. The QIAprep miniprep procedure is a modification of the alkaline lysis method
(Birnboim et al., 1979). Chromosomal DNA and proteins are removed by precipitation and subsequent centrifugation at 14,000 rpm for 10 minutes (Eppendorf 5417C) before the supernatant, containing the plasmid DNA, is passed through a QIAprep spin column containing a matrix to immobilise the plasmid DNA and facilitate purification washes. The plasmid DNA was then eluted from the spin column using approximately 50 μl of sterile distilled water or TE buffer and stored at -20°C. The concentration and yield of DNA was determined by spectrophotometry.

2.2.4.2 Large scale preparation of plasmid DNA from bacteria

Plasmid DNA was isolated from large scale (25 ml to 100 ml) cultures using the QIAGEN Plasmid Midi and Maxi kits (QIAGEN) according to the manufacturer’s instructions. The procedure is a modification of the alkaline lysis method (Birnboim et al., 1979). In brief, a starter culture containing a single inoculated bacterial colony in 2 to 5 ml of LB medium, supplemented with the appropriate selective antibiotic, was incubated for 8 hours at 37°C on a rotary shaker (Innova 4230, New Brunswick Scientific). This starter culture was then diluted 1/500 into 25 ml to 100 ml LB medium containing selective antibiotic (100 μg/ml ampicillin or 30 μg/ml kanamycin) and incubated overnight (12 to 16 hours) at 37°C on a rotary shaker (Innova 4230, New Brunswick Scientific). The recombinant bacterial cells were then harvested by centrifugation at 6,000 x g for 15 minutes (Sorvall RC26 Plus, SLA-1500 rotor) before being resuspended in 4 to 10 ml of resuspension solution supplemented with RNase A (100 μg/ml). The bacterial cells were then lysed by the addition of 4 to 10 ml of lysis solution, gentle agitation and incubation at room temperature for 5 minutes. Chilled neutralisation buffer was then added (4 to 10 ml) and the solution incubated in ice for 20 minutes before the chromosomal DNA and proteins were removed by centrifugation at 20,000 x g for 30 minutes at 4°C (Sorvall RC26 Plus, SLA-1500 rotor). The plasmid-containing supernatant was then removed and centrifuged again for 15 minutes to remove any residual precipitated material. This supernatant was
then applied to an equilibrated-QIAGEN-tip 100 (Midi) or 500 (Maxi) column by gravity flow to immobilise the plasmid DNA. The column was washed and the DNA eluted with 5 to 15 ml of elution buffer. The plasmid DNA was removed by precipitation using 0.7 volumes of isopropanol and centrifugation at 15,000 x g for 30 minutes at 4°C (Sorvall RC26 Plus, SLA-1500 rotor). The resulting DNA pellet was washed with 70% ethanol, air-dried and resuspended in sterile distilled water or TE buffer and stored at -20°C. The concentration and yield of DNA was determined by spectrophotometry.

2.2.4.3 Extraction of genomic DNA from whole zebrafish embryos and caudal fin clips

Extraction of genomic DNA was carried out essentially as described in (Meng et al., 1999). Embryos and whole adults were first anaesthetised by prolonged incubation in 1X E3 medium supplemented with tricaine (16.8 mg tricaine (Sigma)/100 ml E3 medium). Embryos, typically in batches of 100 embryos, were then homogenised in 1 ml 1X DNA extraction buffer by sequential passage through 18-gauge then 22-gauge needles (Terumo) connected to a 5 ml luer taper tip syringe (Terumo). Once the embryos were completely homogenised in the extraction buffer, they were thoroughly vortexed and then incubated at 55°C with gentle agitation for 4 hours. During this incubation, the samples were vigorously vortexed every hour to promote digestion.

When genomic DNA was to be isolated from caudal fin clips of adult zebrafish, a clean razor blade was used to remove the posterior-most 2/3rds of the caudal fin which was then placed into 500 µl of 1X DNA extraction buffer, vortexed, and incubated at 55°C as above. Adult fish were then placed into system water to recover before being introduced back into the system. Following incubation, the samples were centrifuged at 14,000 rpm for 10 minutes at room temperature before two rounds of chloroform extraction. This involved the addition of 1 volume of chloroform (BDH), a vigorous vortex, followed by centrifugation at 14,000 rpm for 10 minutes at room temperature (Eppendorf 5417C) and
the subsequent removal of the upper aqueous phase containing the purified genomic DNA. The DNA was then precipitated by incubation in 1.5 volumes of 100% ethanol overnight (12-16 hours) at -20°C. Once precipitated, the mixture was centrifuged at 14,000 rpm for 30 minutes at 4°C (Beckman GS-15R), washed with 70% (v/v) ethanol, air-dried and resuspended in 30 μl of 1X TE buffer (pH 8.0). The concentration of the genomic DNA was determined by spectrophotometric measurement.

2.2.4.4 Purification of nucleic acids by phenol/chloroform extraction and ethanol precipitation

Phenol/chloroform extraction was used for the removal of proteins from DNA or RNA solutions. Phenol equilibrated with Tris-HCl (pH 8.0) or water was used for DNA or RNA solutions, respectively. In brief, an equal volume of phenol was added to the nucleic acid solution and thoroughly vortexed for 1 minute before the mixture was centrifuged at 14,000 rpm for 10 minutes (Eppendorf 5417C). The upper aqueous phase was then vortexed with an equal volume of phenoform (1:1 phenol:chloroform) and centrifuged again at 14,000 rpm for 10 minutes (Eppendorf 5417C). The upper aqueous phase was then extracted again with an equal volume of chloroform. Following this extraction, the nucleic acids in the upper phase were precipitated by the addition of 0.1 volumes of 3 M sodium acetate (pH 5.2) and 2 volumes of 100% ethanol. This mixture was incubated for 20 minutes at -20°C before centrifugation for 20 minutes at 14,000 rpm and 4°C (Beckman GS-15R). The DNA pellet was then washed with 70% (v/v) ethanol, air-dried and resuspended in sterile distilled water or TE buffer and stored at -20°C. The concentration of DNA was determined by spectrophotometric measurement.
2.2.4.5 Purification of nucleic acids from PCR reactions and restriction endonuclease digests

To purify and remove unwanted proteins, salts, polymerases and oligonucleotides from nucleic acids following PCR reactions and endonuclease digest reactions, the QIAquick PCR purification kit (QIAGEN) was used according to the manufacturer’s instructions. In brief, the products from a PCR or digestion reaction were applied to a QIAquick spin column to immobilise the amplification product or digestion product(s) under centrifugation for 1 minute at 14,000 rpm (Eppendorf 5417C). The DNA was then washed and eluted in 50 µl of sterile distilled water or TE buffer under centrifugation at 14,000 rpm for 1 minute (Eppendorf 5417C) and stored at –20°C.

2.2.4.6 Purification of DNA from agarose gels

DNA was isolated and purified from agarose gels using the GENECLEAN kit (BIO 101) according to the manufacturer’s instructions. In brief, DNA was resolved on a low melt agarose gel (Sigma) containing 0.5 µg/ml ethidium bromide (Invitrogen). The specific DNA fragments of interest were then identified under fluorescence illumination and excised from the gel using a clean razor blade. These gel blocks were then placed into 1.5 ml eppendorf tubes. Approximately 2.5 to 3 volumes of 6 M sodium iodide solution was then added and the mixture incubated at 55°C for 5 minutes to promote melting of the agarose gel slice. GLASSMILK matrix was then added; approximately 5 µl of GLASSMILK per 5 µg of DNA. To facilitate binding of the DNA to the GLASSMILK silica matrix, the mixture was vortexed and incubated on ice for 5 minutes with frequent mixing. The silica matrix-bound DNA was then pelleted by centrifugation at 14,000 rpm for 5 seconds (Eppendorf 5417C). The resulting pellet was washed several times. To elute the DNA from the silica matrix, the pellet was resuspended in 5 µl of sterile distilled water or TE buffer and incubated at 55°C for 3 minutes before centrifugation at 14,000 rpm for 30
seconds. The DNA-containing water or TE buffer was removed and stored at -20°C. This elution step was repeated to elute any DNA not removed from the GLASSMILK matrix during the first elution step.

2.2.5 Analysis of DNA

2.2.5.1 Automated DNA sequencing

Automated DNA sequencing was performed at the Centre for Gene Technology (School of Biological Sciences, The University of Auckland, Auckland, New Zealand), the DNA Sequencing Facility (Faculty of Medicine and Health Sciences, The University of Auckland, Auckland, New Zealand) or the Waikato DNA Sequencing Facility (University of Waikato, Hamilton, New Zealand).

2.2.5.2 Separation of DNA by gel electrophoresis

DNA samples to be resolved on an agarose gel were combined with DNA loading buffer to give a 1X final concentration. Electrophoresis gels were made from 0.5 to 1.5% (w/v) agarose (Sigma), for diagnostic gels, or low melt agarose (Sigma), for gel excision and fragment purification, in 1X TAE buffer containing 0.5 μg/ml ethidium bromide (Invitrogen). The concentration of agarose was dependent upon the size of the DNA fragments that were to be resolved on the gel. Small gels (8.4 cm x 6 cm) for diagnostic analysis were cast using the Horizontal 58 (GibcoBRL) electrophoresis apparatus. For low melt gels (8 cm x 7 cm), the Easy-Cast (model No. B1A) electrophoresis system (Owl Scientific) was used. DNA was separated in the gels by electrophoresis in 1X TAE buffer with 5 V/cm of applied voltage (Model 250 power supply, GibcoBRL). Following electrophoresis, the separated DNA was visualised under ultraviolet (UV) illumination and documented using the Bio-Rad Gel Imager (Bio-Rad) and associated imaging software.
2.2.5.3 Quantification of nucleic acids

DNA and RNA samples were quantified by spectrophotometric measurement using a GeneQuant RNA/DNA calculator (Pharmacia). Readings at 260 nm and 280 nm were taken from 100 µl DNA or RNA samples in a quartz cuvette. The ratio between the OD$_{260}$ (optical density) and OD$_{280}$ was used to estimate the purity of the nucleic acid solutions. Typically, pure RNA and DNA solutions have a ratio of 2.0 and 1.8, respectively.

2.2.6 Manipulation of DNA

2.2.6.1 Restriction endonuclease digests

Restriction endonuclease digests were performed in approximately 10 to 200 µl volumes containing a 1X concentration of the specific digestion buffer recommended by the enzyme manufacturer and 2 to 3 units of restriction enzyme per µg DNA. Reactions were typically at 37°C for 1 to 2 hours. When multiple digestions were performed, digests were sequential with purification steps in-between each digest using the QIAquick PCR purification kit (QIAGEN). The success of endonuclease digestion was assessed by gel electrophoresis.

2.2.6.2 Ligation reactions

For ligations, approximately 30 to 50 ng of CIP-treated linear vector DNA was mixed with a 6-fold molar excess of insert DNA in 1X T4 DNA ligase buffer supplemented with 1 unit of T4 DNA ligase in a total volume of 10 µl. This mixture was incubated at 16°C overnight (12 to 16 hours). Rapid ligation was performed using the Rapid DNA Ligation Kit (Roche Molecular Biochemicals) in accordance with the manufacturer’s
instructions. Amplification products were directly subcloned into the vectors pGEM-T, using the pGEM-T Easy Vector System (Promega) according to the manufacturer’s instructions, or pCR 2.1 (Invitrogen).

2.2.6.3 Dephosphorylation of 5' phosphate groups

Linear vector DNA was dephosphorylated using calf intestinal alkaline phosphatase (CIP)(Roche Molecular Biochemicals). Typically, following digestion and linearisation, 1 to 10 µg of linear vector DNA was incubated for 15 minutes at 37°C in the presence of 1 unit of CIP and 1X TA buffer. The CIP was then heat-inactivated by incubation for 10 minutes at 68°C.

2.2.6.4 Blunt-ending of 3' overhangs

_E. coli_ DNA polymerase I, large (Klenow) fragment was used to blunt-end linear vector DNA resulting from endonuclease digestions that generated 3' overhangs. Approximately 1 to 10 µg of linear DNA was incubated for 15 minutes at room temperature in the presence of 33 µM dNTP and 1 unit/µg DNA Klenow in 1X TA buffer. The blunt-ended DNA was then purified using the QIAquick PCR purification kit (QIAGEN).

2.2.7 Site-directed mutagenesis

Site-directed mutagenesis was performed using the Transformer Site-Directed Mutagenesis Kit (Clontech) according to manufacturer’s instructions. In brief, plasmid DNA was denatured and the mutagenic and selection primers annealed following which the mutant DNA was synthesised and gaps sealed using T4 DNA polymerase and T4 DNA
ligase, respectively. The parental plasmid was then digested with the selection restriction endonuclease and the hybrid mutated/parental plasmid transformed to generate mutated and parental plasmids. Following a second round of digestion screening to linearise the parental plasmid, the mutated plasmid was preferentially transformed. Individual transformants were then screened to confirm the presence of the desired mutations by sequence analysis.

2.2.8 Polymerase chain reaction

2.2.8.1 Amplification of DNA

PCR amplifications were typically performed in 50 or 100 µl volumes using a Robocycler Gradient 96 (Stratagene) thermocycler equipped with a hot top. Diagnostic amplification reactions were performed in 50 µl volumes of 1X PCR buffer supplemented with 2% (v/v) DMSO, 200 µM of each dNTP, 1 µM of each primer, 2.5 units of Taq DNA polymerase and the DNA template (typically, 50 ng for plasmid DNA, 250 ng for genomic DNA and 3 to 5 µl of first strand cDNA). Preparative amplification reactions were performed using the Expand High Fidelity PCR System (Roche Molecular Biochemicals) according to the manufacturer’s instructions. In brief, reactions were performed in 100 µl volumes of 1X Expand High Fidelity PCR buffer, supplemented with 15 mM magnesium chloride, 2.6 units of Expand High Fidelity PCR enzyme mix, 200 µM of each dNTP, 300 nM of each oligonucleotide primer and approximately 0.75 µg of template DNA.

2.2.8.2 Reverse-transcription polymerase chain reaction

To synthesise first strand cDNA, 2.0 µg of purified total RNA was mixed with 500 ng of oligo (dT)₁₂₋₁₈ primers (Invitrogen) and diethyl pyrocarbonate (DEPC)-treated sterile distilled water in a total volume of 12 µl and incubated at 70°C for 10 minutes. This was
followed by a quick chill on ice for 1 minute, then the addition of 1X Superscript II reaction buffer (GibcoBRL), 10 mM DTT and 500 μM of each dNTP in a total volume of 19 μl followed by an incubation for 5 minutes at room temperature. Following this incubation, 200 units of Superscript II RNase H- reverse transcriptase was added and the mixture incubated at 42°C for 2 hours followed by an incubation at 70°C for 20 minutes to inactivate the reverse transcriptase. To degrade the unwanted RNA template, 1 μl of RNase H was added and the mixture incubated at 37°C for 30 minutes followed by inactivation at 70°C for 20 minutes. Samples were processed in parallel without the presence of reverse transcriptase to serve as a control for the detection of genomic DNA contamination (RT -ve). This first strand cDNA (3 to 5 μl) then served as a template for amplification reactions using gene-specific oligonucleotide primers.

2.2.9 Isolation and purification of RNA from zebrafish embryos

Total RNA was extracted from staged zebrafish embryos using a procedure based on the acid-phenol extraction method (Chomczynski and Sacchi, 1987). To prevent RNase contamination, water and solutions were DEPC treated and aerosol barrier pipette tips used (Boehringer Mannheim). Embryos were raised to the desired developmental stage in 1X E3 medium at 28°C and then sacrificed by prolonged incubation in 1X E3 medium supplemented with tricaine (Sigma) (16.8 mg tricaine/100 ml E3 medium). Approximately 50 to 100 embryos were then transferred into 5 to 10 ml of RNA extraction buffer and homogenised by sequential passage through 18-gauge then 22-gauge needles (Terumo) connected to a 5 ml luer taper tip syringe (Terumo). Homogenised embryo extracts could then be stored at -20°C to await further processing. Thawed extracts were then mixed with 0.1 volumes of 2M sodium acetate (pH 4.0), 1 volume of phenol (water-equilibrated) and 0.2 volumes of chloroform with thorough vortexing between additions. This solution was then incubated on ice for 15 minutes before centrifugation at 14,000 rpm for 20 minutes at 4°C (Beckman GS-15R). RNA within the upper aqueous phase was then precipitated by the
addition of an equal volume of isopropanol and incubation for 30 minutes at -20°C before being pelleted by centrifugation at 14,000 rpm for 20 minutes at 4°C (Beckman GS-15R). The RNA pellet was then washed in 70% (v/v) ethanol, air-dried and resuspended in 100 µl of 1X DNase buffer containing approximately 10 units of RNase-free DNase I (Boehringer Mannheim). Incubation at 37°C for 15 minutes resulted in the digestion of any contaminating DNA. RNA was then purified by phenol/chloroform extraction with the addition of an equal volume of phenolform (1:1 phenol:chloroform). The purified RNA was ethanol precipitated, washed with 70% ethanol, air-dried and resuspended in DEPC-treated sterile distilled water and stored at -70°C.

2.2.10 Diethyl pyrocarbonate treatment

To control and minimise RNase contamination of procedures involving RNA, such as whole mount *in situ* hybridisations, RNA isolation and synthetic capped transcript synthesis, solutions and any associated glassware were DEPC treated to inactivate RNA degrading RNases. Generally this involved the addition of 0.1% (v/v) DEPC (Sigma) to sterile distilled water or solutions which were then left overnight with gentle agitation before being autoclaved.

2.2.11 Synthesis of digoxigenin- and fluorescein-labelled RNA probes for whole mount *in situ* hybridisation analysis

For the synthesis of digoxigenin (DIG)- and fluorescein (FLU)-labelled riboprobes, reagents supplied by the DIG RNA Labelling Kit (Boehringer Mannheim) were used. To prepare linear DNA templates, approximately 5 to 10 µg of plasmid DNA was linearised using an appropriate restriction endonuclease. The digestion product was then purified by phenol/chloroform extraction and ethanol precipitation before being resuspended in approximately 15 µl of sterile distilled DEPC-treated water. The success of the digest was
determined through gel electrophoresis and the concentration of the linear template was quantified by spectrophotometric measurement. For the synthesis of DIG-labelled RNA probes, the following reaction mixture was incubated at 37°C for 2 hours, 1X RNA polymerase transcription buffer, approximately 1 to 2 μg of linear DNA template, 1X DIG labelling mix (composed of 1 mM each of ATP, CTP and GTP, 6.5 mM UTP and 3.5 mM DIG-11-UTP), 40 units of RNase inhibitor and approximately 50 units of T3, T7 or SP6 RNA polymerase in a final volume of 20 μl. For FLU-labelled RNA probe synthesis, the same reaction mixture was generated except that a 1X FLU labelling mix (composed of 1 mM each of ATP, CTP and GTP and 3.5 mM FLU-12-UTP) was used instead of the DIG-labelling mix. Following the 2 hour incubation, 10 units of DNase 1 (RNase-free) was added to the reaction mixture and further incubated at 37°C for 10 minutes to digest the DNA template. The RNA probe was then precipitated in 100% ethanol containing 3.5 mM DEPC-treated EDTA (Sharlau) and 100 mM DEPC-treated LiCl (Sigma) in a total volume of 100 μl for 30 minutes at -70°C before being spun at 14,000 rpm for 15 minutes at 4°C (Beckman GS-15R). The resulting pellet was then washed in 70% (v/v) ethanol, air-dried at room temperature and resuspended in 100 μl of DEPC-treated sterile water containing 40 units of RNase inhibitor. To confirm a successful transcription reaction, 5 μl of the RNA probe was run on a 1% (w/v) agarose gel containing 0.5 μg/ml ethidium bromide (Invitrogen). The concentration of the riboprobe was quantified by spectrophotometric measurement before being stored at -70°C.

2.2.12 Whole mount in situ hybridisation analysis

Staged embryos to be used in whole mount in situ hybridisation analysis were hand dechorionated using watch-makers forceps. Alternatively, embryos were placed in a petri dish containing 2 mg/ml Pronase in 1X E3 medium. The staged embryos were then gently agitated until approximately 80% of the embryos had lost their chorions at which point the petri dish was flooded with 1X E3 medium. The embryos were then transferred to a glass
beaker and washed 3 to 4 times with 100 ml of 1X E3 medium by decanting to remove the empty chorions. The embryos were subsequently fixed in a 4% (w/v) PFA solution overnight (12 to 16 hours) at 4°C, then rinsed twice in PBS and dehydrated in 100% methanol for at least 20 minutes at −20°C. Embryos could be stored in 100% methanol at −20°C until required for in situ hybridisation. The whole mount in situ hybridisation protocol employed was based upon that described in Westerfield, 1993. Unless otherwise stated, all washes and rinses were approximately 5 ml in volume and all solutions were DEPC-treated to minimise contamination with RNases. Embryos were rehydrated by sequential 5 minute room temperature washes in 50% (v/v) methanol/PBS, 30% (v/v) methanol/PBS and then two washes in PBS. The embryos were then fixed again in 4% (w/v) PFA solution for 20 minutes and washed twice in PBS for 5 minutes at room temperature. To permeabilise embryos, they were incubated at room temperature in PBST buffer supplemented with proteinase K. The concentration of proteinase K and the duration of treatment is summarised below (Table 2.8).

<table>
<thead>
<tr>
<th>Stage of development (hpf)</th>
<th>Concentration of proteinase K (µg/ml)</th>
<th>Treatment duration (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 to 10</td>
<td>10</td>
<td>2.5</td>
</tr>
<tr>
<td>11 to 18</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>19 to 24</td>
<td>10</td>
<td>7.5</td>
</tr>
<tr>
<td>25 to 36</td>
<td>20</td>
<td>12.5</td>
</tr>
<tr>
<td>37 to 72</td>
<td>20</td>
<td>20</td>
</tr>
</tbody>
</table>

Table 2.8 Summary of proteinase K concentrations and treatment times for different developmental stages.

Following the permeabilisation treatment, embryos were washed twice in PBST buffer for 5 minutes at room temperature then re-fixed in 4% (w/v) PFA solution for 20 minutes at room temperature. After this, embryos were rinsed twice in PBST. Embryos
were transferred into 1 ml of pre-hybridisation solution and incubated at 55 to 65°C (depending upon the particular riboprobe used) for 5 minutes before being incubated at the same temperature for 4 hours in 1 ml of hybridisation buffer for pre-hybridisation treatment. Following pre-hybridisation, embryos were transferred into a riboprobe/hybridisation buffer mixture. First, the DIG- or FLU-labelled riboprobes were denatured by heating to 99°C for 5 minutes, then quickly chilled on ice before being added to 1 ml of hybridisation buffer to give a final concentration of approximately 1 μg probe/ml hybridisation buffer. Embryos were hybridised overnight (12 to 16 hours) at 55 to 65°C then washed twice in a solution composed of 50% (v/v) formamide and 2X SSCT buffer for 30 minutes at 55 to 65°C. This was followed by a wash in 2X SSCT for 15 minutes at 55 to 65°C, two washes in 0.2X SSCT for 30 minutes at 55 to 65°C and two further washes in MABT buffer for 5 minutes at room temperature.

Embryos were transferred into 0.5 ml blocking solution composed of MABT buffer solution containing 10% (v/v) lamb serum and 2% (w/v) blocking reagent (Boehringer Mannheim) for 2 hours at room temperature with gentle agitation on an orbital shaker (Bellco Glass, Inc.). Embryos were then transferred into 1 ml of antibody solution (blocking solution supplemented with anti-DIG or anti-FLU Fab fragments (Boehringer Mannheim)) and incubated at 4°C overnight (12 to 16 hours). The antibodies were diluted 1:5000 and 1:2000 for the anti-DIG and anti-FLU Fab fragments, respectively. Embryos were then rinsed in MABT buffer containing 10% (v/v) lamb serum at room temperature before being washed in the same solution for 25 minutes at room temperature. Embryos were then transferred into MABT buffer for 25 minute and 2 hour washes at room temperature.

For staining, embryos were washed three times in staining buffer for 5 minutes at room temperature before being incubated at room temperature in 1 ml of staining buffer supplemented with 225 μg/ml 4-Nitroblue tetrazolium chloride (NBT) (Boehringer Mannheim) and 175 μg/ml 5-Bromo-4-chloro-3-indoyl-phosphate (BCIP) (Boehringer Mannheim). Embryos were protected from light until the desired level of staining was
achieved, as detected by light microscopy. Once this level was attained, embryos were washed twice in PBST for 5 minutes then fixed in 4% (w/v) PFA solution overnight (12 to 16 hours) at 4°C. Fixed embryos were then rinsed three times in PBS before being transferred into 80% (v/v) glycerol/PBS for further analysis.

2.2.13 Double whole mount in situ hybridisation analysis

For double whole mount in situ hybridisation experiments, essentially the protocol described above for single gene expression analysis was used apart from the following exceptions. Instead of a single riboprobe being hybridised, two probes were simultaneously hybridised to the embryos. One RNA probe was DIG-labelled while the other was FLU-labelled. Generally, the RNA probe designed to detect transcripts of the less abundantly expressed gene was DIG-labelled while the probe that gave a stronger signal was FLU-labelled. Following hybridisation, the probes were washed off as described above and the first antibody bound. Best results were obtained when the anti-DIG (1:5000 dilution) antibody was used first then alkaline phosphatase activity detected using BCIP/NBT substrates at the concentrations described above. When an ideal level of staining was achieved with these chromogenic substrates the reaction was stopped with three washes of PBT for 5 minutes, then fixation in 4% (w/v) PFA solution for 20 minutes at room temperature. Embryos were then washed three times in PBT for 5 minutes before incubation at room temperature in 100 mM glycine (pH 2.2) containing 0.1% (v/v) Tween-20 for 15 minutes, 4 times. This was followed by two rinses in MABT buffer and then three washes in MABT buffer for 5 minutes at room temperature. Embryos were then transferred into blocking solution and the subsequent binding of the second, anti-FLU antibody (1:2000 dilution) was performed. Anti-FLU labelled transcripts were detected using the chromogenic substrate Fast Red (Boehringer Mannheim). Embryos to be stained using Fast Red were equilibrated in staining buffer of 100 mM Tris-HCl (pH 8.2) supplemented with 0.1% (v/v) Tween-20 by three washes for 5 minutes at room temperature. The Fast Red
staining solution comprised one Fast Red tablet dissolved in 2 ml of the staining buffer, any undissolved particles were removed by filtration through a syringe filter. This staining solution was made fresh and used within 30 minutes of preparation. Once the desired level of staining was achieved, embryos were processed as described above.

2.2.14 JB-4 plastic embedding

Stained embryos selected for sectioning were removed from 80% (v/v) glycerol/PBS through a sequential wash in 30% (v/v) glycerol/PBS for 1 hour, then three washes in PBS buffer for 5 minutes. Individual embryos were then placed into wells of a 24-well tissue culture plate (Falcon) containing almost polymerised molten 1.2% (w/v) agarose. Once the agarose containing the embryos had set, embryos were cut out using a clean razor blade and fashioned into a small cube (typically of dimensions, 3-4 mm x 3-4 mm x 3-4 mm) to give the desired sectioning plane. These agarose blocks were dehydrated by sequential 10 minute washes in, 25% (v/v) methanol/PBS, 50% (v/v) methanol/PBS, 80% (v/v) methanol/PBS followed by three washes in 100% methanol at room temperature. The agarose blocks were then left at room temperature in 100% methanol overnight (12-16 hours). These dehydrated agarose blocks were transferred into 25 ml of catalysed JB-4 Plus solution A for 48 hours at 4°C (composed of 0.25 g JB-4 Plus catalyst dissolved, on ice, in 25 ml of JB-4 Plus solution A, Polysciences). Following infiltration, the agarose blocks were transferred to embedding medium composed of 15 ml freshly made infiltration solution supplemented with 1 ml of JB-4 Plus solution B. Once this embedding solution was made, it was kept on ice to retard premature polymerisation. Agarose blocks were orientated using watch-makers forceps in 6 mm x 8 mm moulds (Histomold, Leica), covered with embedding medium and left to polymerise at room temperature. A circular microtome chuck (Leica) was positioned onto the resin-filled mould prior to polymerisation. Once set, the blocks were removed and any excess unpolymerised resin removed by wiping with tissue paper before being mounted into a RM2155 microtome.
Sections, typically 5 μm, were cut and transferred to glass slides which were left to dry on a slide warmer, prior to histological procedures.

2.2.15 Cryosectioning

Embryos that were stained with the chromogenic substrate Fast Red (Boehringer Mannheim) were cryosectioned because of the alcohol solubility of the Fast Red stain which prevented plastic resin sectioning. Selected embryos were transferred from 1X PBT into a 30% (w/v) sucrose solution in 1X PBT by sequential passage through 10%, 20% and 30% (w/v) sucrose/1X PBT solutions. Once equilibrated, embryos were transferred into Tissue-Tek O.C.T. 4583 Compound (Miles) and stored for 2 to 3 days at 4°C until sectioned using a Leica CM3059 cryostat. Once cut, sections were immediately transferred to polysine-coated glass slides (Biolab Scientific) and mounted in Cytoseal 60 (Stephens Scientific).

2.2.16 Histology

JB-4 resin sections that had been dried on a slide warmer were initially stained for 2.5 hours at 45°C in Azocarmine solution A, then washed in sterile distilled water. To remove the stain from the resin, slides were dipped in 50% (v/v) aniline alcohol before being rinsed in tap water, then in sterile distilled water. Sections were then stained for 5 to 6 minutes at 45°C in Aniline blue solution B then rinsed in tap water followed by a rinse in sterile distilled water. Slides were dried on a slide warmer for 1 hour at 60°C then stained in Gills III Haemotoxylin (BDH) for 35 minutes at room temperature. The stain was then thoroughly rinsed off with tap water, then sterile distilled water before being dried on a slide warmer for 1 hour at 60°C, mounted with PolyMount (Polysciences) then dried again.
for several hours at 60°C. The tissue structures that were stained and their respective colours are summarised in Table 2.9.

<table>
<thead>
<tr>
<th>Tissue/Structure</th>
<th>Colour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclei</td>
<td>Red</td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>Red</td>
</tr>
<tr>
<td>Muscle</td>
<td>Orange (red and yellow)</td>
</tr>
<tr>
<td>Glia fibrils</td>
<td>Red</td>
</tr>
<tr>
<td>Mucin</td>
<td>Blue</td>
</tr>
<tr>
<td>Collagen and reticulum</td>
<td>Dark blue</td>
</tr>
<tr>
<td>Glomerula stroma</td>
<td>Dark blue</td>
</tr>
<tr>
<td>Chromophobes</td>
<td>Colourless or light grey</td>
</tr>
</tbody>
</table>

Table 2.9  Tissue structures stained and their respective colours following Azan/Haemotoxylin staining.

2.2.17 Synthesis of synthetic capped transcripts

Synthetic capped mRNA was generated for forced expression analysis using the mMessage mMACHINE SP6 kit (Ambion) according to the manufacturer’s instructions. In brief, 5 to 10 μg of DNA template (typically within the pCS2+ expression vector (Turner and Weintraub, 1994)) was linearised with the appropriate restriction endonuclease. The digestion product was then purified using the QIAquick PCR purification kit (QIAGEN) and resuspended in approximately 15 μl of DEPC-treated sterile distilled water. The concentration of the linearised DNA template was determined by spectrophotometric measurement. Approximately 1 μg was used for the mRNA synthesis reaction. The transcription reaction was composed of 1 μg of linear DNA template, 1X NTP/Cap mix, 1X reaction buffer, 2 μl of SP6 enzyme mix and nuclease-free water in a total volume of 20 μl.
This mixture was then incubated for 2 hours at 37°C followed by a DNase treatment, involving the addition of 2 units of RNase-free DNase I and incubation for a further 15 minutes at 37°C. The synthetic transcripts were then precipitated by the addition of 30 μl of nuclease-free water and 25 μl of lithium chloride precipitation solution (7.5 M lithium chloride, 50 mM EDTA) followed by incubation for 30 minutes at −20°C. The RNA was then pelleted by centrifugation at 14,000 rpm for 15 minutes at 4°C (Beckman GS-15R), washed with 70% (v/v) ethanol, air-dried, resuspended in an appropriate volume of DEPC-treated sterile distilled water and stored at −70°C. The concentration and yield of mRNA was determined by spectrophotometric measurement.

2.2.18 Microinjection

2.2.18.1 Preparation of DNA, RNA and antisense morpholino oligonucleotides for microinjection

DNA was either injected as circular plasmid (for transient forced expression analysis) or as a linear injection construct by liberating the expression cassette from vector backbone with appropriate restriction endonuclease digestion (for transgenesis). Digested plasmid DNA was run on a 1% (w/v) low melting point agarose (Sigma) gel containing 0.5 μg/ml ethidium bromide (Invitrogen) and the expression cassette excised and purified from the agarose gel using the GENE CLEAN kit (Bio 101) in accordance with the manufacturer’s instructions. Plasmid DNA to be injected into zebrafish embryos was purified by phenol/chloroform extraction and ethanol precipitation before being quantified by spectrophotometric measurement. DNA solutions were typically diluted with sterile distilled water to concentrations in the range 50 to 100 ng/μl to facilitate injection doses in the range 50 to 100 pg with a 1 nl injection volume. The integrity of the plasmid or linear DNA to be injected was determined through gel electrophoresis, typically on a 1% (w/v) agarose (Sigma) gel. DNA was injected with 0.125% (w/v) tetramethyl-rhodamine-dextran
(Sigma) supplemented with 100 mM KCl (Sigma). This dye served as an internal control enabling the amount of injected DNA to be monitored in real-time, promoting injection consistency from one embryo to the next. DNA solutions were spun at 14,000 rpm for 5 minutes (Eppendorf 5417C) to pellet undissolved particulate material immediately before injection.

Synthetic capped transcripts to be injected were diluted in DEPC-treated sterile distilled water to concentrations in the range 1 to 50 ng/μl to facilitate injection doses in the range 1 to 50 pg with a 1 nl injection volume. Quantity and quality of the synthetic mRNA was also determined by spectrophotometric measurement and gel electrophoresis analysis, typically on a 1% (w/v) agarose (Sigma) gel. Immediately prior to injection, RNA solutions were spun at 14,000 rpm for 5 minutes at 4°C (Beckman GS-15R) to pellet any residual particulate material.

MOs (Gene Tools, LLC), received as sterile salt-free lyophilised solids were resuspended in sterile distilled water to a concentration of 50 mg/ml and stored as a stock solution at −20°C. For injections, this stock solution was diluted to 3 to 6 mg/ml with 1X Danieau buffer (58 mM NaCl (Sigma), 0.7 mM KCl (Sigma), 0.4 mM MgSO₄ (Sigma), 0.6 mM Ca(NO₃)₂ (Sigma) and 0.5 mM HEPES pH 7.6 (Sigma)) and typically injected at a volume of 2 to 3 nl because of the greater viscosity of MO solutions at higher concentrations.

2.2.18.2 Microinjection of early zebrafish embryos

Early zebrafish embryos (1- to 4-cell stage) were positioned in an injection tray as described in Westerfield, 1993. In brief, approximately 8 borosilicate glass capillaries (1 mm O.D., Clark Electromedical Instruments) were placed within a large petri dish which was then filled with molten 1.5% (w/v) agarose in 1X E3 medium to a thickness of 1 cm. Once set, the agarose was inverted and the capillaries removed, creating wells into which embryos could be securely immobilised and positioned for microinjection. Injection
needles were fashioned from borosilicate glass capillaries (1 mm O.D., Clark Electromedical Instruments) using a Flaming/Brown micropipette puller (Sutter Instrument Co.) and then ground to an angle of 45° using a Narishige microgrinder (model EG-40). Injection needles were back filled, using an eppendorf Microloader, with approximately 2 to 3 μl of DNA, RNA or MO solutions that had been spun at 14,000 rpm in a microcentrifuge (Eppendorf 5417C) to remove particulate material that may block the injection needle. Loaded needles were then fastened into a Narishige micromanipulator connected to an MPPI-2 pressure injector (Applied Scientific Instrumentation). To calibrate individual needles prior to injection, drops of the injection mixture were positioned over a standard haemocytometer chamber (Fortuna, Germany) to determine the volume of the sphere, and subsequently the injection dose, based on its diameter. Typically DNA and RNA solutions were injected at a volume of 1nl, and MO solutions at 2 to 3 nl. Embryos were positioned animal pole upward in the injection tray and injection doses were targeted into the individual blastomeres or at the yolk/cytoplasm interface. Injected embryos were then counted, removed from the injection tray and left to recover in 1X E3 medium at 28°C to await further analysis.

2.2.19 Microangiographic analysis

Microangiographic analysis of zebrafish embryos was performed essentially as described in Weinstein et al., (1995). Yellow-green fluoresceienated carboxylated latex beads, obtained from Molecular Probes (Cat No. F8787), were diluted 1:1 in 2% (w/v) BSA (Sigma). This mixture was then sonicated on ice using a Misonix sonicator equipped with a microprobe for 25 minutes (5 x 5 minute cycles) at maximum power. Following sonication, the solution was centrifuged at 14,000 rpm for 5 minutes (Eppendorf 5417C) to pellet any particulate material that may block the microinjection needle.

Embryos to be injected were collected, dechorionated (either manually using watchmakers forceps or via Pronase digestion) and incubated in 1X E3 medium until the desired
developmental stage was reached. Embryos were then anaesthetised prior to injection by incubation in 1X E3 medium supplemented with tricaine (16.8 mg tricaine (Sigma)/100 ml E3 medium). When microangiography was performed on embryos older than 24 hpf, PTU-treatment was employed to prevent pigmentation (as described in Westerfield, 1993). Holding pipettes and microinjection needles were fashioned from borosilicate glass capillaries (1 mm O.D., Clark Electromedical Instruments). Holding pipettes were generated by partially melting one end of the capillary with a bunsen burner to give an opening of approximately 0.2 mm. Injection needles were generated by first pulling the capillary using a Flaming/Brown micropipette puller (Sutter Instrument Co.) and then grinding the tip to approximately 45° using a microgrinder (Narishige, model EG-40). Microinjection needles were back loaded using an eppendorf Microloader and secured into a Narishige micromanipulator while the holding pipettes were secured into an eppendorf CellTram oil capillary holder. The CellTram was under the control of an eppendorf TransferMan micromanipulator robotic arm and injection pulses were supplied by an eppendorf FemtoJet. Anaesthetised embryos were positioned ventral side up, such that the cardinal vein/sinus venosus was exposed. The bead suspension was injected into the sinus venosus using small pulses over the course of about 1 minute. Care was taken not to inject too much dye initially as short episodes of cardiac arrest were common immediately following the first injection pulse. Once the heart began to beat normally, injection of small doses of bead solution resumed. Fluorescence of the bead solution and the success of the microangiography were monitored by switching between light and dark fields on a Leica MZFLIII fluorescent stereo microscope equipped with a GFP filter set. Injected embryos were immediately removed from the tricaine-containing E3 medium and transferred into 1X E3 medium to recover for approximately 5 minutes before being embedded in 2% (w/v) methyl cellulose (Sigma) in 1X E3 medium for fluorescence analysis and subsequent image capture.
2.2.20 Acridine orange staining

Embryos were stained in AO essentially as described in Furutani-Seiki, (1996). Embryos to be stained were raised to the desired developmental stage in 1X E3 medium at 28°C before being dechorionated either manually using watch-makers forceps, or enzymatically by gentle agitation in 1X E3 medium supplemented with 2 mg/ml Pronase. Embryos were then incubated in 5 μg/ml AO (Sigma) in 1X E3 medium for 30 minutes at room temperature. During this treatment, the degree of staining was monitored under fluorescence microscopy using a Leica MZFLIII fluorescent stereo microscope equipped with a GFP filter set. Once staining was complete, embryos were removed from the staining solution and transferred into 1X E3 medium by three 5 minute washes before being analysed for apoptotic cell death by fluorescence microscopy.

2.2.21 PTU treatment of embryos to inhibit pigmentation

To inhibit pigmentation developing in embryos older than 24 hpf, a PTU treatment was used as described in Westerfield, (1993). A 0.3% (w/v) stock solution of 1-phenyl-2-thiourea (PTU)(Sigma) in 1X E3 medium was made and stored at 4°C. Post 24 hpf stage embryos were then transferred into a 1:100 dilution of this stock solution in 1X E3 medium to inhibit pigmentation.

2.2.22 Heat-induction treatment for transgenic embryos

Hemizygous and homozygous transgenic embryos and their wild-type siblings were harvested from individual matings and stored in 1X E3 medium at 28°C in a plastic petri dish until the desired developmental stage was reached. The petri dish containing the embryos was then placed within an incubator (Spin ‘n’ Stack, Thermo Hybaid) at temperatures ranging from 38 to 40°C. The duration of the heat treatments varied from 1 to
6 hours. At the completion of the heat-induction treatment, embryos were placed back at 28°C to recover before being screened for GFP expression (using a Leica MZFLIII fluorescent stereo microscope equipped with a GFP filter set) and any associated developmental defects throughout embryogenesis.

2.2.23 Photography and imaging

Images of manipulated embryos were captured using a Leica DC200 digital camera connected to either a Leica MZFLIII fluorescent stereo microscope equipped with a GFP filter set (for microangiography, GFP expression and AO-staining), a Leica MZFLIII stereo microscope (imaging of in situ hybridisations) or a Leitz DMR compound microscope (Leica) (for mounted embryo sections).

2.2.24 Care and breeding of zebrafish stocks

Zebrafish used in this study were maintained within an in-house designed zebrafish facility (Faculty of Medical and Health Sciences, The University of Auckland, Auckland, New Zealand). Embryos from pair-wise breedings were harvested 15 to 30 minutes following spawnings and incubated at 28°C in 1X E3 medium for the first 5 days of development. Fry were then transferred to the MaxHatch™ nursery where they were fed paramecia until large enough to be housed within the main zebrafish system (MaxGrow™). Both systems were designed around a recirculating water supply that was kept at a constant 28°C and pH 7.2 to 7.5. Particulate material was removed using particle filtration and contaminating fauna through UV sterilisation. The MaxHatch™ nursery used 5 and 0.2 µm polypropylene depth filters (Contamination Control) while the MaxGrow™ Modular Holding System used 50 and 20 µm pleated paper cartridge filters (Contamination Control). Adult zebrafish were fed ZM200 and ZM300 feeds (ZM Ltd.) and brine shrimp (Brine Shrimp Direct) twice daily and kept on a 14/10 hour light/dark cycle.
Chapter 3

The intimate relationship between *radar* expression and the ICM compartment

I. Introduction

3.1 Spatial relationship between *radar* expression and the zebrafish ICM compartment

The ICM is derived from a population of lateral plate mesoderm cells that express a number of genes implicated in haematopoietic and vascular development (Long *et al.*, 1997; Gering *et al.*, 1998; Thompson *et al.*, 1998; Quinkertz *et al.*, 1999; Brown *et al.*, 2000; Kalev-Zylinska *et al.*, 2002). These cells migrate medially beneath the somites to form the ICM at the embryonic midline where they contribute to both the haematopoietic and vascular lineages. Throughout their migration and the genesis of the ICM, these haemangioblastic precursor cells, that are destined to form the ICM, are tightly bordered by the hypochord and the posterior PGE, tissues that strongly express *radar*. To characterise the spatial relationship between *radar* expression during embryogenesis and the developing ICM region, double whole mount *in situ* hybridisation analysis was conducted, using *radar*- and *scl*-specific RNA probes, on zebrafish embryos of different developmental stages that spanned the genesis of the ICM. The *scl* gene is expressed by haemangioblastic cells within the lateral plate mesoderm throughout their migration to form the ICM, where they differentiate and become committed to the blood lineage (Gering *et al.*, 1998).
II. Results

3.2 Expression of radar in the posterior mesoderm is intimately associated with that of scl

Transcripts for radar are detected transiently by in situ hybridisation as a pair of bilateral stripes in the lateral mesoderm from 11.5 hpf (5 somite) until the 15 hpf (12 somite) stage (Figure 3.1A). To characterise this expression domain, in particular its relationship to scl expression in the lateral plate mesoderm, double whole mount in situ hybridisation analysis was conducted on 12.5 hpf (7 somite) stage embryos using DIG- and FLU-labelled RNA probes specific for radar and scl transcripts, respectively. Embryos were first stained for scl using Fast Red as a substrate, then for radar using BCIP/NBT. Transcripts for both radar and scl appeared to overlap at the posterior-most extremities of their mesodermal expression domains (Figure 3.1B). Their expression patterns then became distinct and separate more rostrally. Where radar expression was clearly distinct from that of scl, transcripts for radar were located more laterally but still bordered the entire posterior scl expression domain (Figure 3.1B and C). Figure 3.1D displays a diagrammatic representation of radar expression in the posterior lateral mesoderm relative to that of scl.

3.3 Expression of radar in the trunk and tail borders the developing ICM compartment and co-localises with scl in the posterior ICM region

Transcripts for radar can be detected by whole mount in situ hybridisation in the hypochord and the closely associated PGE from the time that the hypochord delaminates
Figure 3.1 Expression of radar and scl in the lateral mesoderm during early segmentation. (A) Dorsal posterior view of a whole mount in situ hybridisation staining for radar expression in a 7 somite stage embryo (anterior to top). (Insert) Posterior optical cross-section (posterior down) displaying mesodermal radar expression relative to more dorsal ectodermal expression. Black arrows denote bilateral stripes of lateral mesoderm radar expression and black arrowheads denote radar expression in the ectoderm above the neural keel. (B) Dorsal posterior view of a double whole mount in situ hybridisation staining for radar (purple) and scl (red) in a 7 somite stage embryo. Red and purple arrowheads mark scl and radar expression, respectively. (C) Higher power view of embryo in panel B. (D) Diagrammatic representation (dorsal posterior view) of radar and scl expression in the posterior compartment of a 7 somite stage embryo. Scale bars in A, B and C represent 150 μm.
from the underlying endoderm at around the 16 hpf (14 somite) stage (Davidson, 1998). In 18 hpf stage embryos, transcripts for radar can be detected in the hypochord and PGE while transcripts for scl are sandwiched between these domains (Figure 3.2A). In the posterior ICM compartment of the developing tail, transcripts for radar and scl appear to co-localise, if only in a subset of these cells (Figure 3.2A). Once the ICM has formed, an overlapping of radar and scl expression is clearly detectable in the posterior ICM compartment/VTM (Figure 3.2B). Transverse sections through this region display this co-localisation, which appears to be most significant at the posterior extremities of these expression domains (Figure 3.2C and D), and more separate rostrally (Figure 3.2E). In the developing trunk, a clear separation is evident between scl, expressed in the ICM, and radar, expressed in the overlying hypochord and the underlying posterior gut endoderm (Figure 3.2E). This expression of radar in the hypochord and PGE persists at least until the 36 hpf stage of development, albeit somewhat reduced (Figure 3.2F).

III. Summary

The relationship between radar expression and the ICM compartment was analysed by double whole mount in situ hybridisation for radar and scl transcripts. Commencing from the 11.5 hpf (5 somite) stage until the 15 hpf (12 somite) stage, radar transcripts were detected in a bilateral population of cells within the lateral mesoderm compartment of the early embryo. This expression was temporally coincident with an adjacent domain of scl expression in cells fated to contribute to both the haematopoietic and vascular lineages. These paired bilateral expression domains, although not completely overlapping, closely bordered each other within the mesoderm, with radar expression placed more lateral than that of scl. In later staged embryos (18 hpf to 24 hpf), radar and scl expression was co-localised within the VTM of the posterior ICM. This co-expression became separate more rostrally, where scl transcripts within the ICM compartment were bordered by radar
Figure 3.2 Co-localised expression of radar and scl in the posterior ICM compartment. Double whole mount in situ hybridisation staining of 18 and 24 hpf stage embryos to detect radar (purple) and scl (red) transcripts in the posterior ICM region. (A) Lateral view of double stained 18 hpf stage embryo (anterior to left) displaying overlapping domains of radar and scl expression in the posterior ICM (black arrow). (B) Similar view of a 24 hpf stage embryo with coincident expression in the posterior ICM region (black arrow). (C, D and E) Transverse sections taken through the posterior trunk/tail region of a 24 hpf stage embryo double stained for radar (purple) and scl (red), black arrows indicate overlapping expression domains while purple and red arrows denote exclusive radar and scl expression, respectively. The sectioning planes are represented on panel B. (F) Lateral view (anterior to left) of a whole mount in situ hybridisation for radar expression in a 36 hpf stage embryo. Arrow and arrowhead denotes radar expression in the hypochord and PGE, respectively. N = Notochord, NT = Neural tube. Scale bars in A/B/F and C/D/E represent 100 μm and 50 μm, respectively.
expression in the immediately dorsal hypochord and the immediately ventral PGE. Transcripts for *radar* were still detected in the hypochord and PGE by whole mount *in situ* hybridisation at the 36 hpf stage of development.
Chapter 4

Forced radar expression during embryonic development

I. Introduction

4.1 Insights into gene function by transient forced expression

The delivery of exogenous transcripts into zebrafish embryos has become a standard method for providing functional insight into gene function. The attributes that the zebrafish offers as a developmental model system are highly suited to this technique. Not only do zebrafish embryos develop ex-utero, enabling them to be injected with foreign nucleic acids at very early stages of development, they are also transparent, facilitating the real-time tracking of ectopic expression through reporter constructs.

In the zebrafish embryo there is no physical boundary present between the yolk and the individual blastomeres up to the 8-cell stage. It is not until the next round of replication, the 16-cell stage, that a membrane has formed separating the yolk from a sub-group of blastomeres (Kimmel et al., 1995). This enables the injection of foreign, in vitro-synthesised, capped transcripts or DNA expression cassettes, up until the 8-cell stage. Exogenous nucleic acid can be expected to migrate into every blastomere via cytoplasmic yolk streaming, regardless of whether it was directly injected into an individual blastomere or the yolk. Ectopic gene expression can be effected through both RNA and DNA injections into early zebrafish embryos. Each approach possesses its own advantages and disadvantages. The early delivery of exogenous RNA transcript into embryos can result in a
functional protein being synthesised along with maternal transcripts at an early stage in development. Such an approach is of benefit if the gene is involved in very early embryogenesis events, pre mid-blastula transition.

A major recognised limitation of using RNA for forced gene expression is its limited half-life. RNAs have varied, but limited, stability and are susceptible to degradation by RNases. Elements that determine the stability, localisation and translation efficiency of a transcript are predominantly located in the untranslated regions (UTRs). Indeed, different UTRs can result in substantial differences in the efficiency of protein translation. In *Xenopus* embryos, the addition of a long polyA tail has been demonstrated to increase RNA half-life (Harland and Misher, 1988). This has lead to the development of transcription vectors that incorporate UTR elements believed to confer increased stability to the transcribed RNA. An example of such a vector is the pCS2 vector (Turner and Weintraub, 1994) which contains the SV40 polyadenylation site, resulting in the synthesis of stable, polyadenylated RNAs. This vector and its derivatives have been widely used to synthesise stable transcript for delivery into both *Xenopus* and zebrafish embryos. An upstream promoter element can be engineered into these vectors and likewise injected to provide a constant supply of freshly transcribed RNA throughout embryogenesis. This approach circumvents the stability problems associated with RNA injections and unwanted side-effects that can occur when injected RNA interferes with very early developmental processes.

The transcription of foreign DNA begins with the commencement of zygotic transcription at around the mid-blastula transition (Kane and Kimmel, 1993). Injection of plasmid vectors can provide an additional element of control through the use of inducible and tissue-specific promoter/enhancer elements. Depending on where and when ectopic expression is desired, an enhancer element can be selected to guide misexpression. A number of promoter elements are beginning to be characterised from the zebrafish, providing an increasing ability to guide tissue-specific misexpression, examples include the *gata-1* (Long *et al.*, 1997) and −2 (Meng *et al.*, 1997), *tie-1* (Motoike *et al.*, 2000), *flil*
(Lawson et al., 2002), shh (Muller et al., 1999) and pax2.1 (Picker et al., 2002) regulatory elements. Recently, the isolation and characterisation of the promoter for the zebrafish hsp70 gene has led to the establishment of a stable transgenic line in which the reporter EGFP is driven by this heat-inducible element. Heat-shock can then induce expression of EGFP in the transgenic fish. Such an inducible promoter will provide temporal control for overexpressing a gene of interest, allowing one to target specific events throughout zebrafish development (Halloran et al., 2000). Alternatively, expression can be driven using a ubiquitous promoter to effect non-specific overexpression. In the zebrafish, an example of a powerful promoter that can drive robust expression in most tissue types is the cytomegalovirus (CMV) promoter. Interestingly, some researchers have reported that DNA injections result in a higher degree of mosaicism when compared to RNA injections, due to the plasmid DNA diffusing less readily in the cytoplasm of injected embryos (Westerfield et al., 1992).

In summary, the delivery of exogenous RNAs or cDNAs into zebrafish embryos provides a powerful technique to rapidly identify potential downstream targets in regulatory cascades and thus provide valuable insight into gene function in vivo.

II. Results

4.2 Transient overexpression of the Radar-encoding P2RAD construct

The effect of forced radar overexpression during early zebrafish embryonic development was examined using a radar encoding fusion cDNA construct comprising the mature domain of radar fused, in-frame, with the pro-domain of human BMP2. This was used instead of a full-length Radar-encoding construct for two reasons. Firstly, an antibody was available for the N-terminus region of human BMP2, allowing for the possibility to
report the location of exogenous protein by immunohistochemistry. Secondly, the human BMP2 pro-domain has been reported to facilitate the secretion and processing of a number of TGF-β family members in vitro (Davidson, 1998). The P2RAD construct maintained the predicted human BMP2 cleavage site (Figure 4.1). Cleavage at this site would release a full-length Radar mature domain. To report the location of ectopic radar expression in real-time, the pCS2/EGFP reporter construct was coinjected along with radar encoding cDNA (Figure 4.2A). The P2RAD construct was subcloned into the EcoRI and XbaI sites of the pCS2+ polylinker region to create the pCS2/P2RAD expression construct. This cDNA vector could be injected as a circular plasmid, or a linear construct by releasing the expression cassette from the vector backbone by digestion with the restriction enzymes SalI and NotI (Figure 4.2B).

4.2.1 Forced radar expression results in ventralisation

Injection of pCS2/P2RAD encoding cDNA into 1- to 2-cell stage zebrafish embryos resulted in varying degrees of ventralisation (Figure 4.3). Injected embryos were scored for ventralisation following 24 hours development. For ease of scoring, the degree of ventralisation was classified into the following phenotypic classes: V1 embryos displayed the mildest ventralisation, possessing a reduction of anterior head structures but maintaining a notochord; V2 embryos were slightly more ventralised and displayed a reduced or absent notochord, expanded posterior somites and ICM region but maintained major head structures; V3 embryos possessed little or no head structures, no notochord and an expanded ICM region; V4 embryos were the most ventralised, lacking anterior structures but maintaining some paraxial somitic mesoderm. Injection of a full-length Radar-encoding cDNA construct (also subcloned into pCS2+) resulted in similar ventralisation (Figure 4.3B). A small population of P2RAD-injected embryos also displayed defects in gastrulation and subsequently failed to form a typical embryonic axis. Injected embryos were grouped into either 'normal', 'V1-V2', 'V3-V4' or 'gastrulation
Figure 4.1 Human BMP2-radar (P2RAD) fusion overexpression construct. DNA sequence and conceptual translation of the fusion construct encoding the pro-domain of human BMP2 (normal typeface) fused in-frame to the mature domain of zebrafish radar (bold typeface) while maintaining the predicted R-X-X-R cleavage site (underlined). The seven conserved cysteine residues characteristic of TGF-β family members are boxed in red.
Figure 4.2 Expression constructs for *radar* overexpression analysis. (A) Plasmid map of the constitutively expressed pCS2/EGFP expression vector. (B) Plasmid map of the constitutively expressed pCS2/P2RAD expression vector. (C) Plasmid map of the heat-shock-inducible pCS2/hsp70/EGFP expression vector. (D) Plasmid map of the heat-shock-inducible pCS2/hsp70/P2RAD expression vector.
Figure 4.3 Forced *radar* expression results in ventralisation. (A) Lateral view of a 24 hpf wild-type embryo. (B) Similar view of a 24 hpf ventralised embryo, following the injection of 75 pg pCS2/EGFP and pCS2/RAD (full-length *radar* clone) at the 1-2 cell stage. White arrow denotes reduction of head structures, black arrow denotes fused somites and black arrowhead denotes an expansion of the ICM region. (C) Bright field image of a 24 hpf embryo following the injection of 75 pg pCS2/EGFP at the 1-2 cell stage, panel D is the dark field image of C while panel E is an overlay of panels C and D. (F) Bright field image of a modestly ventralised embryo at the 24 hpf stage following the injection of 75 pg pCS2/EGFP and pCS2/P2RAD. Panel G is the dark field image of F while panel H is an overlay of panels F and G. Arrow in F, G and H denotes expansion of posterior ICM region with associated EGFP expression. (I) Bright field image of a severely ventralised 24 hpf embryo following the injection of 75 pg pCS2/EGFP and pCS2/P2RAD. Panel J is the dark field image of I while panel K is an overlay of panels I and J. Arrowhead in I denotes lack of anterior head structures, arrow in I, J and K denotes expansion of posterior trunk region and associated EGFP expression. Scale bars in A,B,C and I represent 200 µm.
Figure 4.3 Forced *radar* expression results in ventralisation. (A) Lateral view of a 24 hpf wild-type embryo. (B) Similar view of a 24 hpf ventralised embryo, following the injection of 75 pg pCS2/EGFP and pCS2/RAD (full-length *radar* clone) at the 1-2 cell stage. White arrow denotes reduction of head structures, black arrow denotes fused somites and black arrowhead denotes an expansion of the ICM region. (C) Bright field image of a 24 hpf embryo following the injection of 75 pg pCS2/EGFP at the 1-2 cell stage, panel D is the dark field image of C while panel E is an overlay of panels C and D. (F) Bright field image of a modestly ventralised embryo at the 24 hpf stage following the injection of 75 pg pCS2/EGFP and pCS2/P2RAD. Panel G is the dark field image of F while panel H is an overlay of panels F and G. Arrow in F, G and H denotes expansion of posterior ICM region with associated EGFP expression. (I) Bright field image of a severely ventralised 24 hpf embryo following the injection of 75 pg pCS2/EGFP and pCS2/P2RAD. Panel J is the dark field image of I while panel K is an overlay of panels I and J. Arrowhead in I denotes lack of anterior head structures, arrow in I, J and K denotes expansion of posterior trunk region and associated EGFP expression. Scale bars in A,B,C and I represent 200 \( \mu \text{m} \).
24 hpt

pCS2/EGFP+pCS2/RAD

OVERLAY

pCS2/EGFP+pCSZP2RAD

pCS2/EcFP+pCS2/P2RAD

BF

24 hpf

DF

OVERLAY

24 hpf

BF

24 hpf

OVERLAY
defects' classes based on microscopic observation at 24 hpf. In experiments where the pCS2/EGFP reporter construct was coinjected, embryos were also observed under fluorescence to detect EGFP expression levels.

Injection of a 75 pg dose of pCS2/P2RAD along with a similar dose of pCS2/EGFP resulted in the majority of embryos adopting the most severe, 'V3-V4', levels of ventralisation (50 ± 14%; 5 experiments; n = 235) (Figure 4.4A). A significantly smaller fraction (11 ± 6%; 5 experiments; n = 235) possessed the milder, 'V1-V2', ventralised phenotype while 14 ± 6% (5 experiments; n = 235) appeared phenotypically normal. The remainder (25 ± 13%; 5 experiments; n = 235) displayed gastrulation defects that retarded any further growth due to the lack of any recognisable body axis. Furthermore, only those embryos that expressed EGFP developed a ventralised phenotype, all EGFP negative coinjected embryos developed normally. A tight correlation existed between the degree of this ventralisation and the intensity of EGFP expression, validating the reporting strategy. In general, only those embryos that possessed the most robust EGFP expression displayed the highest levels of ventralisation (Figure 4.3C to K).

To determine if this Radar-induced ventralisation displayed a dose response, a range of concentrations of pCS2/P2RAD were injected (5 pg, 25 pg and 75 pg doses). In this set of experiments, only those embryos that developed an embryonic axis were scored. A clear dose response was observed, with the higher injection doses resulting in more embryos displaying the severe classes of ventralisation and fewer phenotypically normal embryos when compared to lower doses (Table 4.1). Almost all control embryos injected with pCS2/EGFP alone (75-100 pg doses) developed normally and displayed no abnormal developmental defects (Table 4.1) (Figure 4.3C to E).
(A) Coinjection of the expression vectors pCS2/P2RAD with pCS2/EGFP (75 pg each) into 1-2 cell stage embryos resulted in a gradation of ventralised phenotypes (scored at 24 hpf). Only those embryos expressing the EGFP reporter construct were characterised ($n = 235$; 5 separate experiments), all EGFP negative embryos were normal. For ease of scoring, the phenotypes were categorised into four groups based on microscopic observation; Normal, embryos that appeared phenotypically normal; V1-V2, moderately ventralised; V3-V4, moderately to severely ventralised; Gastrulation defects, embryos that failed to complete normal gastrulation movements. V1-V4 classification adapted from Kishimoto et al., 1997. 

(B) Typical examples of classification groups.
Normal

V1
Reduction of anterior head structures, notochord present.

V2
Reduced or absent notochord with expanded posterior somites and ICM region, head present.

V3
Little or no head, no notochord and expanded ICM region.

V4
Lacks all anterior structures but maintains somites.
Two experiments.
Five experiments.
Four experiments.

The accompanying error represents the average difference between the average percentage and the individual experimental percentages.

Table 4.1 Phenotype frequencies in zebrafish embryos following forced radar expression.

### 4.2.2 Analysis of vascular and blood development in Radar-ventralised embryos

To analyse the effect of ectopic radar expression on embryonic vascular development and haematopoiesis, whole mount in situ hybridisation analysis was conducted on pCS2/P2RAD-injected embryos (75 pg dose). To mark cells fated to the endothelial and blood lineages, flk-1- and scl-specific RNA probes were used, respectively. Injected embryos were analysed following 12 and 24 hours of development.
Expression of *scl* in 12 hpf injected embryos displayed a range of disruptions to normal expression. These included a shortening of the posterior bilateral stripes of *scl* in the posterior lateral plate mesoderm (Figure 4.5C), apparent fusion of the anterior bilateral expression domains (Figure 4.5D) and a completely disrupted localisation of *scl* transcript in what was believed to be the posterior of the embryo (Figure 4.5E). These aberrant *scl* expression patterns seem to be associated with the abnormal gastrulation movements observed following pCS2/P2RAD injections.

In older injected embryos (24 hpf) that developed an embryonic axis, the only changes to *scl* or *flk-l* expression, that were detected by *in situ* hybridisation, were changes to domains that typically express these genes, or delays in migration of their expression domains. No ectopic expression was detected. The majority of Radar-ventralised embryos possessed an expanded posterior expression compartment for both *scl* (Figure 4.5H) and *flk-l* (Figure 4.5J). Transverse sections through the caudal trunk of pCS2/P2RAD-injected embryos revealed an inhibition or delay in the normal midline convergence of the lateral *scl* expression domains, a feature associated with the absence of a notochord (compare Figure 4.5F with G). Transverse sections through this region also revealed that the expansion of *flk-l*-expressing endothelial precursors in this caudal compartment disrupted the assembly of the caudal vasculature (compare Figure 4.5I with J).

### 4.3 An inducible system to provide temporal control of ubiquitous *radar* overexpression

To provide a more controlled strategy for transient *radar* misexpression, the promoter of the zebrafish heat-inducible *hsp70* gene (Halloran *et al.*, 2000) was employed to drive inducible expression of the P2RAD construct. The zebrafish *hsp70* promoter/enhancer element has been demonstrated to provide a reliable and robust means to temporally control the expression of EGFP in a transgenic line harbouring an *hsp70*-driven EGFP reporter transgene (Halloran *et al.*, 2000). Heating these transgenic embryos
Figure 4.5 Blood and vascular development in P2RAD-injected embryos. Whole mount in situ hybridisation analysis to detect scl transcripts in P2RAD-injected embryos (75 pg) at 12 hpf (C, D and E) and 24 hpf (G and H). (A) Dorsal posterior view of a wild-type 12 hpf embryo displaying scl-expressing bilateral stripes in the lateral plate mesoderm. (B) Dorsal anterior view of a similar staged wild-type embryo displaying anterior scl-expressing bilateral stripes. (C) Dorsal posterior view of a P2RAD-injected embryo at 12 hpf displaying a slightly reduced posterior scl expression domain. (D) A P2RAD-injected embryo displaying a disrupted anterior scl expression pattern. (E) Dorsal posterior view of a P2RAD-injected embryo showing disrupted organisation of scl transcripts. (F) Lateral view (posterior to left) of scl expression in a 24 hpf wild-type embryo, and associated sections (α and β). (G) Similar view of scl expression in a 24 hpf P2RAD-injected embryo, displaying a lack of convergence of scl-expressing bilateral stripes and the absence of a notochord. (H) Similar view of a 24 hpf P2RAD-injected embryo displaying an expansion of scl expression in the posterior ICM. Red arrows in sections highlight the location of scl transcripts. (I) Expression of flk-1 in a 24 hpf wild-type embryo and its associated transverse section through the caudal vasculature displaying flk-1-expressing cells of the primitive caudal artery and caudal vein (red arrow and arrowhead, respectively). (J) Expression of flk-1 in a 24 hpf embryo following the injection of P2RAD cDNA, and its associated transverse section through the caudal vasculature displaying an expansion of flk-1-expressing cells (red arrows). Black arrowhead denotes notochord. Scale bars in A and F represent 250 μm.
in a 37 to 40°C water bath for 1 hour resulted in faithful EGFP expression throughout the embryonic tissues. Furthermore, an hsp70:Gal4 transgenic activator line displays high levels of Gal4 expression following 40°C heat-shocks of varying durations. Significant levels of Gal4 transcript were detected by semi-quantitative RT-PCR following heat-shocks lasting only 5 minutes (Scheer et al., 2002).

4.3.1 Optimisation of induction parameters for the zebrafish hsp70 promoter/enhancer element

To evaluate the potential of the hsp70 promoter/enhancer element to drive inducible expression within zebrafish embryos, in our own hands, the EGFP reporter gene was subcloned downstream of the hsp70 promoter/enhancer element. This construct was then injected into 1- to 2-cell stage zebrafish embryos which were subsequently screened for EGFP expression by fluorescence microscopy, before, and following heat-induction treatment. To generate the hsp70/EGFP construct, a 1.02 kb SmaI (blunt-cutter), XhoI fragment containing the complete EGFP coding region, along with the bovine growth hormone polyA signal, was released from the pIRES-EGFP expression vector (Clonetech). This was then directionally subcloned into the EcoRV (blunt-cutter) and XhoI sites of the pzHSP70/4prom (clone 20) plasmid polylinker region. The pzHSP70/4prom (clone 20) plasmid contains the zebrafish hsp70 promoter/enhancer element within a pBluescript SK-vector backbone (Halloran et al., 2000). Injection of a 50 pg dose of hsp70/EGFP into 1- to 2-cell stage embryos (n = 150; 2 experiments) resulted in no detectable levels of EGFP expression following a 1 hour, 38°C heat-shock treatment at the 10-12 hpf stage. Injected embryos were monitored under fluorescence for EGFP expression for up to 20 hours following induction treatment. To overcome this lack of expression, the hsp70 promoter/enhancer element was subcloned into the pCS2+ expression vector already containing the EGFP reporter gene (pCS2/EGFP) to create the expression vector pCS2/hsp70/EGFP (Figure 4.2C). The pCS2/hsp70/EGFP vector was constructed by
releasing a 1.5 kb *SmaI* (blunt-cutter), *HindIII* fragment containing the *hsp70* promoter/enhancer element from pzHSP70/4prom (clone 20). This was subcloned into the *SalI* (blunt-ended), *HindIII* linearised pCS2/EGFP expression vector. Digestion of pCS2/EGFP with *SalI* and *HindIII* resulted in the release of the pCS2/EGFP CMV promoter allowing for its replacement with the *hsp70* promoter/enhancer. The injection of this expression construct did result in inducible expression of EGFP.

To test the efficiency of the pCS2/*hsp70*/EGFP vector during zebrafish embryogenesis, 1- to 2-cell stage embryos were injected and induced at around the 75% epiboly stage (7.5-8.5 hpf) for 1 hour at 38°C. Injected embryos were divided into two groups, an induced group and a non-induced group. All embryos were kept at 28°C unless undergoing a heat-induction treatment. Embryos were screened for EGFP expression under fluorescence before heat-induction treatment, to detect any leakage from the *hsp70* promoter/enhancer, and at 24 hpf, following the heat treatment. Injection of a 30 pg dose resulted in no leaky expression (0 ± 0%; 2 experiments; *n* = 157) (Figure 4.6). However, following a 1 hour, 38°C heat treatment approximately 1/3 (32 ± 7%; 2 experiments; *n* = 157) (Figure 4.6) of the induced pool displayed extremely weak mosaic EGFP expression. No embryos in the non-induced group displayed leaky EGFP expression. Injection of a higher dose (50 pg) resulted in 1/10 (10 ± 7%; 2 experiments; *n* = 139) of the induced group expressing EGFP promiscuously before heat-induction treatment. Following heat-induction, 7/10 (70 ± 16%; 2 experiments; *n* = 139) (Figure 4.6) of the induced group displayed very low-level mosaic expression in a similar manner to those injected with the lower, 30 pg, dose. At this higher dose leaky expression was also observed in the non-induced group (6 ± 6%; 2 experiments; *n* = 139). Typically, EGFP expression was detected 1.5-2 hours following the end of the heat-induction treatment.
Figure 4.6 The zebrafish *hsp70* promoter can drive inducible expression during embryogenesis. Graph representing the frequency of EGFP expressing embryos following the injection of 30 pg (*n* = 157; 2 experiments) and 50 pg (*n* = 139; 2 experiments) doses of pCS2/hsp70/EGFP at the 1-2 cell stage. Embryos were screened for EGFP expression prior to, and following, heat-induction treatment (38°C for 1 hour) at the 7.5 to 8.5 hpf stage of development.
4.3.2 Ubiquitous *radar* overexpression during late gastrulation

To examine the effects of driving ubiquitous *radar* overexpression during the late gastrula stages of embryonic development an inducible *radar* expression vector was developed (pCS2/hsp70/P2RAD) (Figure 4.2D). This expression vector encoded the P2RAD fusion construct downstream of the *hsp70* promoter/enhancer element within the pCS2+ vector backbone. The pCS2/hsp70/P2RAD vector was assembled in two steps. First the creation of a pCS2/hsp70 vector in which the CMV promoter was replaced by the *hsp70* promoter/enhancer element. This was followed by the ligation of P2RAD into the poly linker region of the pCS2/hsp70 vector to create pCS2/hsp70/P2RAD. The intermediate pCS2/hsp70 vector was generated by first releasing the CMV promoter from the pCS2+ vector by digesting with *SalI* (blunt-ended) and *HindIII* restriction enzymes. Then ligating in a 1.5 kb *SalI*, *HindIII* *hsp70* promoter/enhancer containing fragment isolated from the pzHSP70/4prom (clone 20) vector. Next, a 1.4 kb P2RAD containing *ClaI*, *XbaI* fragment, released from the pCS2/P2RAD expression vector, was ligated into the corresponding restriction sites of the pCS2/hsp70 vector to create the pCS2/hsp70/P2RAD inducible expression vector. The pCS2/hsp70/P2RAD expression cassette could be linearised by digestion with *ScaI* and *NotI* restriction enzymes. This construct was coinjected into 1- to 2-cell stage zebrafish embryos with the pCS2/hsp70/EGFP inducible reporter construct. Injected embryos were again randomly divided into 2 equal groups, an induced and non-induced group. All embryos were then screened for leaky EGFP expression under fluorescence microscopy prior to heat-induction treatment, and then monitored for induced EGFP expression and any phenotypes following induction.

Embryos coinjected with between 30 and 40 pg of pCS2/hsp70/P2RAD and pCS2/hsp70/EGFP were left to recover from the injections at 28°C until around the 75 to 90% epiboly stage (8 to 9 hpf) when they were given a 1 hour heat treatment at 38°C. Prior to the heat treatment, approximately 1/3 (32 ± 1%; 2 experiments: n = 154) of the injected embryos displayed leaky EGFP expression when observed under fluorescence microscopy.
This leaky expression was very low and extremely mosaic. Following approximately 12 hours recovery at 28°C (24 hpf stage), both the induced and non-induced groups were screened for EGFP expression and the presence of ventralised phenotypes.

When comparing phenotype frequencies between the non-induced and induced groups, a significant decrease in normal embryos and an associated sharp increase in those displaying the most severe ventralisation (V3-V4) was observed (Figure 4.7). In the non-induced group, 70 ± 4% (2 experiments; n = 154) displayed a normal phenotype at 24 hpf while 18 ± 10% (2 experiments; n = 154) were severely ventralised. However, only 19 ± 2% (2 experiments; n = 154) of those embryos that received the heat-shock appeared normal at the same stage and 62 ± 9% (2 experiments; n = 154) were severely ventralised. Only a small increase in those embryos that displayed mild levels of ventralisation (V1-V2) was observed following heat-induction treatment when compared to the non-induced group (10 ± 4%, non-induced group; 18 ± 6%, induced group; 2 experiments; n = 154). This increase in ventralisation following heat-induction was also associated with an elevation in EGFP expression levels. Induced embryos displaying the most extreme levels of ventralisation generally also possessed the greatest levels of EGFP expression. All embryos that appeared normal expressed EGFP at extremely low levels or not at all, as detected by fluorescence microscopy. No ventralisation was observed in wild-type or pCS2/hsp70/EGFP-injected control embryos heated to 38°C for 1 hour.

III. Summary

Forced radar expression during early zebrafish embryogenesis resulted in varying degrees of ventralisation in a dose-dependent manner. Furthermore, coinjection of an EGFP reporter construct demonstrated, in real-time, that elevated EGFP expression was tightly correlated with increased degrees of ventralisation. In general, early delivery of a Radar-encoding cDNA construct led to the expansion of ventral blood and vascular mesoderm. This was detected by expanded domains of scl and flk-1 expression in the posterior ICM.
Figure 4.7 Radar induces ventralisation during gastrulation. Graph representing degrees of ventralisation (scored at 24 hpf) following the injection of a 30-40 pg dose of pCS2/hsp70/P2RAD at the 1-2 cell stage ($n = 154$; 2 experiments) and subsequent induction treatment (38°C for 1 hour) at the 8 to 9 hpf stage of development. Injected embryos that were not subjected to a heat treatment provided a non-induced control group.
compartment. This expansion was at the expense of more dorsal mesoderm such as the notochord, which was often absent in radar-injected embryos.

To temporally control ectopic radar expression, an inducible expression construct driven by the zebrafish heat-shock promoter/enhancer, hsp70, was employed. This inducible expression system was demonstrated to induce expression of an EGFP reporter within injected zebrafish embryos following a heat-shock treatment of between 38 to 40°C for 1 hour. When used to drive radar expression, induction of ectopic radar during the latter stages of gastrulation (75% epiboly) led to ventralisation. Unfortunately, leaky promoter activity, which resulted in ventralisation of non heat-treated injected embryos, prevented the use of this transient system to explore the function of radar following gastrulation. This was because the downstream effects of ventralisation masked and confused any interpretation of latter phenotypes. However, the potential of the hsp70 promoter/enhancer to drive inducible expression was demonstrated in these transient experiments. Furthermore, previous experiments have demonstrated that following the stable integration of the hsp70 promoter/enhancer into the zebrafish genome, the promoter displays faithful activity, with no detectable leakage (Halloran et al., 2000). This suggests that a stable zebrafish line could be developed carrying an hsp70-driven radar transgene to facilitate the temporal control of radar overexpression. This would allow one to by-pass the ventralising activity of Radar by inducing expression following gastrulation, allowing for functional dissection of latter domains of expression, in particular those that flank the trunk vasculature.
Chapter 5

Generating an inducible radar transgenic line

I. Introduction

5.1 Transgenesis in the zebrafish

The development of transgenic organisms has provided a valuable tool allowing the developmental geneticist to dissect embryonic development in a number of model systems (McMahon et al., 1985; Jaenisch, 1988; Rubin et al., 1988; Spieth et al., 1988). The use of germ-line transformation allows for the ectopic misexpression of transgenes in whole animals throughout embryonic development. The stable integration of exogenous DNA sequences into the zebrafish can be used to address a number of important biological questions in the following ways: 1) to identify cis-acting regulatory elements by generating stable transgenic lines expressing promoter/enhancer-driven reporter genes; 2) perform candidate gene rescue of mutant phenotypes; 3) to study genetic regulatory pathways; and 4) provides a means of insertional mutagenesis, generating new mutations that can be easily identified and subsequently cloned (Jowett et al., 1999). Since the first transgenic zebrafish line was generated through the microinjection of plasmid DNA into 1- to 4-cell stage embryos (Stuart et al., 1988), a number of transgenic fish have been reported by employing a number of transgenesis approaches. These techniques include electroporation (Powers et al., 1992; Muller et al., 1993), microprojectiles (Zelenin et al., 1991) and retroviral-mediated infection (Lin et al., 1994; Linney et al., 1999) (summarised in appendix 3, Table A3.1).
Another recently described method for generating transgenic zebrafish is through sperm nuclear transplantation (Jesuthasan et al., 2002). Zebrafish sperm nuclei are pre-incubated with DNA encoding the expression cassette/reporter construct, before being injected directly into the eggs. Through this technique it is feasible to generate non-mosaic transgenic zebrafish directly from microinjection. Furthermore, DNA introduced into zebrafish using this technique has been demonstrated to contribute to the germ-line, resulting in transgenic F1 progeny (Jesuthasan et al., 2002).

5.1.1 Transgenesis strategies

The injection of foreign plasmid DNA into early zebrafish embryos can result in one of the following scenarios: 1) it may be lost from the embryo; 2) it may replicate and be expressed in the injected cell and subsequently in all of that cells descendants; 3) it may integrate into the genomic DNA of the cell, generating a clone of transgenic somatic cells; or 4) it may integrate into the chromosomal DNA of a germ cell progenitor, generating a subset of transgenic germ cells that can then pass on the transgene to subsequent generations (Jowett et al., 1999). The method of choice for generating transgenic zebrafish remains microinjection (Stuart et al., 1990; Culp et al., 1991; Westerfield et al., 1992; Gibbs et al., 1994; Amsterdam et al., 1995; Amsterdam et al., 1996; Higashijima et al., 1997; Long et al., 1997; Halloran et al., 2000), although the efficiency for germ-line transmission is often quite low, and transgenic founders are generally highly mosaic in the germ-line. However, the benefits that the zebrafish offers as a model system, in particular its large clutch size and the ability to raise large numbers of injected embryos, easily overcomes these problems resulting in a reliable method to generate transgenic zebrafish.

Transgenic zebrafish have been generated through the injection of both circular plasmid (Stuart et al., 1990) and linear DNA (Long et al., 1997). Therefore, the stable integration and transmission of foreign transgenic DNA sequences from transgenic founders, seems not to be heavily dependent upon whether plasmid vector sequence is
present, or whether the DNA is circular or linear. Despite this, some researchers have reported that the excision of vector sequence, especially that 5' or upstream of the transgene, results in more efficient integration (Higashijima et al., 1997). In almost all cases however, the transgene integrates in a highly random fashion, usually at a single chromosomal location and in a tandem array of multiple copies (Jowett et al., 1999).

Although transgenesis has been demonstrated successfully through the generation of a number of zebrafish transgenic lines, complications with the application of this technology to the zebrafish do exist. The high germ-line mosaicism in the founder population following transgene microinjection is the predominant problem. It results in only a small proportion of F₁ that harbour the transgene, typically in the range of 1 to 20%. A recently characterised meganuclease-mediated transgenesis approach represents an exception to this rule. This technique demonstrated highly efficient transgene integration into injected zebrafish embryos, apparently overcoming mosaicism problems (Thermes et al., 2002). In brief, a reporter construct flanked by two I-SceI meganuclease recognition sites, when injected with the I-SceI enzyme, resulted in a transgenesis frequency of 30.5%. Furthermore, the germ-lines of most of these transgenic founders were non-mosaic and typically generated 50% transgenic F₁ offspring (Thermes et al., 2002). As mentioned above, the large numbers of injected embryos that can be injected and raised to maturity can ensure that, even with high mosaicism in the germ-line, a transgenic founder will most likely be present. However, this still requires the relatively time-consuming identification and isolation of these rare transgenic F₁. This genotyping is often through PCR analysis of embryos resulting from an intercross between mature founder fish (F₀), and then an outcross of each of these F₀ fish (of a positive pair) to identify which founder carries the transgene.

An alternative to this PCR-based genotyping is through the use of a reporter such as EGFP that can report, in real-time, the presence of the transgene in the F₁ progeny (Long et al., 1997; Motoike et al., 2000). This can be accomplished by generating a fusion construct comprising the transgene and EGFP. Not only will this facilitate the rapid detection and
isolation of transgenic F_1, it will also act as a valuable reporter, to highlight regions of transgene expression in living embryos. A further complication experienced with transgenic technology in the zebrafish is the lack of transgene expression in some transgenic lines (Gibbs et al., 1994; Long et al., 1997; Halloran et al., 2000). In a transgenic line that carried a CMV promoter/enhancer-driven luciferase transgene, a lack of expression was believed to be due to high levels of methylation of the transgene (Gibbs et al., 1994).

II. Results

5.2 Strategy for generating an inducible radar transgenic line

To generate an inducible transgenic line in which ectopic radar expression can be temporally regulated, and in which promiscuous promoter activity can be more tightly controlled, we endeavoured to create an inducible transgene containing an EGFP-tagged radar fusion construct downstream of the hsp70 promoter/enhancer element (pCS2/hsp70/EGFP-rdr). Fluorescence detection of EGFP expression could then be used as a real-time, non-invasive means of monitoring the induction and location of exogenous Radar. We have demonstrated the ability of the hsp70 promoter/enhancer element to drive inducible EGFP and radar overexpression in vivo in transient forced expression experiments. However, the degree of leakage from the hsp70 heat-inducible promoter/enhancer complicated the analysis of these results. The stable integration of hsp70-driven transgenes into the zebrafish genome has resulted in stable, tightly regulated, and faithful inducible expression of the transgene without any promiscuous, non-induced leakage (Scheer and Caminos-Ortega, 1999; Halloran et al., 2000). To reproduce this tight regulation of the hsp70 promoter/enhancer we generated a transgenic zebrafish line harbouring the pCS2/hsp70/EGFP-rdr transgene employing a traditional microinjection strategy.
5.2.1 Construction of a heat-inducible radar transgene

The pCS2/hsp70/EGFP-rdr transgene construct was designed such that EGFP was located downstream of the radar signal sequence within the pro-domain. This would ensure that the fusion construct would still be targeted for secretion and that the EGFP would not interfere with the Radar mature domain once it was cleaved from its pro-domain. Once constructed, this fusion was inserted into the pCS2+ vector containing the ubiquitous CMV promoter and injected into 1- to 2-cell stage zebrafish embryos to ensure that the EGFP was expressed, and did not interfere with Radar function. Injected embryos were screened for ventralisation and associated EGFP expression to confirm the success of this transgene design. Pending this confirmation, the EGFP-radar fusion was subcloned into the pCS2+ expression vector containing the hsp70 promoter/enhancer element and injected into zebrafish embryos. These potential transgenic founders were then raised to sexual maturity and intercrossed to identify transgenic founders that harboured the transgene in their germ-line.

To generate the EGFP-radar fusion, three sets of primers were used to amplify three PCR products using the 'Expand High Fidelity PCR System' (Boehringer Mannheim). One containing radar sequence 5’ to the EGFP insertion point with an engineered KpnI restriction site (using primers SP6RAD5’ and RADKpnI), another containing EGFP-encoding sequence with flanking engineered KpnI and BglII restriction sites to facilitate directional subcloning (using primers EGFP5’KpnI and EGFP3’BglII), and the last containing radar sequence 3’ to the EGFP integration site with an engineered BglII restriction site (using primers RABglII and RADSmal) (Figure 5.1). The cycling conditions consisted of an initial denaturation step for 3 minutes at 95°C, then 25 cycles of, 1 minute at 96°C, 30 seconds at either 65°C (for EGFP primers EGFP5’KpnI and EGFP3’BglII) or 57°C (for radar primers SP6RAD5’, RADKnpI, RABglII and RADSmal) and 1 minute at 72°C. This was followed by an elongation step for 5 minutes at 72°C. The templates for the PCR reactions were pCS2/EGFP (full-length EGFP cDNA
Figure 5.1 Subcloning strategy and assembly of the EGFP-radar fusion. Three sets of primers were used to generate three amplification products, two encoding radar sequence that flank the EGFP integration site (just downstream of the putative signal sequence in the radar pro-domain) that have the engineered restriction sites KpnI and BglII to facilitate the ligation of the EGFP-encoding PCR product with similar engineered restriction sites. The ligation product of these three fragments was integrated into the full-length radar cDNA and then into the pCS2+ expression vector containing the zebrafish hsp70 promoter/enhancer element.
Radar

EGFP

Radar

CAG TCA GCT CGG

GTC AGT CGA CGG

GA TCT CAG AAA AGG

A GCT TTT TCC

Q S A A V P K G / / E L Y R S Q K R

Ligation

HindIII KpnI

BglII SmaI

Pro-domain Mature domain XhoI

RRRRR (Predicted cleavage site)

Subcloning

hsp 70 promoter

SpeI

EGFP-radar

pCS2/hsp70/EGFP-rdr (7.8 kb)

polyA

NcoI
within pCS2+ vector), for the amplification of EGFP sequences, and pCS2/RAD (full-length radar cDNA clone within pCS2+ vector) for the amplification of radar sequences. The resulting PCR products were subcloned into the pCR2.1 TA cloning vector (Invitrogen) and sequenced. The ligation product of these three PCR fragments was then subcloned into the HindIII restriction site within the pCS2+ polylinker region and the SmaI restriction site within the radar pro-domain of either pCS2/RAD (to create pCS2/EGFP-rdr) or pCS2/hsp70/RAD (to create pCS2/hsp70/EGFP-rdr) expression vectors (Figure 5.1). The vector pCS2/RAD encodes a full-length radar cDNA clone driven by the constitutive CMV promoter within the pCS2+ vector while pCS2/hsp70/RAD, also containing a full-length radar, possesses the hsp70 promoter/enhancer element.

To accommodate the integration of EGFP-encoding cDNA into the radar pro-domain, a couple of alterations within the coding regions of radar and EGFP were necessary that translated into two amino acid substitutions and one deletion (Figure 5.2). The changes to EGFP altered regions outside of the critical minimal domain required for EGFP fluorescence, identified through deletion analysis as amino acids 7 through to 229 of the EGFP polypeptide (Li et al., 1997). Two substitutions were necessary in the EGFP coding region, a serine to proline conversion at amino acid position 28, and a lysine to arginine conversion at position 264. In addition to these changes, a leucine and a neighbouring isoleucine residue were deleted from amino acid positions 27 and 28 of the Radar polypeptide.

5.2.2 Ability of EGFP-radar fusion to elicit Radar-induced ventralisation and associated EGFP reporter expression

To test if the EGFP-radar fusion could elicit a Radar-induced ventralised phenotype coupled with detectable EGFP expression, the pCS2/EGFP-rdr expression construct was injected into 1- to 2-cell stage zebrafish embryos which were monitored under fluorescence and scored for ventralisation following 24 hours development. Of the
Figure 5.2 EGFP-radar fusion construct. DNA sequence and conceptual translation of the EGFP-radar fusion. EGFP (green typeface) is integrated just downstream of the putative radar signal sequence (underlined). The predicted cleavage site is in bold text and the conserved cysteine residues are highlighted in red.
embryos injected with a 150 pg dose of pCS2/EGFP-rdr, 88% (n = 68) expressed EGFP with varying, low levels of intensity. Furthermore, this EGFP expression was coupled with ventralisation (28%; n = 68). All phenotypically normal injected embryos (12%; n = 68) displayed no EGFP expression detectable by fluorescence microscopy, validating the reliability of a coupled EGFP reporter.

A PCR induced error was detected, following sequencing, within the EGFP-encoding region of the EGFP-radar fusion construct resulting in an aspartic acid to glycine conversion at residue 260. Although this error was outside of the critical minimal domain for EGFP fluorescence (Li et al., 1997) it was corrected via site-directed mutagenesis along with the serine to proline conversion at residue 28, that was required for the cloning strategy. This was performed using the Transformer Site-Directed Mutagenesis Kit (Clontech) with the primers Trans:XmnI-EcoRV, GFP:Pro-Ser and GFP:Gly-Asp. Following these changes, and sequencing conformation, the fusion construct was injected again to ascertain if these two conversions enhanced the efficiency of the fusion to express EGFP. Injection of the fusion with the incorporated changes made no significant difference to the expression levels of EGFP, detected under fluorescence (data not shown).

5.2.3 Microinjection-based transgenesis strategy

To generate an inducible radar transgenic line, microinjection was selected as the delivery method of choice due to the simplicity and reported success of this technique (Stuart et al., 1990; Culp et al., 1991; Westerfield et al., 1992; Gibbs et al., 1994; Amsterdam et al., 1995; Amsterdam et al., 1996; Higashijima et al., 1997; Long et al., 1997; Halloran et al., 2000). The pCS2/hsp70/EGFP-rdr transgene construct was injected into 1- to 2-cell stage zebrafish embryos that were subsequently raised to sexual maturity and intercrossed. Genomic DNA was isolated from embryos from each of these crosses and primers spanning the hsp70-EGFP domain used to detect the presence of the transgene by PCR amplification. Primers for the wnt-5a gene (Wnt5aUP and Wnt5aDO) were also used,
serving as an internal control for PCR efficiency and genomic DNA quality. Once a positive founder pair was identified, each F₀ parent was outcrossed to a wild-type strain and the resulting embryos genotyped via PCR again to identify which F₀ parent was the germ-line transgenic founder. This fish was outcrossed again and the resulting fry raised to sexual maturity. Genotyping these F₁ fish through PCR amplification of genomic DNA isolated from caudal fin clips identified hemizygous transgenic F₁ that should pass on the transgene in a Mendelian fashion to subsequent generations (Figure 5.3).

5.3 Creating a heat-inducible radar transgenic line

The pCS2/hsp70/EGFP-rdr inducible expression construct was linearised with NotI and SpeI restriction enzymes (Figure 5.1) and injected into 991 1- to 2-cell stage zebrafish embryos (5 separate experiments) at doses ranging from 50 to 100 pg. DNA was injected with 0.125% tetramethyl-rhodamine-dextran (Sigma) in 100 mM KCl (Sigma). This dye served as an internal control enabling the amount of microinjected DNA to be monitored, promoting injection consistency from embryo to embryo (Meng et al., 1999). Injected embryos were counted and left to recover at 28°C until approximately 5 dpf when they were introduced into the zebrafish nursery until they grew large enough to be contained in the main zebrafish system. Once sexually mature (5-7 months of age), founder pairs were intercrossed and genomic DNA was isolated from 30-32 hpf stage embryos to serve as a template for PCR-based detection of the transgene. The primers used for transgene detection (PHSP70UP and EGFPDO) were expected to give a PCR product of approximately 930 bp spanning from the hsp70 promoter/enhancer into the EGFP-encoding region of the transgene (Figure 5.4A). The internal control primers to detect the wnt-5a gene (Wnt5aUP and Wnt5aDO) were expected to give a product size of 387 bp. As a positive control, 30 hpf genomic DNA isolated from wild-type embryos was spiked with approximately 10 fg of the pCS2/hsp70/EGFP-rdr plasmid. The cycling conditions were, an initial denaturation step for 5 minutes at 94°C, then 35 cycles of, 1 minute at 94°C, 45
**Figure 5.3** Transgenesis strategy for generating an inducible *radar* transgenic line. Embryos are injected with the linear hsp70-driven EGFP-radar expression cassette and raised to sexual maturity. Founder pairs are interbred and genomic DNA isolated from the resulting progeny for PCR-based genotyping. Founders of a positive pair are then individually outcrossed with wild-type and the progeny genotyped again to identify the transgenic founder. This transgenic founder is again outcrossed and the $F_1$ progeny raised to sexual maturity. Genomic DNA isolated from caudal fin clips from individual $F_1$ fish is then genotyped to identify hemizygous transgenic $F_1$. $F_2$ progeny, generated from an outcross involving transgenic $F_1$, should display Mendelian inheritance of the transgene.
Infect transgene

**F₀:** Interbreed founder pairs

Interbreed founder pairs

**F₁:**

Identification of +ve founder from a +ve pair

**F₂:**

(Expect Mendelian inheritance: 50% hemizygous for transgene, 50% WT)
Figure 5.4 Primer design for genotyping and RT-PCR analysis and genotyping strategy. (A) Primers spanning the hsp70 promoter/enhancer element and EGFP-encoding sequence (PHSP70UP and EGFPDO) were employed for PCR-based genotyping of putative transgenics. Primers spanning the radar signal sequence and EGFP-encoding sequence (TRCHECKUP and TRCHECKDO) were employed to detect transgene transcript in RT-PCR analysis. (B) Strategy for genotyping putative transgenic fish. Individual founder pairs are interbred and the progeny genotyped for the presence of the transgene, a negative result from this genotyping analysis is only acknowledged when more than approximately 75 good quality embryos result from the mating. If less than 75 embryos result from the spawn and the PCR result is negative, these founders are recycled to be screened again. Furthermore, because of a male sex bias experienced with zebrafish, all transgene-negative females are recycled to provide mating partners for untested male founders.
Figure A: Diagram showing the hsp70 promoter attached to an EGFP-radar sequence.

Figure B: Flowchart illustrating the process of crossing founder pairs and generating PCR genomic DNA from 30-32 hpf embryos. The process involves recycling F₀ females, PCR genomic DNA, and determining the presence (⁺ve) or absence (-ve) of specific markers. The flowchart includes decision points for selecting individuals with desired genotypes or discarding them.
seconds at 66°C and 1.5 minutes at 72°C. To address the potential of encountering severe mosaicism within the founder population, a genotyping strategy was designed to ensure that any fish that produced low numbers of embryos, that were negative for the transgene, were re-crossed until sufficient numbers of embryos were obtained to be confident in the negative result (Figure 5.4B). We found that the production of low numbers of poor quality embryos is a problem often encountered with initial spawnings from zebrafish. The transmission of a transgene to the $F_1$ generation has been reported as being within the range of 2 to 90% (Meng et al., 1999), taking this into account, the lower limit for the number of embryos required from an intercross between $F_0$ fish, to be confident in a negative result, was 75. Therefore, any spawnings that gave less than 75 good quality embryos, that were negative for the transgene, were placed into a separate tank to recover before being crossed again. Another feature that was required to be built into the genotyping strategy was the recycling of founder females that were negative for the transgene because of a strong sex bias towards male zebrafish (Figure 5.4B). This meant that transgene-negative female founder fish were crossed again with unscreened founder males until the entire founder population had been genotyped.

In total, 349 crosses were performed with founder fish, of which 212 gave rise to embryos (61%). These crosses generated 17,600 embryos, an average of around 85 good quality embryos per successful mating. In total, approximately 330 of the original 991 injected embryos survived to sexual maturity and constituted the $F_0$ founder population. From the 212 crosses that gave rise to embryos, 3 resulted in embryos harbouring the transgene. Outcrossing each of the founders, of each positive pair, to wild-type and genotyping the embryos from these spawns confirmed the presence of the transgene in the germ-line of three founder males, 56, 85 and 318. Founder male 56 died before the transgene could be secured in the $F_1$ generation. Males 85 and 318 were then outcrossed to wild-type females and the resulting embryos grown to sexual maturity. In order to determine the degree of mosaicism with which founder males 85 and 318 passed on their transgenes and to identify $F_1$ offspring hemizygous for the transgene, genomic DNA was
isolated from caudal fin clips and PCR analysis conducted to determine the presence of the transgene. Cycling conditions were the same as mentioned previously. To monitor the quality of the extracted genomic DNA and the success of the PCR reaction, the internal control primers, Wnt5aUP and Wnt5aDO, were once again used to amplify the wnt-5a gene. From 155 individual 85F₁ fish tested, 2 hemizygous transgenics were identified, males 85F₁,No.62 (Figure 5.5A) and 85F₁,No.85 (Figure 5.5B). A similarly high degree of mosaicism was experienced when genotyping the offspring from founder male 318, from 133 individual 318F₁ fish genotyped, only 1 was identified as possessing the transgene, male 318F₁,No.119 (Figure 5.5C).

To determine if transgenic males 85F₁,No.62 and 85F₁,No.85 were hemizygous for the transgene and subsequently could pass the transgene on to their progeny with the expected 50% Mendelian ratio (when outcrossed to a wild-type female). Both males were outcrossed and genomic DNA isolated from 24 individual 30-32 hpf stage F₂ embryos to be genotyped by PCR. Males 85F₁,No.62 and 85F₁,No.85 passed the transgene on to 58% (14/24) and 38% (9/24) of their progeny from an outcross to a wild-type female, respectively. The remainder of the F₂ embryos from these two sets of outcrosses were raised to sexual maturity and genotyped to identify sexually mature hemizygous transgenic F₂ fish. It was important to identify hemizygous transgenic F₂ to facilitate generating homozygotes. From the sexually mature F₂ fish generated from an outcross with male 85F₁,No.62, 5 were identified as possessing the transgene from 9 that were genotyped (56%), males 85F₁,No.62F₂,No.1, 4, 7 and 9 and female 85F₁,No.62F₂,No.8 (Figure 5.5D). Similarly, from an outcross with male 85F₁,No.85, 5 were identified as possessing the transgene from 8 that were genotyped (63%), males 85F₁,No.85F₂,No.1, 2, 3 and 6 and female 85F₁,No.85F₂,No.8 (Figure 5.5E).
Figure 5.5 Genotyping by PCR. Positive PCR result for males 85F1No.62 (A) and 85F1No.85 (B) from founder male 85. (C) Positive PCR result for male 318F1No.119 from founder male 318. (D) Positive PCR results for F2 fish (85F1No.62F2No.1, 4, 7, 8 and 9) from 85F1No.62 male. (E) Positive PCR results for F2 fish (85F1No.85F2No.1, 2, 3, 6 and 8) from 85F1No.85 male.
5.3.1 Transgene is expressed in an inducible manner

To determine if the heat-inducible transgene, \textit{hsp70:EGFP-radar}, was expressed within the transgenic lines descendent from founder male 85, RT-PCR analysis was conducted on heat-treated F\textsubscript{2} progeny from an outcross between wild-type females and transgenic males 85F\textsubscript{1},No.62 and 85F\textsubscript{1},No.85. Furthermore, to determine the degree of regulatory control from the \textit{hsp70} promoter/enhancer, a similar analysis was conducted on non-induced sibling embryos.

Transgenic males 85F\textsubscript{1},No.62 and 85F\textsubscript{1},No.85 were crossed with wild-type females and their progeny given a 2 hour/40°C heat-induction treatment at the 10-12 somite stage (14-15 hpf). Following this treatment, the embryos were left to recover at 28°C and randomly divided into 4 groups, \( t = 0, 2, 4 \) and 6. Each group consisted of 50 embryos. Immediately following the heat-shock treatment, total RNA was extracted from the embryos of group \( t = 0 \), and likewise following \( 2, 4 \) and \( 6 \) hour intervals for groups, \( t = 2, 4 \) and 6, respectively. First strand cDNA was then synthesised from total RNA using oligo (dT) primers and reverse transcriptase. To detect the transgene from these cDNA pools, primers spanning the \textit{EGFP-radar} fusion were used, TRCHECKUP and TRCHECKDO (Figure 5.4A). These primers were expected to yield a 524 bp PCR product. As an internal control, primers specific for \textit{ef1-\( \alpha \)} (EF1\( \alpha \)3UP and EF1\( \alpha \)3DO) were expected to give a 299 bp amplification product. Furthermore, to detect any genomic contamination within the extracted RNA, reverse transcriptase negative samples were also amplified. The cycling conditions were, 1 minute at 94°C, 1 minute at 60°C and 1.5 minutes at 72°C for 30 cycles. One-fifth of the PCR product was resolved on a 1% agarose gel (Figure 5.6).

Immediately following the induction treatment, large amounts of transcript for the transgene were detected from both lines 85F\textsubscript{1},No.62 (Figure 5.6A) and 85F\textsubscript{1},No.85 (Figure 5.6B). Interestingly, the level of transcript detected declined rapidly, suggesting a decline in expression and a rapid rate of transcript degradation following the heat treatment. Also of significant interest was the detection of transgene transcript in non-induced embryos. This
Figure 5.6 RT-PCR analysis of transgene expression in transgenic embryos. Time course of transgene expression in F2 embryos from males 85F1No.62 (A), 85F1No.85 (B) and in wild-type embryos (C) following heat treatment (40°C for 2 hours) at the 10 to 12 somite stage (14 to 15 hpf). Embryos not subjected to a heat-induction treatment provided a non-induced control. The time course ranged from 0 hours (immediately following the conclusion of the heat treatment) to 6 hours. RT–ve refers to samples processed without reverse transcriptase.
A  

Line 62:  

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suggests promiscuous, leaky expression of the \textit{hsp70:EGFP-radar} transgene from the \textit{hsp70} promoter/enhancer under non-stressed conditions. All reverse transcriptase negative samples were negative for the transgene, as were similarly heat-treated wild-type embryos (Figure 5.6C).

To determine whether this leaky expression from the \textit{hsp70} promoter/enhancer could be eliminated, transgenic embryos resulting from the same outcrosses as described above were kept at approximately 22°C or the typical 28°C. Total RNA was then extracted from 100 24 hpf stage embryos for RT-PCR analysis. The cDNA synthesis and cycling conditions were identical to those described above. Significant amounts of transcript were detected from both the 22°C and 28°C incubation groups. This demonstrated that leaky expression occurred from the progeny of both transgenic males 85F, No. 62 and 85F, No. 85 when raised at the typical 28°C and at the lower 22°C temperatures (Figure 5.7).

\subsection*{5.3.2 Transgenic embryos develop circulatory defects}

To determine if this ectopic \textit{EGFP-radar} expression interfered with normal embryonic development and led to any detectable EGFP expression, transgenic embryos were subjected to a range of induction treatments at various developmental stages. Induction temperatures ranged from 38 to 40°C over periods ranging from 1 to 6 hours. Multiple inductions were also conducted on the same embryos, separated by a rest period. Transgenic embryos were induced before, during, and after gastrulation. Induction experiments typically consisted of randomly dividing transgenic siblings into 2 groups, an induced group and a non-induced group. In addition, wild-type embryos were likewise divided into similar groups and subjected to identical treatments. Groups were of equal size, typically 75 to 100 embryos.

To determine if induction prior to, or during, gastrulation could induce ventralisation similar to that observed following forced \textit{radar} overexpression, hemizygous transgenic embryos were subjected to induction treatments as early as the 8- to 16-cell and
Figure 5.7 RT-PCR analysis of leaky promoter activity at lower temperatures. RT-PCR analysis to detect transgene expression in non-induced F2 embryos from males 85F1No.62 (line 62) and 85F1No.85 (line 85) raised at 28°C and 22°C. RT-ve refers to samples processed without reverse transcriptase.
32- to 64-cell stages for periods ranging from 2 to 6 hours. Hemizygous embryos were the progeny resulting from an outcross involving either males 85F\textsubscript{1} No.62 or 85F\textsubscript{1} No.85. Hence, 1/2 of these F\textsubscript{2} embryos were hemizygous for the transgene while the remainder were wild-type. No consistent phenotype resulted from these induction experiments. In addition, embryos were particularly sensitive to heat-induction prior to gastrulation movements. Wild-type embryos subjected to long (6 hours) or very early (8- to 16-cell stage) induction treatments resulted in a wide variety of embryonic defects making the analysis of transgene-related embryonic defects inconclusive. No ventralised embryos resulted from these induction experiments and no EGFP expression was detected by fluorescence microscopy. Post gastrulation-stage embryos (12-16 somites/15-17 hpf) were more tolerant of the heat-induction treatments. However, no consistent phenotype was observed and no EGFP expression detected following induction.

When the progeny from two hemizygous transgenic parents (85F\textsubscript{1} No.85F\textsubscript{2} No.6 and 85F\textsubscript{1} No.85F\textsubscript{2} No.8) were observed throughout development, a highly specific and reproducible phenotype was observed, despite the lack of any heat-induction treatments. Embryos generated from such a cross, expected to give 25% wild-type, 50% hemizygous and 25% homozygous genotypes, were indistinguishable from one another during the first 1.5 days of development (Figure 5.8A). All developed normally and initiated a typical primitive circulation. However, by 2 days development approximately 25% (23 ± 2%; \( n = 1,179 \); 6 separate experiments) lacked circulation throughout the developing trunk and head. In such embryos blood cells were either restricted to passage between the atrium and ventricle (Figure 5.8D, F and H) of the heart or pooled in the vicinity the anterior hypochord (Figure 5.8D insert) or the developing tail. By 3 days development the pericardium became oedematous (Figure 5.8H) and circulation was still absent throughout the entire embryo. Following 5 days development, severe pericardial and yolk sac oedemas continued to develop (Figure 5.8B) until death, at around day 7 to 8, due to substantial necrosis throughout the entire embryo. All other sibling embryos (77 ± 2%; \( n = 1,179 \); 6 separate experiments) displayed a typical developmental program. Interestingly, this
Figure 5.8 Progeny from hemizygous transgenic parents display defects in circulation. (A) Morphologically normal looking 24 hpf stage embryos generated from hemizygous transgenic parents. (B) Progeny from the same parents following 124 hours development. A subgroup of embryos displayed pericardial and yolk oedemas (denoted by arrow and arrowhead, respectively) associated with a complete lack of circulation. (C) Lateral view of a wild-type 50 hpf stage embryo, arrow denotes developing heart. (D) Similar lateral view of a 50 hpf stage putative homozygous transgenic embryo displaying a lack of trunk circulation and an accumulation of blood in the developing atrium and ventricle (arrow). Insert displays a similar embryo at the 74 hpf stage displaying blood pooling in the vicinity of the anterior hypochord (arrow). (E) Close-up of a normal looking 50 hpf stage embryo generated from hemizygous transgenic parents displaying a morphologically normal heart (arrow). (F) Similar close-up view of embryo in panel D displaying an accumulation of blood in the developing heart (arrow). (G) Close-up of a normal looking 74 hpf stage embryo generated from hemizygous transgenic parents displaying a morphologically normal heart (arrow). (H) Similar view of a putative homozygous transgenic sibling that lacks circulation and possesses pericardial oedema (small arrow) and an accumulation of blood in the developing heart. Large arrow and arrowhead denotes atrium and ventricle, respectively. (I) PCR genotyping to detect transgene from 12 randomly selected embryos demonstrating the lack of circulation phenotype. Scale bars in A/B and C/D/E/F/G/H represent 1 mm and 250 μm, respectively.
phenotype presented itself with essentially the same frequency regardless of whether the embryos received an induction treatment (1.5 hours/40°C at the shield stage) or not (23 ± 1%; n = 359; 2 experiments compared to 24 ± 2%; n = 820; 4 experiments in heat-treated and non-heat-treated embryos, respectively). To determine if this phenotype was linked to the presence of the transgene, genomic DNA was isolated from 12 individual 4 dpf embryos possessing this lack of circulation phenotype and genotyped by PCR. PCR conditions were identical to those used previously for amplification of the transgene using the primers PHSP70UP and EGFPDO. All 12 embryos were demonstrated to carry the transgene in their genomes (Figure 5.8I). At no stage was EGFP expression detected from either induced or non-induced embryos generated from hemizygous transgenic parents.

III. Summary

We endeavoured to generate an inducible radar transgenic line to facilitate the study of Radar function from the hypochord and PGE. An EGFP-tagged Radar-encoding cDNA construct was engineered downstream of the zebrafish heat-inducible hsp70 promoter/enhancer element. This EGFP-radar fusion construct, when driven by the constitutive CMV promoter, was demonstrated to induce EGFP-coupled ventralisation when transiently overexpressed in early zebrafish embryos.

Of 991 1- to 2-cell stage zebrafish embryos injected with the linearised pCS2/hsp70/EGFP-rdr transgene, 330 survived to sexual maturity to constitute the F₀ founder population. From 212 individual intercrosses within this founder pool, 3 sexually mature fish capable of transmitting the transgene within their germ-lines were identified through a PCR-based screening strategy. These were founder males, 56, 85 and 318. Transgenic F₁ were generated from founders 85 and 318 from an outcross with wild-type females, but not from founder male 56.

From 155 individual 85F₁ fish, 2 transgenic F₁ fish were identified, males 85F₁No.62 and 85F₁No.85. Similarly, from 133 individual 318F₁ fish genotyped, 1
transgenic F₁ fish was identified, male 318F,No.119. Outcrosses with wild-type females for both males 85F₁,No.62 and 85F₁,No.85 gave an average 48% transmission rate (n = 48) to the F₂ generation (expected Mendelian ratio, 50%), confirming their hemizygous genotype.

The ability of the transgenic zebrafish to induce transgene expression following heat treatment was assayed by RT-PCR analysis following a 2 hour heat-induction treatment at 40°C. Hemizygous transgenic F₂ embryos generated from an outcross from both males 85F₁,No.62 and 85F₁,No.85 displayed elevated transgene transcript levels following heat-induction, followed by rapid transcript degradation over the time course analysed (0 to 6 hours post induction). However, significant levels of expression was also detected from both transgenic lines under non-stressed conditions. Raising transgenic embryos at lower temperatures (22°C) failed to eliminate this leaky promoter activity.

Multiple induction experiments involving transgenic hemizygotes heated at various developmental stages and using different induction treatment times and temperatures, failed to elicit any consistent phenotype that was linked with the presence of the hsp70:EGFP-radar transgene. However, approximately 25% of induced and non-induced embryos, generated from hemizygous parents, developed a highly specific and reproducible phenotype. Such embryos were characterised by a complete absence of blood flow following 1.5 days development. Furthermore, this phenotype was demonstrated to be linked with the presence of the hsp70:EGFP-radar transgene.
Chapter 6

Generating a Radar loss-of-function model

I. Introduction

6.1 Morpholino antisense technology

The zebrafish has provided the developmental biologist and geneticist with an excellent model system for studying the genetics of vertebrate development. However, until recently, there was a limited potential to conduct targeted loss-of-function studies similar to those routinely used in the mouse. With the ease of finding novel genes from the zebrafish, a knock-out technology was needed to relate genes to function. In the zebrafish, the injection of dominant negative constructs has been extensively used to provide insight into gene function. This approach requires the engineering of specific mutations into the gene of interest to abolish the function of the encoded protein and to inhibit the function of simultaneously expressed endogenous protein. This technology, however, is not applicable to all genes and tends to be most effective for proteins that assemble into functional multimers, such as receptor complexes (Lagna and Hemmati-Brivanlou, 1998). Furthermore, substantial knowledge of protein structure is required to design an effective mutation that will result in an inactive protein. Another alternative explored in the zebrafish is the use of double-stranded RNA. This method provides a sequence-dependent means to reduce gene function in vivo and relies on the double-stranded RNA being homologous to the gene of interest. RNAi has been used successfully to disable gene function in a number of development systems (Kuwabara et al., 2000; Fjose et al., 2001; Maine et al., 2001). However, its use in the zebrafish has generated mixed results. Some researchers have had
success in specifically inhibiting gene function (Wargelius et al., 1999; Li et al., 2000) while others have reported the non-specific down regulation of transcripts following the injection of double-stranded RNA (Oates et al., 2000; Zhao et al., 2001).

Recently, the use of morpholino phosphorodiamidate oligonucleotides (morpholinos; MOs) has proved successful in specifically inhibiting targeted genetic loci in a number of developmental systems (Table 6.1), including the zebrafish (Table 6.2). MOs are synthetic DNA analogues that possess a neutral-charged backbone and a morpholine ring instead of the typical ribose sugar moiety (Ekker and Larson, 2001).

<table>
<thead>
<tr>
<th>Developmental model system</th>
<th>Delivery method</th>
<th>Effective duration (h)</th>
<th>Developmental temperature (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>X. laevis</em> (Frog)</td>
<td>Microinjection</td>
<td>&lt;26</td>
<td>15-22</td>
<td>(Heasman et al., 2000)</td>
</tr>
<tr>
<td><em>G. gallus</em> (Chicken)</td>
<td>Electroporation</td>
<td>24</td>
<td>37</td>
<td>(Kos et al., 2001)</td>
</tr>
<tr>
<td><em>D. rerio</em> (Zebrafish)</td>
<td>Microinjection</td>
<td>50+</td>
<td>22-29</td>
<td>(Nasevicius and Ekker, 2000)</td>
</tr>
<tr>
<td><em>S. purpuratus</em> (Sea urchin)</td>
<td>Microinjection</td>
<td>18+</td>
<td>13-22</td>
<td>(Howard et al., 2001)</td>
</tr>
<tr>
<td><em>D. melanogaster</em> (Fruit fly)</td>
<td>Microinjection</td>
<td>8+</td>
<td>18-29</td>
<td>(Ekker and Larson, 2001)</td>
</tr>
</tbody>
</table>

Table 6.1 Morpholino-mediated gene knock-down in different model systems, adapted from Ekker and Larson, 2001.

This modification confers an increased stability against nuclease degradation. MOs have a very high affinity for RNA, acting as efficient antisense reagents. This new, efficient and most importantly sequence-specific loss-of-function technology is providing zebrafish developmental biologists with a means to conduct knock-out style experiments in the fish.
Table 6.2 Morpholino-mediated phenocopies of known zebrafish mutants.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>no tail, chordin, one-eyed-pinhead, nacre and sparse</td>
<td>(Nasevicius and Ekker, 2000)</td>
</tr>
<tr>
<td>cyclops</td>
<td>(Karlen et al., 2001)</td>
</tr>
<tr>
<td>bmp2/swirl and bmp7/snailhouse</td>
<td>(Imai et al., 2001)</td>
</tr>
<tr>
<td>squint, one-eyed-pinhead and no tail</td>
<td>(Feldman et al., 2001)</td>
</tr>
<tr>
<td>endothelin1/sucker</td>
<td>(Miller et al., 2001)</td>
</tr>
<tr>
<td>colourless</td>
<td>(Dutton et al., 2001)</td>
</tr>
<tr>
<td>swirl, snailhouse, somitabun, minifin, silberblick and pipetail</td>
<td>(Lele et al., 2001)</td>
</tr>
<tr>
<td>lost-a-fin</td>
<td>(Bauer et al., 2001)</td>
</tr>
</tbody>
</table>

6.1.1 Mechanism of action

MOs injected into early stage zebrafish embryos (1- to 16-cell stage (Nasevicius and Ekker, 2000)) act via an RNase H-independent mechanism to inhibit translation by binding to specific complementary regions of mRNAs. The efficiency of morpholino-mediated knock-down is highly dependent upon where the MO is designed to bind the transcript. MOs must be designed against the leader sequence or nearby bases to effect translation inhibition (Figure 6.1) (Ekker and Larson, 2001). The binding of the modified oligonucleotide to these sequences sterically prevents the 40S ribosomal subunit from identifying the start codon and subsequently recruiting the 60S subunit for translation initiation (Figure 6.1A and B) (Ekker and Larson, 2001). MOs, being DNA analogues, are ideally suited to targeted in vivo translation inhibition because of their superior resistance to enzymatic degradation. This results in enhanced stability throughout embryonic
Figure 6.1 Mechanism of morpholino-mediated inhibition of translation. (A) Diagrammatic representation of normal translation of a transcript. The 40S ribosomal subunit reads the 5' leader sequence to identify the start codon, it then recruits the 60S ribosomal subunit and translation initiates. (B) MOs bind to their complementary target sequence near the start codon of the transcript, preventing the 40S subunit from identifying the start codon, resulting in translation inhibition. (C) Inhibition efficiency of MOs plotted against their target site on the transcript. MOs become less efficient at specifically inhibiting translation of a target gene when they are designed to target sequence 3' to the start codon. Adapted from Heasman et al., 2002.
development, allowing for the possibility to target genes that may not be expressed until organogenesis stages. The duration of effectiveness is likely to be gene-specific and rely on the individual transcription and translation characteristics of a given gene. However, at least in the zebrafish, MOs appear to provide efficient and specific translation inhibition at least until the hatching stage (approximately 48 hpf) (Nasevicius and Ekker, 2000; Dutton et al., 2001) and in some cases beyond (van der Sar et al., 2002).

6.1.2 Mistargeting and non-specific side-effects of morpholino oligonucleotides

Although the use of MOs to target and specifically inactivate gene function has been widely reported in the zebrafish and Xenopus, many researchers have also reported on the unexpected and presumably non-specific phenotypes resulting from MO injections, particularly at higher MO doses (Ekker and Larson, 2001; Heasman et al., 2002). These unexpected phenotypes include neural degeneration (Nasevicius and Ekker, 2000; Braat et al., 2001; Karlen et al., 2001; Lele et al., 2001) and epiboly defects (Imai et al., 2001). The reason why some MOs appear to produce non-specific developmental defects when injected at high concentrations is not understood. This requires stringent controls to be implemented to delineate defects specific to a down regulation of the gene function of interest. Some researchers hypothesise that the high incidence of neural degeneration defects associated with high dose MO use is a result of promiscuous mistargeting of MOs to genes required for neural cell survival (Ekker and Larson, 2001). This would result in the undesired simultaneous inactivation of the target gene and the neural survival gene that shares sufficient sequence homology with the MO to effect binding. Interestingly, neural degeneration was one of the most common defects observed from the zebrafish mutagenesis screens, suggesting the existence of many genes whose functions are required for neural cell survival (Furutani-Seiki et al., 1996).
6.1.3 Specificity controls

The use of MO technology to gain insight into gene function requires the incorporation of stringent controls. These controls become even more significant when functional studies on completely novel genes are carried out. When using MOs to effect targeted knock-down of a specific gene function, a number of phenotypes can result. Some embryos may display a phenotype that is completely specific to the down regulation of only the targeted genetic loci, or, a combination of non-specific and specific phenotypes. Alternatively, a completely non-specific phenotype may result, or no phenotype at all. Indeed, not all genes are critical for early developmental processes. It is crucial that this signal-to-noise ratio is determined and controlled in order to retrieve valuable information from knock-down experiments. This can be achieved by implementing one or more of the following specificity controls (reviewed in Ekker and Larson, 2001 and Heasman et al., 2002): 1) Western analysis to confirm successful polypeptide down regulation; 2) complementary phenotypes following the injection of independent non-overlapping MOs targeting the same transcript; 3) simultaneous targeting, using 2 non-overlapping MOs that target the same transcript, resulting in a synergistically enhanced efficacy with an associated reduction in non-specific mistargeting defects; 4) rescue experiments using synthetic transcripts that are devoid of the MO target sequence; and 5) the use of a mismatch control MO that differs from the targeting MO by a series of mispaired bases.

6.1.4 Other limitations

Despite the advantages that MO technology offers the developmental biologist, there are limitations to its use. A clear example of this is the inferior penetrance of MOs designed to target the one-eyed-pinhead (oep) transcript. Nasevicius and Ekker reported that a MO specifically designed to target oep, when injected into zebrafish embryos, elicited the oep phenotype in only half of the injected embryos (Nasevicius and Ekker,
Furthermore, the efficacy of some MOs may be strain dependent, as demonstrated by an *oep*-targeting MO that does not function in a wild-type strain of zebrafish from Japan (Nasevicius and Ekker, 2000). The researchers suggest that this may be a result of polymorphisms that are present in the targeted sequence.

II. Results

6.2 Using morpholino oligonucleotides to phenocopy the *one-eyed-pinhead* mutant, a technical control

To determine the ability of morpholino-technology to phenocopy a characterised zebrafish mutant, a MO designed to target *oep* transcripts was injected into 1- to 4-cell stage zebrafish embryos. The zebrafish *oep* gene encodes a maternally and zygotically expressed EGF-related ligand that possesses a functional signal sequence and is membrane-bound. It is these properties that are disrupted in the *oep* mutant alleles *oep<sup>c57</sup>* and *oep<sup>m134</sup>* (Zhang *et al.*, 1998). The *oep* phenotype is recognisable by the tail bud stage of development (10 hpf). The defects include a reduced body axis and the absence of a prechordal plate. By the 24 hpf stage, the eye vesicles are fused (cyclopia) and the anterior part of the forebrain is reduced, while in the trunk, the notochord is slightly undulating and the yolk tube thicker and shorter. In addition, the body axis of *oep* mutants is curved down and there is no detectable floor plate or hatching gland (Hammerschmidt *et al.*, 1996). These disruptions to normal embryonic development are due to defects in endoderm, prechordal plate, and ventral neuroectoderm patterning and formation (Zhang *et al.*, 1998).
6.2.1 An oep-targeting morpholino can phenocopy the oep mutant

The oep-targeting MO (oep MO) was a 25-mer oligonucleotide designed to target within the 5’ UTR of the oep transcript from nucleotide position -8 to -32 (Figure 6.2A). Injection of a 6 ng dose of oep MO into 1- to 4-cell stage embryos (n = 140) resulted in no observable phenotype following 24 hours development. A 12 ng dose was then injected into 1- to 4-cell stage zebrafish embryos. These embryos were left to recover and scored for any disruptions to normal development at 24 hpf. Based on the original description of the oep mutant morphology (Hammerschmidt et al., 1996), injected embryos were scored for the following defects, fused eyes (cyclopia), undulating notochord, extended somites, shorter and thicker yolk tube and curled tail. In addition to these defects, another phenotype was observed in oep MO-injected embryos at the 24 hpf stage that had not been described in the oep mutant. This was a complete absence of eye development. This defect was also scored, as a non-specific phenotype. The most common phenotype class (67%; n = 165) possessed all the oep defects except for cyclopia, instead there was a complete absence of eye development and other anterior head structures (Figure 6.2C and D). This is most likely due to non-specific targeting of another gene involved in eye development. Approximately 1/10th (9%; n = 165) of injected embryos possessed cyclopia (Figure 6.2E and F), along with almost all of the other defects characteristic of the oep mutant. A similar frequency of oep MO-injected embryos (8%; n = 165) appeared phenotypically normal under microscopic observation at 24 hpf.

Other studies involving morpholino-mediated targeting of oep transcripts has revealed that this non-specific, absent eyes, phenotype is common with high-end doses of MO (Nasevicius and Ekker, 2000; Feldman et al., 2001). Furthermore, researchers reported inefficient penetrance of the oep MO-induced phenotype using a MO of the same sequence as the one used in our study (Nasevicius and Ekker, 2000). In addition, it has been reported that the early delivery of oep-targeting MOs into a Japanese strain of wild-type fish failed to elicit any phenotype (Nasevicius and Ekker, 2000). This may result from polymorphisms
Figure 6.2 Injection of oep-targeting MOs into zebrafish embryos phenocopies the one-eyed-pinhead mutant phenotype. (A) Partial cDNA sequence and conceptual translation of oep displaying the location of the oep MO target sequence within the 5' UTR domain (underlined). The 5' UTR domain is highlighted in bold text. Genbank accession number = AF041440. (B) Wild-type 24 hpf stage zebrafish embryo (anterior to left). (C) 24 hpf stage zebrafish embryo (anterior up) following the injection of oep MO (12 ng approx.) displaying a lack of anterior head structures (arrow) and a disrupted notochord (arrowhead). (D) Dorsal anterior view (anterior down) of embryo in panel C, showing a lack of eye development (arrow). (E) Lateral view of an oep MO-injected embryo (anterior up) possessing a single fused eye (arrow) and a disrupted notochord (arrowhead). (F) Dorsal anterior view (anterior to left) of embryo in panel E, displaying a single fused eye (arrow). Scale bars in B, C and D represent 250 μm.
in the 5' UTR to which the oligo was designed to bind. Although the oep mutant may not represent the most ideal candidate mutant to phenocopy using MO technology, the injection of oep-targeting MOs did phenocopy the oep mutant and highlighted some interesting considerations. Most important was the need to identify and characterise non-specific phenotypes through employing a number of carefully designed specificity controls.

6.3 Generating a Radar-specific loss-of-function model using morpholino technology

For loss-of-function analyses, a radar deletion mutant, \( rdr^{D1} \), has been previously generated to assess Radar's role in dorsal neuroectoderm cells where it may function as a maintenance factor (Delot et al., 1999). Although the deletion spanning the radar locus was large, encompassing 27.4 cM of LG 16 (Delot et al., 1999), and may have abrogated other genes essential in embryogenesis, we used the description of the mutant to initially benchmark the specificity of the radar-targeting MOs. In brief, the \( rdr^{D1} \) phenotype was described as possessing a shortened axis and a reduction of head structures, presumably as a result of cell death in this region, particularly posterior to the eyes. Apoptosis, as detected by acridine orange (AO) staining, was reported in the head, dorsal neural tube and tail (Delot et al., 1999). Furthermore, the \( rdr^{D1} \) mutant embryos die around 2 dpf, precluding identification of any phenotype that may not manifest until later in development.

6.3.1 Early delivery of radar-targeting morpholino oligonucleotides phenocopies the \( rdr^{D1} \) neurodegenerative defects

Two non-overlapping antisense MOs (rad-MO1 and rad-MO2) were designed to target the 5' region of the radar transcript (Figure 6.3). These MOs were then delivered via microinjection into 1- to 4-cell stage zebrafish embryos that were monitored for
**Figure 6.3** Partial cDNA sequence and conceptual translation of *radar* highlighting morpholino oligo target sites. Two non-overlapping MOs (rad-MO1 and rad-MO2) have been designed to target *radar* transcripts just downstream of the start codon (rad-MO1, red) and within the 5’ UTR (rad-MO2, green). The predicted signal peptide is underlined and the 5’ UTR is in bold typeface.
developmental defects throughout embryogenesis. Similar phenotypes resulting from the injection of both rad-MO1 and rad-MO2 provides a reliable test of specificity of the gene knock-down mechanism (Ekker, 2000). We present here the data using rad-MO1.

When 12 ng of rad-MO1 was injected into 1-4 cell-stage embryos, a number of developmental abnormalities were observed at the 18 somite stage of development. An expansive cellular opacity symptomatic of cell death was easily detectable in morphant embryos. In particular, neural degeneration accumulated rapidly in the course of developmental stages analysed (18, 20, 22 and 24 somites) (Figure 6.4). By 24 hpf (approximately 31 somites), a large proportion of embryos (94%) displayed this distinct neurodegenerative phenotype (Table 6.3). These radar morphants displayed a modest developmental retardation, a characteristic feature of zebrafish neural degenerative mutants (Furutani-Seiki et al., 1996). Injection of rad-MO2 resulted in similar developmental defects suggesting that these defects were due to a reduction in the radar gene product. AO staining was performed on the radar morphants to ascertain if the cell death observed in the head, tailbud and dorsal neural tube was due to apoptosis or necrosis (Figure 6.4). AO is a vital dye that has been reported to selectively label apoptotic cells, thus differentiating apoptotic from necrotic cell death (Abrams et al., 1993; Furutani-Seiki et al., 1996). Following 30 minutes of staining, substantial apoptotic activity was detected in the eyes, head and a narrow dorsal population of cells extending the length of the neural tube (Figure 6.4B, F, J and N). Occasionally, apoptotic cells were also detected in the developing tailbud (Figure 6.4B and F). In uninjected controls, limited apoptotic activity was detected sporadically in varying locations throughout the developing embryo (Figure 6.4D, H, L and P).
**Figure 6.4** Injection of rad-MOs results in a specific pattern of apoptotic cell death. The vital dye acridine orange (AO) was used to selectively stain apoptotic cell death in *radar* morphant (A,B,E,F,I,J,M and N; injected with 12 ng rad-MO1) and wild-type (C,D,G,H,K,L,O and P) embryos at 18 somite (A-D), 20 somite (E-H), 22 somite (I-L) and 24 somite (M-P) stages. Panels B,D,F,H,J,L,N and P are the AO-stained embryos of A,C,E,G,I,K,M and O, respectively, viewed under fluorescence. Although cell death was visible under Nomarski optics, AO staining allowed a more precise localisation. *Radar* morphant embryos exhibited apoptotic cell death throughout the developing head, in regions of the forebrain, midbrain and hindbrain (small arrow heads in B, F and J), and occasionally in the developing tail bud (large arrow in B and F). Apoptotic activity was also detected in the developing eye of *radar* morphant embryos (small arrows in B, F and J). Perhaps the most striking observation was a row of apoptotic cells extending the length of the dorsal neural tube (large arrow head in B, F, J and N). *Radar* morphant embryos often displayed a modest developmental retardation (compare A with C, E with G and M with O). Scale bars in A and M represent 100 μm.
Table 6.3 Rescue of radar morphant neurodegenerative phenotype.

6.3.1.1 RNA rescue of radar morphant neurodegenerative phenotype

To further demonstrate that the phenotype observed was Radar-specific, the dose of the MO was varied, and a phenotype rescue was conducted. Injection of 8 ng of MO reduced the affected phenotype frequency by half and resulted in less severe cellular apoptosis, indicative of a dose response (data not shown). To effect rescue, *in vitro* transcribed P2RAD transcripts were injected along with radar targeting MOs. P2RAD encodes a human BMP2 pro-domain, *radar* mature domain fusion construct that is devoid of the rad-MO1 target sequence. This was necessary to ensure that *radar*-targeting MOs did not bind to, and sequester, the injected transcripts, and that the rescue strategy operated by actual restoration of functional amounts of Radar protein. Injection of capped P2RAD mRNA induced ventralisation (data not shown) indicative of functional Radar.
Unfortunately, this ventralisation effect prevented optimal doses of mRNA to effect complete rescue. Nevertheless, the partial rescue achieved was still very informative. A range of amounts of P2RAD mRNA (5 pg to 25 pg/embryo) was coinjected with 12 ng rad-MO1 to rescue the neural degeneration. Coinjection of 12 ng rad-MO1 with P2RAD mRNA higher than 5 pg resulted in the majority of injected embryos ventralising, hence preventing rescue analysis. However, when rescue was attempted with 5 pg of P2RAD transcript, only 4% of the injected embryos appeared ventralised. When these embryos were scored for neural degeneration following 24 hours development, there was a significant 8-fold decrease in embryos displaying severe cell death, together with a 5-fold increase in those embryos that appeared phenotypically normal (Table 6.3).

Other phenotypes, following the injection of high doses of rad-MO1, included undulations of varying degrees in the notochord (20%; n = 139). Eye development was also notably disrupted due to cellular degeneration just posterior to the developing eye. This resulted in significantly reduced eyes at later stages of development (data not shown).

6.3.2 Radar morphants display specific circulatory defects following 2 days development

Following 50 hours development, embryos injected with moderate to low doses of radar-targeting MOs (6 and 8 ng doses) demonstrated significant and severe circulatory defects. The vast majority of these embryos displayed no aberrant embryogenesis prior to the detection of these circulatory defects, appearing morphologically normal. The most severely affected embryos demonstrated a complete absence of axial circulation throughout the trunk, often despite a normal anterior circuitry that supplied blood, in a simple loop, over the yolk, through the heart and into the developing head. No circulating blood cells were observed in the DA, PCV, CA, CV and the intersegmental vessels. A more subtle class of phenotype was also present in which intersegmental circulation was absent and blood was observed to leak out of the axial vasculature at apparently random locations
throughout the trunk. Under light microscope, blood cells could be clearly seen leaking from the trunk vasculature and often moving dorsally and pooling in extravascular tissues in the dorso-lateral extremities of the trunk. The early delivery of both rad-MOl and rad-MO2 resulted in these disruptions to normal circulation. To observe these defects in detail, microangiography was performed by injecting fluorescently-labelled latex beads into the cardinal vein or sinus venosus (Figure 6.5) of anaesthetised 50 hpf stage rad-MO1-injected embryos.

6.3.2.1 Classification and microangiographic description of Radar-specific circulatory defects

For ease of scoring, the circulatory defects were characterised using two distinct criteria: classifying the pattern of circulation in the main axial vessels of the trunk, the DA and PCV, and noting the presence or absence of intersegmental circulation (Table 6.4). Based on this criteria, rad-MO1-injected embryos displaying circulatory defects were classified into one of three phenotypic classes, class I, II or III. In class I morphants, blood passed down the DA but returned rostrally via the PCV prematurely at random locations along the yolk extension (Figure 6.6C, D, E and F). This short-circuiting resulted in an absence of blood circulation in the CA and CV plexus as well as in the posterior extremities of the DA and PCV. Microangiographic analysis of this class of morphant revealed typical anterior circuitry (Figure 6.6C and E) with the injected fluorescent dye restricted to and highlighting the major and minor head vessels as well as the CCV and heart. At the location of the axial short-circuit, extensive haemorrhaging was observed under light microscopy. This verified the leakage of the fluorescent dye out of the trunk vasculature (Figure 6.6C, D, E and F).
Figure 6.5 Microangiographic analysis of zebrafish embryos. An anaesthetised embryo is held ventral side up by a holding pipette while fluorescently-labelled latex beads are injected into the sinus venosus (just posterior to the heart). These beads are then restricted to, and highlight, the developing vascular circuitry (denoted by superimposed green arrows). Scale bar represents 250 μm.
Figure 6.6 Visualisation of Radar-specific circulation defects by microangiography. (A) Normal trunk circulation in a 50 hpf wild-type embryo (anterior to left). (B) Normal caudal circulation in the tail of a 50 hpf wild-type embryo (anterior to left). (C-J) Disrupted circulation observed in 50 hpf radar morphant embryos (injected with 8 ng rad-MO1). Panels D, F and H are the higher power views of embryos C, E and G, respectively. Embryos C/E and G represent morphants with class I and II circulatory defects, respectively. Red arrows denote haemorrhages. Red arrowhead in G denotes cranial haemorrhage. DLAV = Dorsal longitudinal anastomotic vessel, DA = Dorsal aorta, CCV = Common cardinal vein, PCV = Posterior cardinal vein, Se = Intersegmental vessel, CA = Caudal artery, CV = Caudal vein. Scale bars in A and B represent 200 µm.
<table>
<thead>
<tr>
<th>Circulation phenotype at 50 hpf</th>
<th>Injection doses</th>
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<tbody>
<tr>
<td></td>
<td>6 ng rad-MO1(^a)</td>
</tr>
<tr>
<td>Normal axial circulation(^a)</td>
<td>55 ± 9</td>
</tr>
<tr>
<td>Class I</td>
<td>42 ± 7</td>
</tr>
<tr>
<td>Class II</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>Class III</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>No intersegmental circulation</td>
<td>40 ± 6</td>
</tr>
<tr>
<td>Total embryo number</td>
<td>148</td>
</tr>
</tbody>
</table>

\(^a\)Two experiments.
\(^b\)Three experiments.
\(^c\)‘Normal axial circulation’ refers to typical blood flow through the main trunk vessels (DA, CA, CV and PCV), exclusive of intersegmental circulation.

The phenotype percentages from each experiment were averaged and entered into the above table. The accompanying error represents the average difference between the average percentage and the individual experimental percentages.

### Table 6.4 Frequencies of radar morphant circulation defects.

In class II morphants, blood cells were observed to accumulate in the trunk or pulsate back and forth with the heart beat (Figure 6.6G and H). In such embryos, no distinct DA or PCV was detected through microangiographic analysis (Figure 6.6G and H). The class III embryos possessed the most extreme circulatory defect, lacking circulation in the trunk altogether, often despite circulation through the head and around the yolk in a simple rudimentary network. The class II and III phenotypes were only observed in a handful of injected embryos.
A reproducible dose-dependent effect of rad-MO1 injection was observed (Table 6.4). Injection of 8 ng of rad-MO1 resulted in more embryos possessing the various classes of circulatory phenotypes when compared with injection of 6 ng. At the 6 and 8 ng doses, the class I circulatory phenotype was the most common abnormality, accounting for more than 90% of embryos affected by a circulatory defect (Table 6.4). Injection of 6 ng rad-MO1 resulted in 42% possessing the class I phenotype, compared to 67% when injected with the higher (8 ng) dose (Table 6.4). The majority of these morphants had a normal circulation at 30 hpf (80 ± 2%; n = 198; 8 ng dose; 2 separate experiments) and looked morphologically normal until the circulation defects occurred. This increase in morphant embryos displaying leaky circulation following an elevation in MO dosage was complemented by a decrease in the number of injected embryos that possessed typical axial circulation through the DA, PCV, CA and CV plexus.

Interestingly, the vasculature posterior to sites of circulatory short-circuiting were often highlighted by the fluorescent dye, suggesting that caudal vasculature was present (Figure 6.6E, F and I). In such embryos, sections of the DA (Figure 6.6E) and CA (Figure 6.6I) posterior to the site of short-circuiting and haemorrhaging were often visible through microangiographic detection. In addition, some *radar* morphants displayed typical DA, CA, CV and PCV circulation despite the presence of haemorrhaging along the trunk (Figure 6.6J). In all phenotypic classes, blood was often seen pooling at various locations, including the head (Figure 6.6G). In these cases, blood was observed to take divergent paths throughout the embryo to bypass the apparent obstruction. A significant number of embryos, (40% with 6 ng dose and 80% with 8 ng dose) possessed no intersegmental circulation following 50 hours development, irrespective of the phenotype class to which they were assigned (compare Figure 6.6A and B with C, D, G, H, I and J) (Table 6.4).
6.3.2.2 Specificity controls

As mentioned previously, the early delivery of both rad-MO1 and rad-MO2 into zebrafish embryos resulted in vascular leakage from the axial vessels. Similar phenotypes resulting from non-overlapping MOs designed to target a single transcript is a good measure of target specificity. However, to further evaluate the specificity of this \textit{radar} morphant circulatory phenotype we employed a number of other controls including a mismatch and negative control MO (Table 6.4), as well as attempting to rescue the leakage phenotype through the injection of \textit{radar} transcripts and a \textit{radar} encoding DNA construct (Table 6.5).

Embryos injected with 8 ng of either a negative control MO (reported to possess no target specificity) or a 4-base mismatch control MO (rad-MO1-mm, a 4-base mismatch control oligo of rad-MO1) elicited no abnormal developmental defects when analysed by light microscopy at 50 hpf (Table 6.4). To effect rescue of the circulation defects, both P2RAD mRNA and cDNA was injected along with \textit{radar}-targeting MOs (rad-MO1). Transcripts encoded by P2RAD are devoid of the rad-MO target sequence. Only a partial rescue was obtained using this strategy because of the well documented early ventralising activity of \textit{radar} that masked the detection of any rescue of the later vascular defect. Injection of 8 ng rad-MO1 with 2 pg P2RAD mRNA increased the frequency of normal embryos by 2-fold (27\% to 49\%), which correlated with a decrease in the proportion of class I morphants (67\% to 45\%) (Table 6.5). Delivery of 10 pg P2RAD mRNA further decreased this phenotype class (20\%), however, this was not reflected in the proportion of normal embryos rescued; instead a representation of severely ventralised embryos (23\%) was produced by this treatment (Table 6.5). In an attempt to circumvent this interfering ventralisation, P2RAD cDNA was used. However, similar results were obtained with 10, 30 and 50 pg doses (Table 6.5).
<table>
<thead>
<tr>
<th>Circulation phenotype at 50 hpf</th>
<th>Injection doses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8 ng rad-MOI&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Normal axial circulation&lt;sup&gt;4&lt;/sup&gt;</td>
<td>27 ± 8 (%)</td>
</tr>
<tr>
<td>Class I</td>
<td>67 ± 8 (%)</td>
</tr>
<tr>
<td>Class II</td>
<td>1 ± 0 (%)</td>
</tr>
<tr>
<td>Class III</td>
<td>5 ± 1 (%)</td>
</tr>
<tr>
<td>Ventralised (V3-V4)</td>
<td>0 (%)</td>
</tr>
<tr>
<td>No intersegmental circulation</td>
<td>80 ± 8 (%)</td>
</tr>
<tr>
<td>Total embryo number</td>
<td>273</td>
</tr>
</tbody>
</table>

1 Two experiments.
2 Three experiments.
3 'Normal axial circulation' refers to typical blood flow through the main trunk vessels (DA, CA, CV and PCV), exclusive of intersegmental circulation.
4 The phenotype percentages from each experiment were averaged and entered into the above table. The accompanying error represents the average difference between the average percentage and the individual experimental percentages.
5 ) Corrected values when severe ventralisation (V3-V4) phenotype was excluded from the calculation of 'rescued' frequencies.

Table 6.5 Partial rescue of radar morphant circulation defects.
To normalise the data sets against ventralisation, embryos displaying this phenotype were excluded from the calculation, resulting in a corrected value for rescue (Table 6.5). Severely ventralised embryos (V3 and V4) possessing extremely disrupted trunk vasculature as a downstream effect of ventralisation, in particular the absence of a notochord, were separated from the cohort with normal circulation and notochord at 24 hpf. These remaining embryos were then scored for vascular defects at 50 hpf. Using this correction strategy, a dose dependent rescue was evident using P2RAD mRNA and cDNA (Table 6.5). Early delivery of 8 ng rad-MOl with the highest doses of P2RAD mRNA and cDNA (10 pg and 50 pg, respectively), increased the proportion of embryos displaying normal axial circulation from 27% (8 ng rad-MOl alone) to 73% and 67%, respectively.

6.3.2.3 Radar-specific defects occur following the initiation of a normal circulation

Prior to vascular leakage, circulation in radar morphant embryos was normal. This was clearly evident under light microscope, whole mount in situ hybridisation for the blood cell-specific marker βEγ-globin and by microangiographic analysis (Figure 6.7A and B). βEγ-globin expression in radar morphants during the initiation of circulation (approximately 25 hpf) revealed a typical pattern, with blood cells restricted to and highlighting a normal rudimentary vascular network. Blood cells were observed in the axial vasculature (DA, PCV, CA and CV plexus) as well as the anterior blood vessels, including the anterior extremities of the PCV, the left and right LDA and the CCV (Figure 6.7A). Microangiographic analysis of these embryos confirmed this normal circulation throughout the developing trunk and tail (Figure 6.7B). Although leakage was most evident at the 50 hpf stage, its inception was often observed as early as 35 hpf (Figure 6.7C and D). During this period, blood cells travelled throughout the length of the embryo in a typical fashion apart from minor leakages that occurred from the axial vasculature at random locations along the trunk (Figure 6.7C). Blood cells that escaped from the vessels could be observed moving dorsally past the notochord, contributing to the genesis of a haemorrhage (Figure
Figure 6.7 The progression of vascular leaks from radar morphants that initiate normal circulation at 25 hpf. (A) Whole mount in situ hybridisation analysis for βE3-globin expression in 25 hpf radar morphant embryos. Large black arrows and arrowheads in A denote blood cells in the common cardinal vein and the anterior cardinal vein, respectively. Small black arrows in A denote blood cells in the left and right lateral dorsal aorta. (B) Microangiographic analysis of a 25 hpf radar morphant embryo displaying typical circulation through the trunk and tail (anterior to left). (C) Lateral view of a 35 hpf radar morphant embryo (anterior to left) displaying the genesis of a vascular leak. Red arrows denote direction of blood flow. (D) A higher power view of embryo in panel C. Red arrowheads denote blood cells just dorsal to the notochord that have leaked from the DA. (E) Cross-section of a 50 hpf wild-type embryo (dorsal to top). (F) Magnification of section in panel E showing axial vasculature (DA and PCV). (G) Magnification of section in panel E showing dorso-lateral trunk region. (H) Cross-section of a 50 hpf radar morphant embryo (dorsal to top) at the location of an axial haemorrhage, displaying ectopic blood cells and physical destruction of axial vasculature (DA and PCV). (I) Magnification of section in panel H showing blood cells pooling lateral to the notochord. (J) Magnification of section in panel H showing blood cells pooling in dorso-lateral regions of the trunk. White arrows denote pronephric ducts. DA = Dorsal aorta, PCV = Posterior cardinal vein, CA = Caudal artery, CV = Caudal vein, NT = Neural tube, N = Notochord, RBC = Red blood cells. Scale bars in A, B, C, E and F/G represent 200 μm, 250 μm, 100 μm, 50 μm and 20 μm, respectively.
6.7D). As development proceeded and blood pressure increased, this haemorrhage grew and progressively restricted axial circulation until a blockage formed that completely inhibited caudal blood flow. This resulted in a short-circuiting of circulation, clearly evident by 50 hpf, forcing the blood to return prematurely to the heart via the PCV.

Cross-sections through the trunk of 50 hpf stage radar morphant embryos at sites of axial short-circuits revealed extensive leakage of blood cells from the axial vasculature (Figure 6.7H, I and J). Cross-sections through the trunk of 50 hpf wild-type embryos displayed blood cells restricted to the DA and PCV (Figure 6.7E and F). Occasionally, blood cells were also present in the intersegmental vessels that flank the notochord and neural tube, and in the dorsal longitudinal anastomotic vessels that flank the dorsal region of the neural tube (data not shown). Radar morphant embryo cross-sections revealed that haemorrhaging resulted in extensive physical damage to the DA and PCV (Figure 6.7H). Blood cells would often travel dorsally, past the notochord and neural tube (Figure 6.7I), where they would pool in extravascular locations (Figure 6.7J). The movement of blood seemed to follow a path of least resistance, passing between tissue boundaries rather than through the somitic tissue.

6.3.2.4 *Radar* morphants display typical initiation of vascular patterning and haematopoiesis

To determine if vascular patterning was affected in the radar morphant background, *in situ* hybridisation analysis of genes known to be involved in early mammalian vascular patterning was conducted. These genes included the endothelially-expressed flk-1 and flt1, which encode a receptor tyrosine kinase (Liao *et al.*, 1997) and transcription factor (Thompson *et al.*, 1998), respectively, as well as the endothelially-expressed receptor tyrosine kinase tie2 (Lyons *et al.*, 1998) and its ligand ang1 (Pham *et al.*, 2001), whose expression highlights the fish hypochord.
Interestingly, each of these genes displayed a typical expression pattern in 24 to 27 hpf stage *radar* morphant embryos (Figure 6.8). In the *radar* morphant, *angl* expression highlighted a morphologically normal looking hypochord in the trunk (Figure 6.8D) while the endothelial-specific markers *flk-1* (Figure 6.8B), *flil* (Figure 6.8F) and *tie2* (Figure 6.8H), labelled typical vascular circuitry throughout the trunk. This demonstrated that, in *radar* morphants, angioblasts migrated to the embryonic midline and assembled into a morphologically normal DA and PCV. Furthermore, *flk-1* and *flil* expression highlighted the genesis of phenotypically normal intersegmental vasculature (Figure 6.8B and F).

To determine if haematopoiesis was affected in *radar* morphant embryos, whole mount *in situ* hybridisation analysis was conducted using the blood cell-specific marker \( \beta E_{r}\text{globin} \). \( \beta E_{r}\text{globin} \) expression was normal within the *radar* morphant background, with strong expression restricted to blood cells throughout the entire ICM compartment (Figure 6.8J). Furthermore, these \( \beta E_{r}\text{globin} \)-expressing cells were demonstrated to contribute to an initially normal circulation in the early morphant embryo (Figure 6.7A).

### 6.4 Similarities and differences between the targeted knockdown of Radar and Vegf-A in the zebrafish

Of all the vasculogenic and angiogenic regulators known, perhaps the most compelling evidence supports the critical requirement for the PDGF family member VEGF in this process. Studies in a number of model systems have demonstrated this molecule to be involved in a number of key events during the establishment of the vascular system (Drake and Little, 1995; Flamme *et al.*, 1995; Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996; Cleaver and Krieg, 1998; Schmidt *et al.*, 1998; Carmeliet *et al.*, 1999b; Nasevicius *et al.*, 2000). Targeted knock-down of the *vegf-A* gene product in the zebrafish highlighted its role in the establishment of a functional axial vascular circuit (Nasevicius *et al.*, 2000). The *vegf-A* morphant possessed vascular defects ranging from the complete absence of any patent blood vessels throughout the trunk, to a more subtle phenotype, in which blood
Figure 6.8 Normal vascular patterning and haematopoiesis in radar morphant embryos. Whole mount in situ hybridisation analysis of flk-1 (A and B), ang1 (C and D), fltl (E and F), tie2 (G and H) and $\beta E_\alpha$-globin (I and J) in wild-type (A, C, E, G and I) and radar morphant (B, D, F, H and J, injected with 8 ng rad-MO1) embryos. DA = Dorsal aorta, PCV = Posterior cardinal vein, Se = Intersegmental vessel. Scale bars in A and I represent 250 μm and 200 μm, respectively.
circulated in a typical fashion throughout the major trunk vessels, the DA and PCV, but was reduced or absent in the minor axial vessels (Nasevicius et al., 2000). Interestingly, these morphant embryos revealed that Vegf-A is not required for the initial establishment of axial vascular patterning. Whole mount in situ hybridisation analysis of flk-1- and fli1-expressing angioblasts showed that these endothelial precursor cells migrated to the embryonic midline and contributed to a rudimentary DA and PCV (Nasevicius et al., 2000). However, consistent with the lack of microangiographic detection, the genesis of the intersegmental vessels does appear to be dependent upon functional Vegf-A, as revealed by a deficiency in flk-1 and fli1 expression in these vessels.

This vegf-A morphant possessed deficiencies that were somewhat reminiscent of those observed in the radar morphant. To further characterise the vegf-A morphant and explore any potential interaction between these two molecules or their respective signalling cascades, we generated a vegf-A morphant loss-of-function model and analysed its circulatory defects through microangiography. Furthermore, we conducted synergy experiments to determine if the targeted knock-down of Radar and Vegf-A had an enhanced effect on axial vascular development, supporting a potential interaction between their signalling pathways.

6.4.1 Early delivery of vegf-A-targeting morpholino oligonucleotides disrupts vascular development

A vegf-A-targeting MO (of the same sequence as that used by Nasevicius et al.) was injected into early, 1- to 4-cell stage, zebrafish embryos that were subsequently observed throughout early embryogenesis under light microscope for developmental deficiencies. The MO was designed to target the first 25 bases of the predicted signal peptide of the vegfiso isoform (Nasevicius et al., 2000) (Figure 6.9A). Injection of 6 and 9 ng doses resulted in substantial circulatory defects clearly evident following 2 days development (Figure 6.9). In the most extreme class of phenotype observed, pericardial oedema was
Figure 6.9 Phenotypes following the targeted inactivation of Vegf-A signalling. (A) Partial annotated sequence of the zebrafish vegf$_{165}$ gene displaying the vegf-A MO target site (underlined nucleotides). The putative signal peptide is underlined and the 5' UTR region is highlighted in bold typeface. Sequence adapted from Liang et al., 1998. (B) Lateral view (anterior to left) of a 50 hpf wild-type embryo. (C-F) Phenotypes observed in 50 hpf embryos following the injection of a 6 ng dose of vegf-A MO. (C) Vegf-A MO-injected embryo displaying pericardial oedema (arrow) and an accumulation of blood around the anterior hypochord (arrowhead). Haemorrhaging was also observed in vegf-A MO-injected embryos in the head (arrows in D) and the trunk (arrow in E). (F) Lateral view of the tail of a vegf-A MO-injected embryo (anterior to left) displaying an accumulation of blood in the developing tail region (arrow). Scale bars in B/E/F and D represent 250 μm and 200 μm, respectively.
significant, as was pooling of blood cells in the vicinity of the anterior hypochord and the developing tail region (Figure 6.9C and F). In *vegf*-A morphants that possessed these phenotypes, no axial circulation was evident under light microscope, despite a beating heart and a typical anterior vascular circuit carrying blood throughout the developing head. Although most evident at the 50 hpf stage of development due to the accumulation of pooled blood, the lack of trunk circulation was detectable at the 30 hpf stage, with such morphant embryos never establishing any axial blood flow. Of those embryos injected with 6 and 9 ng doses of *vegf*-A MO, 14 ± 3% (2 experiments; *n* = 224) and 20 ± 1% (2 experiments; *n* = 208) failed to initiate any axial circulation through the trunk. Of the remainder of embryos that did initiate and maintain axial circulation, many lacked or demonstrated reduced blood flow through the minor trunk vessels, the intersegmental vessels. Interestingly, a very small subgroup of *vegf*-A morphant embryos exhibited cerebral and axial haemorrhages, detected as blood pools under light microscope (Figure 6.9D and E).

### 6.4.1.1 Microangiographic analysis of Vegf-A-specific circulatory defects

To confirm and further characterise the circulatory defects observed following the targeted knock-down of Vegf-A, microangiography was performed on 50 hpf embryos that had received a 9 ng dose of *vegf*-A MO. In embryos that completely lacked any trunk circulation, the injected fluorescent dye did not highlight the DA and PCV (Figure 6.10C). At most, only the anterior extremities of the DA and PCV were highlighted (Figure 6.10D). This was in contrast to the normal blood flow within the developing head circuitry, including the central artery (CtA), the PHBC, the basilar artery (BA) and the ventral aorta (VA) (Figure 6.10C). This lack of caudal circulation down the DA resulted in the accumulation of blood cells in the anterior trunk, in the vicinity of the anterior hypochord, clearly observable under light microscope. Furthermore, blood cells were stranded in the developing tail. Microangiography also confirmed the lack of, or reduction in,
Figure 6.10 Targeted knock-down of Vegf-A signalling results in specific circulatory defects as revealed by microangiography. (A) Microangiogram of axial circulation throughout the trunk of a 50 hpf wild-type zebrafish embryo (anterior to left). (B) Microangiogram of circulation throughout the tail of a 50 hpf stage wild-type zebrafish embryo (anterior to left). (C-H) Microangiograms of 50 hpf zebrafish embryos, that have received a 9 ng dose of vegf-A MO, displaying varying degrees of perturbed axial circulation (all lateral views, anterior to left). (C) The most severe phenotype resulted in the complete loss of axial circulation while cranial circulation was unaffected. (D) Axial vasculature was sometimes highlighted (DA and PCV), however it did not extend the length of the trunk. (E-H) Less severe phenotypes included a reduction in intersegmental circulation (asterix in E), vascular leaks (arrowhead in F) and a lack of PCV circulation (G and H). Whole mount in situ hybridisation staining to detect flk-1 transcripts in 27 hpf wild-type (I) and vegf-A MO-injected (J) embryos. (J) vegf MO-injected embryos often displayed an absence of intersegmental flk-1 staining. DA = Dorsal aorta, PCV = Posterior cardinal vein, Se = Intersegmental vessels, CA = Caudal artery, CV = Caudal vein, CtA = Central artery, PHBC = Primordial hindbrain channel, VA = Ventral aorta, CCV = Common cardinal vein, BA = Basilar artery. Scale bars in A/B and I represent 200 μm and 250 μm, respectively.
intersegmental blood flow (Figure 6.10E to H). In those vegf-A morphant embryos that demonstrated the most subtle phenotype, only a slight reduction in circulation through the intersegmental vessels was detected (Figure 6.10E). In such embryos, gaps were commonly observed in an apparent random fashion, with some intersegmental vessels capable of carrying blood cells while others could not (Figure 6.10E). In slightly more severe morphants, there was a complete absence of circulation through these minor trunk vessels (Figure 6.10F, G and H). Some of these embryos also lacked rostral blood flow through the PCV, with the injected fluorescent beads only highlighting the DA and CA vasculature of the trunk (Figure 6.10G and H). The haemorrhages that were observed under light microscope in vegf-A morphants were also confirmed through microangiographic analysis, with the injected beads escaping from the axial vasculature (Figure 6.10F).

The lack of blood flow through the intersegmental vessels was supported by whole mount in situ hybridisation analysis for the endothelial-specific marker flk-1 within the vegf-A morphant background. In vegf-A morphants, flk-1-expressing cells constituted the DA, CA, CV and PCV (albeit reduced) but not the intersegmental vessels (Figure 6.10J).

6.4.2 Cumulative action of Radar and Vegf-A knock-down on axial vascular development

To explore a potential interaction between Radar and Vegf-A in the establishment of functional blood vessels in the trunk, synergy experiments were performed. This required the coinjection of sub-optimal doses of radar- and vegf-A-targeting MOs. Coinjected embryos were then monitored at 30 hpf for vascular defects which were compared to those elicited by single injections of each MO alone.

Sub-optimal doses were determined as being 6 ng for both MOs. A 6 ng dose of rad-MO1 and vegf-A MO resulted in only 14 ± 1% (2 experiments; n = 149) and 14 ± 3% (2 experiments; n = 224) of injected embryos that lacked trunk circulation at 30 hpf (Figure 6.11). However, when 6 ng doses of both MOs were coinjected, 96 ± 2% (2 experiments; n
Figure 6.11 Simultaneous knock-down of Radar and Vegf-A function results in a synergistic effect on axial vascular development. Graph representing the frequency of embryos with or without axial circulation in the DA and PCV, at the 30 hpf stage, that have received sub-optimal doses of radar- and vegf-A-targeting MOs alone, or combined doses.
of injected embryos lacked circulation through the trunk (Figure 6.11). Interestingly, almost all of these embryos possessed an enhanced version of the extreme vegf-A morphant phenotype, characterised by a lack of trunk circulation, pericardial oedema (compare Figure 6.12B with C) and the accumulation of blood in the vicinity of the anterior hypochord and developing tail (compare Figure 6.12B with C and E). The only morphologically noticeable difference between the severe vegf-A and vegf-Atlradar morphants was a minor inhibition in eye development in the double knock-downs, a feature of some radar morphants (Figure 6.12C).

6.5 Alk-6 as a candidate target of Radar signalling

The early ventralising activity of Radar has been proposed to act through an Alk-6/BMPR-related mechanism (Goutel et al., 2000). This is based on the inhibition of Radar-induced bmp2b expression in the zebrafish blastula by injecting a truncated version of the Alk-6 receptor (Goutel et al., 2000). The only previous description of alk-6 expression in the zebrafish trunk was as somitic expression (Nikaido et al., 1999). To visualise the expression pattern of alk-6 in the trunk of zebrafish embryos, in particular its proximity to radar expressed by the hypochord and PGE, a PCR product was generated from a 24 hpf zebrafish cDNA library that served as a template for antisense riboprobe synthesis.

6.5.1 Expression of alk-6 in 24 hpf stage zebrafish embryos

To generate a first strand 24 hpf cDNA library, total RNA was extracted from approximately 100 24 hpf stage zebrafish embryos and first strand cDNA generated using oligo (dT) primers and reverse transcriptase. The cDNA was then amplified using the primers ZFALK6U1 and ZKALK6L1. These primers were designed to generate a 1.22 kb amplification product that spanned from bases +40 to +1259 (Genbank accession number AB020758). The cycling conditions were 1 minute at 94°C, 1 minute at 55°C and 1.5
Figure 6.12 Cumulative effects following the coinjection of vegf-A- and radartargeting MOs. (A) lateral view (anterior to left) of a 50 hpf wild-type embryo. (B) Lateral view of a 50 hpf embryo injected with a 6 ng dose of vegf-A MO displaying a lack of trunk circulation, pericardial oedema (arrow) and an accumulation of blood at the anterior hypochord (arrowhead). (C) Lateral view of a 50 hpf embryo that has received a 6 ng dose of vegf-A MO and a 6 ng dose of rad-MO1 displaying a similar lack of circulation, pericardial oedema (black arrow) and a more severe anterior hypochord blood pool (black arrowhead). White arrow in C denotes aberrant eye development. Panels D and E are the higher power lateral views of the cranio-trunk regions of embryos in panels A and C, respectively. Scale bars in A and D represent 250 μm and 200 μm, respectively.
minutes at 72°C for 30 cycles. The PCR product was then resolved on a 1% agarose low melt gel and isolated using the GENECLEAN kit (Bio 101) in accordance with the manufacturer’s instructions. The partial \textit{alk-6} cDNA was then ligated into the pGEM-T rapid ligation vector (Promega) before being orientated through restriction digest analysis. Once the orientation of the clone within the vector was established, it was linearised (using the restriction enzyme Apal) and employed as a template for riboprobe synthesis using SP6 RNA polymerase (Promega). Whole mount \textit{in situ} hybridisation analysis was then conducted on 24 hpf wild-type zebrafish embryos.

Transcripts for \textit{alk-6} were located throughout the 24 hpf stage zebrafish embryo, in the cerebellum, otic vesicle (Figure 6.13A) and throughout the somites in a caudal to rostral expression gradient (Figure 6.13A, B and C).

**III. Summary**

A Radar-specific loss-of-function model was generated by the early delivery of radar-targeting MOs into zebrafish embryos. The specificity of this knock-down model was confirmed by employing the following controls: 1) the use of two non-overlapping MOs to target radar transcripts; 2) phenotype rescue using Radar-encoding capped synthetic transcripts and cDNAs; 3) a 4-base mismatch control MO; and 4) a negative control MO.

The radar morphant phenocopied neurodegenerative aspects of the \textit{rdt}^{31} radar deletion mutant. Apoptotic activity was observed in the developing head, a dorsal population of cells in the neural tube, and in the developing tail bud of 18 to 24 somite-stage radar morphant embryos.

Microangiographic analysis of 2 dpf radar morphants revealed extensive vascular leakage from the axial blood vessels. Circulation commenced normally in radar morphants with blood cells passing through the DA and PCV and the caudal vasculature (CA and CV plexus). The onset of vascular leakage was first detected at around 35 hpf, from which
Figure 6.13 Expression of alk-6 in 24 hpf stage zebrafish embryos. (A) Lateral view of a whole mount in situ hybridisation staining for alk-6 expression in a 24 hpf wild-type embryo. Transcripts are detected throughout the trunk in the somites (large arrow), in the otic vesicle (arrowhead) and in the cerebellum (small arrow). (B) Higher power view of the trunk region of embryo in panel A, showing alk-6 expression in the trunk somites. (C) Dorsal view of panel B demonstrating somitic alk-6 expression (arrows) either side of the medially located notochord and neural tube. Scale bars in A and B represent 250 μm.
point haemorrhages developed and grew to eventually block circulation to the caudal extremities of the zebrafish trunk. In such radar morphants, circulating blood cells short-circuited from the DA to the underlying PCV in the trunk and returned prematurely to the heart. Radar-depleted embryos demonstrated typical expression of flk-1, fli1, tie2 and ang1 by whole mount in situ hybridisation analysis, revealing a normal looking primitive vascular morphology.

_Radar_ morphants displayed typical expression of the blood cell-specific gene \(\beta E_5\)-globin. Morphants possessed blood cells in a typical pattern, restricted to and highlighting the ICM compartment of pre-circulation embryos. Furthermore, in post-circulation radar morphants, these \(\beta E_5\)-globin-expressing cells were demonstrated to contribute to an initially typical circulation in the anterior (LDA, CCV and ACV) and posterior (DA, PCV, CA and CV plexus) blood vessels.

To compare and contrast phenotypes resulting from the knock-down of Radar and Vegf-A signalling, a _vegf-A_-targeting MO was used to generate a zebrafish Vegf-A loss-of-function model. The early delivery of _vegf-A_ MOs into zebrafish embryos resulted in vascular deficiencies characterised by a complete lack of trunk circulation, as detected by microangiography, following 2 days development. In a more subtle phenotype, axial circulation within the major trunk vessels (DA and PCV) was normal despite a graded reduction in circulation through the minor trunk intersegmental vessels. Whole mount in situ hybridisation analysis of the vascular endothelial marker flk-1, within Vegf-A-depleted embryos, demonstrated a lack of expression in the intersegmental vasculature.

Coinjection of sub-optimal doses of both radar- and _vegf-A_-targeting MOs into zebrafish embryos resulted in a synergistic disruption to axial vascular development. Coinjected embryos displayed a significant increase in the most severe class of _vegf-A_ morphant phenotype, typified by an accumulation of blood cells in the vicinity of the anterior hypochord, developing tail and a complete lack of trunk circulation.
Whole mount *in situ* hybridisation analysis for the type I BMP receptor, *alk-6/bmpr-1b* was conducted on wild-type 24 hpf embryos. Transcripts for *alk-6* were detected anteriorly, in the cerebellum and otic vesicle and throughout the trunk in the somites.
Chapter 7

Discussion

I. The Radar signal is well positioned, both spatially and temporally, to influence vascular events during zebrafish embryogenesis

7.1 Mesodermal radar-expressing cells border early progenitors of the ICM compartment

In the early zebrafish embryo, the lateral mesoderm of the developing trunk and tail gives rise to three different types of tissues, the haematopoietic, vascular and pronephric lineages (Gering et al., 1998; Davidson and Zon, 2000; Horsfield et al., 2002). The spatial location of these progenitors within the lateral mesoderm is tightly regulated, with cells destined to contribute to both the haematopoietic and vascular lineages, termed the haemangioblast, restricted to more medial regions of the lateral mesoderm. Transcripts for radar were detected, by whole mount in situ hybridisation, within the lateral plate mesoderm compartment. This domain of expression commenced from the 5 somite (11.5 hpf) stage and persisted until the 12 somite (15 hpf) stage of development. This transient radar expression was demonstrated to be mesodermal, occupying a more ventro-lateral position relative to a neighbouring domain of radar expression in the more dorso-medial ectoderm. Although radar expression within this mesodermal compartment was too weak
to be detected by cross-sectioning, optical cross-sections were adequate to position these bilateral stripes of radar-expressing cells within the mesoderm.

Double whole mount in situ hybridisation analysis demonstrated that this posterior mesodermal radar expression was more lateral than that for the haematopoietic/vascular gene, scl. Apart from some superficial overlap of expression at the posterior-most extremities, radar expression was clearly more lateral compared to scl (Figure 7.1A). However, radar closely bordered the entire posterior mesodermal expression domain of scl. The scl gene is expressed by progenitors of the zebrafish ICM compartment (Gering et al., 1998) along with a number of other genes critical for blood and/or vascular development, including gata-1 (Long et al., 1997), gata-2 (Thompson et al., 1998), fli4 (Thompson et al., 1998), fli1 (Brown et al., 2000), c-myb (Thompson et al., 1998), runx1 (Kalev-Zylinska et al., 2002), βE-globin (Amemiya et al., 1998), hhex (Liao et al., 2000b), flk-1 (Thompson et al., 1998) and lmo2 (Thompson et al., 1998).

The function that Radar performs from this mesodermal compartment is unclear. Although radar expression within this region is only transient and does not significantly overlap with expression domains of genes that highlight ICM progenitors, it does encode a signalling protein that is likely to influence neighbouring cells in a paracrine fashion. Indeed, many BMPs are believed to function by establishing activity gradients that can regulate activity based upon the position of a cell within the gradient and that cells threshold for the particular signal. In the Xenopus and zebrafish gastrula, a ventral to dorsal gradient of BMP activity is believed to specify cell fate (Piccolo et al., 1996; Jones et al., 1998; Myers et al., 2002; Wagner and Mullins, 2002). By studying convergence and extension movements in dorsalised and ventralised zebrafish mutants, a model has been developed in which high BMP activity can specify ventral regions of the early embryo that possess restricted convergence and extension movements. This is in contrast to more lateral embryonic domains that receive limited BMP activity resulting in the specification of regions that possess elevated levels of convergence and extension (Myers et al., 2002).
**Figure 7.1** Diagrammatic representations of *radar* expression domains throughout early embryogenesis relative to sites of active vascular development. (A) Diagrammatic representation of a cross-section through the posterior region of a 6 somite (12 hpf) stage embryo, *radar*-expressing posterior lateral mesoderm is highlighted in red. (B) Diagrammatic representation of a cross-section through the posterior region of a 14 somite (16 hpf) stage embryo, *radar*-expressing endoderm is highlighted in pink. (C) Diagrammatic representation of a cross-section through the trunk of a 26 somite (22 hpf) stage embryo. Expression of *radar* in the PGE and hypochord is highlighted in pink. D = Dorsal, V = Ventral.
**A**  
6 somites (12 hpf)

- Neural tube
- Notochord
- Paraxial mesoderm
- scl +ve mesoderm
- radar +ve mesoderm

Lateral mesoderm (LM)

**B**  
14 somites (16 hpf)

- radar +ve endoderm

**C**  
26 somites (22 hpf)

- Primitive axial vasculature (DA & PCV)
Alternatively, radar expressed from this mesodermal region may act in an autocrine fashion on the same cells that secrete the signal. Given the more lateral position of radar relative to scl, it is possible that these cells represent pronephric mesoderm that are destined to contribute to the zebrafish kidney (Gering et al., 1998; Horsfield et al., 2002). We believe that such an autocrine activity is unlikely given that targeted knock-down of the Radar signal did not result in impaired pronephric development. In radar morphants, transverse sections through the developing trunk revealed morphologically normal looking pronephric ducts following 2 days development (data not shown). However, the possibility still exists that Radar may perform a non-essential or redundant role in these lateral mesodermal cells.

### 7.2 Expression of radar during segmentation stages shadows the formation of the axial vasculature

From the 9 somite stage of development the hypochord exists as a distinct dorso-medial population of endodermal cells in the developing trunk of the zebrafish. Around the 15 to 16 somite stage this population of cells delaminates from the underlying PGE and migrates dorsally, along with the notochord, as a single cell layer (Eriksson and Lofberg, 2000). It is this migration that creates a gap to which haematopoietic and vascular precursors converge, eventually coalescing to form the ICM compartment. Within the ICM, the haematopoietic and vascular lineages are specified to establish a functional axial vascular circuit carrying primitive blood cells throughout the trunk.

Both the hypochord and the PGE strongly express radar during this migration of ICM progenitors and the subsequent differentiation of the blood and vascular lineages (Figure 7.1B and C). Double whole mount in situ hybridisation analysis of radar and scl expression in zebrafish embryos during somitogenesis clearly displayed scl-expressing ICM progenitor cells sandwiched dorsally and ventrally between domains of radar expression. This expression of radar in the hypochord and gut endoderm continued during
the differentiation and assembly of the DA and PCV, respectively. Although somewhat reduced, expression persisted in these tissues until the 36 hpf stage (latest time point tested), spanning both vasculogenic and angiogenic events. This intimate relationship between vascular development and domains of \textit{radar} expression was not restricted to the trunk. The developing tail also possessed \textit{radar} expression that bordered the specification and formation of the caudal circuitry, the CA and CV plexus. Expression of \textit{radar} in the posterior hypochord is immediately dorsal to the developing CA, while expression in the posterior ICM/VTM region is intimately associated with the CV honeycomb-like plexus. Double whole mount \textit{in situ} hybridisation analysis of \textit{radar} and \textit{scl} within the posterior ICM compartment revealed that both are co-localised within a subset of cells in this region. These cells may represent posterior ICM angioblasts destined to form the CA and/or CV plexus. This pattern of \textit{radar} expression throughout the ventral trunk and tail closely borders the formation of all of the major axial blood vessels, the DA, CA, CV and PCV (Figure 7.2A and B).

A number of lines of evidence implicate both the hypochord and the PGE in the construction of a functional axial vascular circuit. The hypochord has been demonstrated to express a number of genes including \textit{vegf} (in \textit{Xenopus} and zebrafish) and \textit{angl} (in zebrafish), two potent modulators of axial vascular development (Cleaver and Krieg, 1998; Eriksson and Lofberg, 2000; Pham \textit{et al.}, 2001; Lawson \textit{et al.}, 2002). Tissue ablation experiments in \textit{Xenopus}, and reciprocal gain-of-function studies, have revealed that the genesis of the hypochord is dependent upon signals secreted from the immediately dorsal notochord (Cleaver \textit{et al.}, 2000). In the zebrafish midline mutants \textit{floating head} (flh), \textit{squint} (sqt) and \textit{no tail} (ntl), that lack a completely functional notochord and subsequently a functional hypochord, although angioblasts expressing the ETS-domain transcription factor \textit{flil} and the receptor tyrosine kinase \textit{flk-1} migrate to the midline, a typical DA does not form (Fouquet \textit{et al.}, 1997; Sumoy \textit{et al.}, 1997; Brown \textit{et al.}, 2000). Similar analysis of the \textit{one-eyed-pinhead} (\textit{oep}) zebrafish mutant, that lacks posterior endoderm, demonstrated that signals from this region are not required for angioblast specification but are essential for the
Figure 7.2 Diagrammatic representation of the intimate relationship between radar expression and axial blood vessels throughout the trunk of the zebrafish embryo. (A) Lateral view of a 25 hpf stage zebrafish embryo displaying the axial vasculature (DA, CA, CV and PCV) relative to radar expression in the hypochord, PGE and VTM. (B) Lateral view of the trunk (anterior to left) of a flt1:EGFP transgenic zebrafish, displaying EGFP-expressing endothelial cells of the DA, PCV and intersegmental vessels along with superimposed radar-expressing domains (overlying hypochord and underlying gut endoderm). Se = Intersegmental vessels, DA = Dorsal aorta, PCV = Posterior cardinal vein. Graphics for B adapted from Weinstein, 2002.
assembly of a typical PCV (Brown et al., 2000). Endoderm ablation experiments in Xenopus confirm this endothelial assembly function. In endoderm-depleted embryos, serial sections throughout the trunk revealed that angioblasts assembled into dense cord-like aggregates but failed to form tubular blood vessels (Vokes and Krieg, 2002).

Taken together these studies highlight a role for the zebrafish hypochord and PGE in the formation of a functional DA and PCV, respectively. The signalling BMP radar represents an example of a gene expressed by both of these tissues. Given the intimate relationship between radar expression in the hypochord, endoderm and VTM and the developing trunk and tail vasculature, axial endothelial cells that constitute the DA, CA, CV and PCV represent an ideal, and likely, target of Radar activity. Another gene expressed in both of these domains is neuropilin-1, a cell-surface receptor required for Vegf-dependent angiogenesis (Lee et al., 2002).

It is interesting to note that the hypochord is a structure exclusive to fishes and amphibians (Lofberg and Collazo, 1997). It is not clear whether an equivalent structure exists in the embryos of other vertebrates. If such a structure does not exists in other vertebrates, what tissue plays an analogous role in organising the assembly of the DA? It is quite possible that completely different tissue types function in the assembly of the axial vasculature in different organisms. In amniote embryos, a structure similar to the epibranchial groove in Amphioxus is present at the midline and may participate in the formation of the embryonic DA (Cleaver and Krieg, 1998). However, the functional conservation displayed by a number of gene products during the assembly of the embryonic vasculature (reviewed in Chapter 1), in a number of organisms, would suggest that the signals and mechanisms underlying these processes are highly conserved.
II. An early ventralising activity for the *radar* gene product

7.3 Transient forced expression analysis confirms an early ventralising activity for the Radar signal

Transient overexpression analysis of *radar* confirmed an early ventralising activity for this pleiotropic gene. Embryos injected with varying doses of a Radar-encoding constitutive expression cassette displayed a dose-dependent commitment of dorsal mesoderm to more ventral fates. To visualise the dose-dependency of this Radar-induced ventralisation phenotype, in real-time, an EGFP construct driven by the same constitutive promoter was coinjected with *radar*. Only EGFP-expressing embryos became ventralised and there was a tight correlation between the degree of expression and the severity of the ventralisation phenotype, validating the reporting strategy. Embryos with low level EGFP expression only exhibited a modest ventralisation, characterised by a slight reduction in anterior head structures, a reduction in notochord mesoderm and a slight expansion of the posterior ICM compartment (a ventral mesoderm derivative). In contrast, those coinjected embryos that demonstrated expansive EGFP expression lacked a notochord and possessed fused somites, a complete lack of anterior head structures and a significant expansion of the haematopoietic/vasculogenic compartment. Early delivery of a heat-inducible Radar-encoding construct into zebrafish embryos demonstrated that this ventralising activity could still be induced during gastrulation movements. This is consistent with previous reports regarding *radar*'s ventralising activity (Goutel *et al.*, 2000). It has been suggested that a maternal *radar* transcript is involved in this regulation of early ventral patterning through an Alk-6/BMP receptor-related mechanism during gastrulation (Goutel *et al.*, 2000). This activity appears to be specific given that similar ectopic expression of *radar*'s closest
relative dynamo, both of which are gene duplicates of murine Gdf6 (Davidson et al., 1999), does not induce such a ventralised phenotype (Goutel et al., 2000).

Interestingly, morpholino-mediated depletion of the Radar signal did not result in dorsalisation. This suggests that although Radar may possess a ventralising activity when overexpressed, the endogenous levels of Radar are not required for the formation and patterning of ventral mesoderm. In support of this, a radar deletion mutant, rdr\textsuperscript{D1}, that possesses a 27.4 cM deletion encompassing the radar locus, is not characterised as possessing a dorsalised phenotype (Delot et al., 1999). Alternatively, redundancy may exist between radar and one or more of the closely related genes, bmp2b, 4 or 7, all of which similarly result in ventralisation when ectopically expressed (Neave et al., 1997; Goutel et al., 2000; Schmid et al., 2000). The overriding masking effects of radar’s early ventralising activity prevented any insightful conclusions to be drawn from these transient forced expression experiments regarding a function for radar expressed in the hypochord and PGE.

To circumvent this early activity, a transgenic line, carrying an inducible EGFP-tagged radar transgene, was generated to facilitate the temporal control of ectopic radar delivery. Transient experiments demonstrated the ability of the hsp70 promoter/enhancer element to drive heat-inducible expression of both EGFP and P2RAD in the zebrafish embryo. Generating a transgenic line carrying a stably integrated hsp70:radar transgene would facilitate forced radar expression following gastrulation, thereby bypassing Radar’s ventralising activity. Such an analysis would provide insight into the function(s) Radar performs during somitogenesis, in particular, those functions transduced from sites that flank the developing axial vasculature.
III. A late angiogenic role for the *radar* gene product

7.4 Transgenic zebrafish carrying an inducible copy of the *radar* gene develop a phenotype consistent with an angiogenic role for the Radar signal

Transgenic embryos were generated by microinjection of an EGFP-tagged, *hsp70* promoter-driven, *radar* expression cassette. Three founder fish were identified that were capable of transmitting the transgene through their germ-line to their progeny (founders 56, 85 and 318). These three fish represented approximately 1% of injected embryos that survived to sexual maturity and were screened. This frequency is in accordance with that reported in a previous microinjection-based transgenesis study that employed the zebrafish *hsp70* promoter (Halloran et al., 2000). The genomes of 2 of these 3 fish were secured within F₁ populations. RT-PCR analysis confirmed that the transgene was expressed by both of the F₁ lines generated from transgenic founder 85. The transgene was robustly expressed immediately following heat-induction treatment, after which transcripts were rapidly degraded by *in vivo* RNases. There was severe mosaicism in the germ-line of both transgenic founders 85 and 318, which transmitted the transgene to approximately 1.3 and 0.8% of their progeny, respectively.

Severe germ-line mosaicism is a well documented problem with microinjection-based transgenesis in the zebrafish (Bayer and Campos-Ortega, 1992; Lin et al., 1994; Gaiano et al., 1996; Long et al., 1997; Jowett et al., 1999; Meng et al., 1999; Halloran et al., 2000). Interestingly, leakage from the *hsp70* promoter continued even after being stably integrated within the zebrafish genome. Furthermore, this promiscuous promoter activity could not be controlled by raising the fish at lower temperatures. This was not expected given the tight regulatory control demonstrated by this promoter when used to generate an *hsp70:EGFP* transgenic line (Halloran et al., 2000). The transgenic F₁ fish that were
generated were demonstrated to be hemizygous by the Mendelian inheritance that the transgene displayed in their progeny from an outcross, as assayed for by PCR-based genotyping. In total, 48% \( (n = 48) \) carried the transgene in their genome, the expected Mendelian ratio was 50%.

Heat-induction treatments performed on hemizygous transgenics failed to elicit any aberrant phenotype or EGFP expression. This was interesting given that the transgene had the potential to induce EGFP expression and ventralisation in early zebrafish embryos, as demonstrated by a transient analysis of the transgene. This would suggest that the EGFP can fold properly when expressed, and that the incorporation of the EGFP tag into the pro-domain of Radar did not interfere with Radar function. However, it was noted that a high dose of the transgene (150 pg/embryo) was required to elicit ventralisation during this transient analysis. This may suggest that these modifications could have slightly reduced Radar activity.

When the progeny from two hemizygous transgenic fish (generated from \( F_1 \) line 85, from founder 85) were observed throughout early development, approximately 23 ± 2% \( (n = 1,179; \) 6 separate experiments) demonstrated a highly specific and reproducible phenotype. Embryos from such a cross would be expected to contain 25% wild-type, 50% hemizygous and 25% homozygous genotypes, assuming Mendelian inheritance. It was interesting that hemizygous embryos from \( F_1 \) line 62 (from founder 85) failed to produce this phenotype in their offspring. This may be a result of epigenetic DNA methylation of the transgene leading to a silencing of expression (Collas, 1998; Attwood et al., 2002). Given that the hemizygotes from previous experiments failed to develop any defects, we assume the 23 ± 2% to represent homozygous transgenic embryos. Indeed, 12 randomly selected embryos that displayed the phenotype were genotyped by PCR and characterised as carrying the transgene. Hence, the phenotype was strongly linked with the presence of the transgene. Embryos that possessed the phenotype were indistinguishable from their putative hemizygous and wild-type siblings during early development. It was not until approximately 1.5 dpf that the putative homozygotes displayed laboured circulation
through their axial vasculature. This circulation became progressively restricted until, at 2 dpf, no blood cells were observed to travel throughout the trunk, and the developing head. These embryos then became progressively oedematous in the pericardium and yolk sac. Blood cells were often visible in the beating heart, but with restricted movement only between the heart chambers. Other embryos that possessed this lack of whole-body circulation possessed blood pools in the anterior region of the hypochord and the developing tail. Given that these embryos initially demonstrated a normal primitive circulation, one would assume that they possess a typical vasculogenic program. At 1.5 dpf, when the circulatory defect is first detectable, the trunk vasculature is undergoing angiogenic remodelling (Isogai et al., 2001; Childs et al., 2002). This would imply that the defect responsible for the aberrant circulation is due to an interference with the angiogenic program. Further research is required to pinpoint exactly how this ectopic Radar signal elicits this phenotype. Interestingly, this defect was observed in approximately 25% of induced and non-induced sibling embryos. This suggests that the phenotype is due to leakage of radar expression from the hsp70 promoter rather than an induction of expression. Sporadic constitutive expression of the hsp70 gene has been reported in zebrafish under non-stress conditions (Blechinger et al., 2002), suggesting that the hsp70 promoter may possess a basal activity. However, this contradicts the tight regulatory control previously reported for this promoter in a zebrafish system (Halloran et al., 2000).

One question that is raised by this leaky promoter-driven phenotype is, why was no ventralisation induced? A possible explanation may lie in the fact that Radar is a BMP signalling molecule. Many BMPs are known to signal by the establishment of an activity gradient, and hence the activity is dependent upon the dosage of the signal (Barth et al., 1999; Hild et al., 2000). The leaky radar expression in these putative homozygous embryos may be below the threshold required to induce ventralisation but adequate to elicit the later circulation defect. Indeed, such a view is supported by the lack of any phenotype observed from the hemizygous transgenics, that demonstrated transgene expression via RT-PCR and would theoretically possess half of the expression levels demonstrated by the homozygotes.
7.5 Radar is required for the establishment of axial vascular integrity in the zebrafish

Morpholino-mediated Radar knock-down experiments highlighted a critical role for the Radar signal in establishing the integrity of the major axial vessels, the DA and PCV, during zebrafish embryogenesis. Interestingly, this was despite an apparently normal looking early haematopoietic and vascular program. To visualise circulatory defects we employed microangiography and observed that the vascular integrity throughout the developing trunk was compromised in 50 hpf embryos that been injected with radar-targeting MOs. The outstanding defect in Radar-depleted embryos was the inability to sustain blood flow in the trunk following the commencement of a normal circulation. To analyse the developing vasculature in the radar morphant background, whole mount in situ hybridisation analysis was conducted to detect the expression of genes known to be involved during the assembly of vertebrate blood vessels. These genes included the endothelial-specific flk-1 (Liao et al., 1997), flil (Brown et al., 2000) and tie2 (Lyons et al., 1998), as well as angl (Pham et al., 2001), expressed by the hypochord. Targeted disruptions to these genes in the mouse have demonstrated critical roles for each in the assembly of a mature, functional vascular system. Mice engineered to be deficient in functional Flk-1 die due to a complete lack of vascular structures and haematopoietic precursors, this suggests a role in endothelial and haematopoietic cell development, perhaps at the level of the haemangioblast (Shalaby et al., 1995). Ang1-deficient mice also die due to defective vascular development. However, in these mice a primary vascular system does form but is not remodelled by angiogenic events, specifically the recruitment of periendothelial support cells (Suri et al., 1996). Not surprisingly, a similar phenotype results from inactivation of the Ang1 receptor, Tie2 (Sato et al., 1995). Targeted inactivation of the transcription factor Flil results in impaired haematopoiesis and haemorrhaging from the DA (Spyropoulos et al., 2000). In radar morphant embryos the expression patterns of each of these genes was unaltered, suggesting that the initial
establishment of vascular patterning is unperturbed. Indeed, the endothelial-specific markers flk-1 and flil highlighted a morphologically normal looking DA, PCV, CA and CV plexus as well as the intersegmental blood vessels. These typical expression patterns, highlighting a normal looking primitive vascular system, would be expected given that Radar-depleted embryos initiate a normal circulation.

While vascular instability of the major axial vessels was obvious when detected by microangiography, only absence or a reduction in intersegmental circulation was noted in the majority of morphants. This perturbed blood flow through these minor trunk vessels may not be due to a specific role for Radar in their angiogenic modelling. When leakage occurred towards the anterior region of the trunk, no blood travelled caudally through the DA. As it is this circulation that feeds the intersegmental vasculature, it is possible that the absence of intersegmental blood flow is an indirect effect of insufficient circulation through the DA.

It is interesting that although transcripts for radar are detected by in situ hybridisation as early as 12 hpf at sites that border vascular events, a loss-of-function circulatory phenotype was not observed until approximately 1.5 days development. A potential explanation for this may be functional redundancy between Radar and other related TGF-β superfamily members such as Dynamo/Gdf6b, which is also expressed by the hypochord during zebrafish somitogenesis (Bruneau and Rosa, 1997). Phylogenetic analysis of the TGF-β superfamily suggests that both radar and dynamo represent orthologues of murine Gdf6, generated from a genome duplication event that occurred during zebrafish evolution (Davidson et al., 1999). Hence, it is plausible that Dynamo may compensate for aspects of Radar function in its absence. Alternatively, and more likely, Radar may function early to induce gene products required to establish endothelial stability, but the phenotype arising from the absence of these proteins does not manifest until later in development. For example, the structural integrity of the axial vasculature may only be compromised in response to an increase in blood pressure and its associated fluid shear stress that occurs during the early larval stage. Fluid shear stress is believed to play a role in
influencing genetic pathways that control endothelial morphogenesis and function during embryonic development (Wilson et al., 1993; Ohno et al., 1995; Resnick and Gimbrone, 1995; Wilson et al., 1995; Owens et al., 1996; Reusch et al., 1996; Skalak and Price, 1996; Resnick et al., 1997; Hungerford and Little, 1999).

This phenomenon of early expression and late effect is not uncommon with regards to blood vessel development. For example, much like radar, neuropilin-1 is expressed early in the lateral mesoderm, then later in the PGE, hypochord and posterior ICM compartment (Lee et al., 2002). However, Neuropilin-1 function does not seem to be required until angiogenic sprouting of the intersegmental blood vessels (Lee et al., 2002). It is quite possible that genetic programs that govern later developmental events are initiated quite early during embryogenesis.

Radar is not the first example of a TGF-β superfamily member believed to function in embryonic vascular assembly. TGF-β1 possesses a number of functions relating to vascular development, including the regulation of VSMC differentiation (Grainger et al., 1998) and the modulation of endothelial-mural cell interactions (Dickson et al., 1995; Carmeliet, 2000). Further evidence for the involvement of the TGF-β signalling cascade in vascular development comes from targeted inactivation of the genes encoding TGF-βRII (Oshima et al., 1996), endoglin (Li et al., 1999), Alk-1 (Oh et al., 2000; Urness et al., 2000) and the intracellular signalling molecule Smad5 (Chang et al., 1999). The outstanding defect demonstrated by each of these mutants relates to the recruitment and/or differentiation of VSMCs during vascular development, despite normal differentiation of endothelial cells and normal early vasculogenic events. These phenotypes are reminiscent of those displayed by Radar-depleted embryos, suggesting that Radar may signal through a pathway common to one, or more, of the zebrafish orthologues of the aforementioned genes.

If Radar does function in the recruitment of periendothelial support cells (such as VSMCs) then one would expect a complementary phenotype from the transgenic radar knock-in model. It would seem that the knock-in phenotype is due to a vascular
maturation/angiogenic defect as circulation commences normally. This complements the Radar knock-down phenotype. Hence, both phenotypes are likely to be due to an interference in the same vascular developmental event. If this event is the recruitment of VSMCs, the knock-in phenotype could be explained by an over-recruitment of VSMCs around the vasculature which may lead to a progressive constriction of trunk blood flow. However, a similar scenario in another model system has not been documented.

Despite the fact that a number of genes believed to be involved in angiogenic maturation of the vertebrate vasculature have yet to be characterised in the zebrafish, genetic screens in this model system have revealed a number of vascular integrity mutants. These mutants are characterised by the genesis of localised haemorrhages following the initiation of circulation (Stainier et al., 1996). Such integrity mutants include bubble head (BBH), leaky heart (leh), mush for brains (mfb), migrane (mig) (Stainier et al., 1996) and gridlock (gdl) (Weinstein et al., 1995; Zhong et al., 2000). The shared vascular phenotypes between these mutants and the radar morphant may represent shared signalling pathways necessary during vascular development.

In summary, Radar signalling appears to be essential for establishing the integrity of the zebrafish axial vasculature. It appears most feasible that this activity is via Radar signalling from the hypochord, PGE and VTM due to the intimate spatio-temporal relationship between these expression domains and the developing trunk vasculature. In addition, a number of studies have implicated these tissues in the assembly of a functional vascular system in the zebrafish and Xenopus trunk (Lofberg and Collazo, 1997; Brown et al., 2000; Cleaver et al., 2000; Eriksson and Lofberg, 2000; Pham et al., 2001; Vokes and Krieg, 2002). It is noted though, that the early radar expression in the lateral mesoderm could possibly play a part in this role. Indeed, a subset of VSMCs are believed to differentiate from an, as yet, unidentified population of mesodermal cells (Hungerford and Little, 1999). However, given the lack of any published VSMC marker in the zebrafish, an analysis of their differentiation and development within the radar morphant background was not possible.
7.6 Could Radar function in a laminar shear stress-induced signalling cascade?

In the early embryo, the initial primitive vascular circuit is established via vasculogenesis in the absence of any blood flow and associated blood pressure. However, subsequent angiogenic remodelling of this primary vascular network and further vascularisation of the developing embryo via angiogenic sprouting, splitting and elongation is established in the presence of blood flow and pressure (Hungerford and Little, 1999). The relationship between this haemodynamic stress and the assembly of a multilayered mature vascular system has lead to a model by which changes in haemodynamics within the developing vasculature play a role in the maturation of vessel wall cells (Wilson et al., 1993; Ohno et al., 1995; Resnick and Gimbrone, 1995; Wilson et al., 1995; Owens et al., 1996; Reusch et al., 1996; Skalak and Price, 1996; Resnick et al., 1997; Hungerford and Little, 1999). Probably the most interesting aspect of the radar morphant phenotype, and indeed the knock-in phenotype, was that the circulatory defect was observed following the commencement of circulation. Indeed, blood flowed throughout the primitive vascular circuitry for a number of hours before any evidence of a circulatory defect was detected. This raises the possibility that the radar gene product may participate in a regulatory cascade that is involved in establishing and/or maintaining haemodynamic-dependent maturation of the axial blood vessels in the zebrafish.

The VSMC represents a good example of how changes in the local microenvironment, such as an increase in mechanical strain, can influence genetic programs and ultimately cell phenotype. The smooth muscle cell is not terminally differentiated and can undergo significant changes in phenotype in response to changes in the local microenvironment (Owens et al., 1996). It is these cues that typically govern its differentiation and maturation. An in vitro study investigating the effects of cyclic mechanical strain on rat aortic smooth muscle cells, cultured on silicone elastomer plates, demonstrated that the application of mechanical stress lead to a substantial increase in the
expression of smooth muscle myosin heavy chain isoforms SM-1 and SM-2 (Reusch et al., 1996). This mechanical stress-induced gene expression was also found to be matrix dependent. The authors suggest that these different matrix-dependent responses are transduced through different types of mechanoreceptors that are specific to different populations of VSMC. In related studies, neonatal rat VSMCs, examined following the application of mechanical strain, demonstrated an increase in PDGF protein and mRNA levels (Wilson et al., 1993).

Precedent does exist for the involvement of a TGF-β superfamily member in shear stress-induced gene regulation. Transcription and production of radar’s relative TGF-β1 has been demonstrated to increase following mechanical stress (Ohno et al., 1995). The exposure of large populations of bovine aortic endothelial cells to steady laminar shear stress resulted in an increased production of biologically active TGF-β1. A tight correlation was observed between the intensity of the laminar shear stress and TGF-β1 mRNA expression (Ohno et al., 1995). Furthermore, inhibiting endothelial K⁺ channels was demonstrated to abrogate this shear stress-induced production of active TGF-β1, implicating changes in K⁺ ion currents in the TGF-β1 signalling mechanotransduction pathway (Ohno et al., 1995). This involvement of ion flux in transducing the laminar shear stress signal has also been shown in whole-cell patch-clamp studies on single arterial endothelial cells exposed to shear stress (Olesen et al., 1988). In such studies, a K⁺ selective ionic current was demonstrated to be proportional to applied shear stress resulting in shear stress-activated hyperpolarisation of the endothelium (Olesen et al., 1988). Targeted inactivation of TGF-β1 in the mouse results in the assembly of fragile blood vessels (Dickson et al., 1995). Not surprisingly, mice engineered to be deficient in the TGF-β1 receptor, TGF-βRII, which is expressed by VSMCs, demonstrate a similar vascular defect. This would suggest that fluid shear stress can lead to the differentiation and/or recruitment of periendothelial support cells and the establishment of vascular integrity. These results support a highly dynamic model in which the vasculature can modulate changes it requires to maintain its function based on cues from its microenvironment.
From the initiation of circulation, endothelial cells that constitute the embryonic vasculature are constantly exposed to fluid shear stress derived from circulating blood. During embryonic development significant changes in this fluid shear stress occur as the vasculature matures and can support elevated blood pressures. It is therefore likely that vascular maturation is dependent upon, in part, signalling cascades initiated by changes in fluid shear stress. This has lead to a general model by which flow-induced vascular remodelling and maturation is mediated by shear stress-induced expression of endothelium-derived autocrine and paracrine molecules. Although it is not clear whether such a mechanisms exists in the zebrafish, the vascular leakage defect that occurs following a reduction in Radar signalling raises the intriguing possibility that Radar may participate in such a shear stress-induced mechanism. The vascular leaks observed in radar morphant embryos strongly correlated with an increase in blood flow during zebrafish development. However, how Radar could function within such a network is unclear. The expression of radar is clearly not dependent upon fluid shear stress. Indeed, expression levels, as detected by whole mount in situ hybridisation analysis, were reduced in later stages of development when blood flow increased and vascular leaks first became evident. However, Radar may be involved in a complex pathway initiated by an endothelium-derived regulatory molecule whose expression is dependent upon fluid shear stress. Further research is required to determine if this is the case, and whether such a mechanism exists in the fish leading to the recruitment and/or differentiation of periendothelial support cells, such as VSMCs, to establish vascular integrity.

7.7 Does the Radar signal interact with Vegf?

The PDGF family member VEGF represents perhaps the most studied modulator of vascular development. Initially, VEGF was isolated and characterised for its ability to induce vascular leak and permeability, as well as its proliferative effects on vascular endothelial cells (Ferrara, 1999). More recent evidence demonstrates a critical
developmental role for VEGF in the assembly of functional vascular endothelial tubes and for arterial-specific gene expression (Drake and Little, 1995; Flamme et al., 1995; Carmeliet et al., 1996; Ferrara et al., 1996; Cleaver and Krieg, 1998; Schmidt et al., 1998; Carmeliet et al., 1999b; Nasevicius et al., 2000; Lawson et al., 2002). Different isoforms of VEGF are believed to function in different vasculogenic/angiogenic processes. The more diffusible splice variants are believed to act at some distance to attract vascular endothelial precursors (Cleaver and Krieg, 1998). In contrast, non-diffusible isoforms function more locally in the assembly of these angioblast aggregates into patent endothelial tubes. In the developing zebrafish embryo, transcripts encoding two vegf isoforms (vegf,121 and vegf,165) are detected by whole mount in situ hybridisation in the ventro-medial somite region (Liang et al., 1998; Liang et al., 2001). Another study has demonstrated a further domain of axial expression in the hypochord (Lawson et al., 2002). This places the Vegf signal in close proximity to that of Radar from the hypochord and PGE. Moreover, targeted knock-down of Vegf-A in the zebrafish using MO technology generated a Vegf loss-of-function model with similarities to the radar morphant described here (Nasevicius et al., 2000). This vegf-A morphant demonstrated a critical role for vegf,165 in the assembly of functional axial blood vessels, the DA and PCV, as well as in angiogenic sprouting of the minor intersegmental blood vessels (Nasevicius et al., 2000). To study a potential relationship between Radar and Vegf we generated our own vegf morphant to compare and contrast their respective knock-down phenotypes.

The most striking similarity between the radar and vegf-A morphant phenotypes was that, in both backgrounds, angioblasts migrated to the midline of the embryo and aggregated into a typical DA and PCV. This suggests that both signals are not necessary for the initial establishment of axial vascular patterning and primary vasculogenic events, but are critical for the subsequent assembly of these medially located angioblasts into functional blood vessels. This was consistent with the previously characterised vegf-A morphant phenotype (Nasevicius et al., 2000). A significant difference between the two morphants was that intersegmental vasculature failed to develop, as detected by whole
mount in situ hybridisation analysis for flk-1, in embryos depleted of the Vegf-A signal. However, these vessels typically formed, as detected by the same marker, in the radar morphant background. This would suggest that vegf-A, but not radar is essential for the formation of the intersegmental vasculature.

Both the radar and vegf-A morphants displayed significant vascular defects following 2 days development. However, these defects in vascular development possessed similarities and differences in the extent to which they affected normal circulation. In the radar morphant, the circulatory defects, as detected through microangiography, appeared to be a result of fragile axial vasculature that was prone to vascular leakage when subjected to elevations in blood flow. In the vegf-A morphant embryos, there was a gradation in the severity of the vascular phenotype. The most severe morphants never possessed circulation throughout the developing trunk while other morphants possessed more subtle circulation defects, lacking circulation in all, or a subset of, intersegmental blood vessels. The vascular defects appeared to be quite specific for the axial blood vessels with both morphants typically possessing a normal anterior vascular circuit, facilitating blood flow through the CCV, over the yolk, into the heart and subsequently into the developing head. This is not so surprising given that it is the axial expression domains of both genes that borders vascular development throughout the trunk. Transverse sections through the more severe vegf-A morphants revealed that tubular axial blood vessels did not form, suggesting a function for Vegf-A in their assembly (Nasevicius et al., 2000).

Microangiography of the vegf-A morphant embryos also demonstrated a very small population that exhibited cranial and axial haemorrhaging, a phenotype not described in the previously characterised vegf-A morphant (Nasevicius et al., 2000). The microangiograms displayed by the severe vegf-A morphants was supported by the accumulation of blood cells in the tail and anterior hypochord regions as well as substantial pericardial oedema, phenotypes typical of disrupted trunk circulation. The foremost similarity between the two morphant circulatory phenotypes was restricted to the developing intersegmental vasculature. Both morphants displayed limited flow through these minor trunk vessels to
varying degrees, suggesting a potential role for each in their angiogenic development. However, in radar morphants, the lack of intersegmental blood flow was most likely due to insufficient circulation through the DA, which feeds the intersegmental vessels. Such a view is supported by the in situ hybridisation analysis that confirmed the initial formation of these minor trunk vessels in radar morphant embryos. In contrast, embryos with depleted Vegf-A signalling lacked endothelial-specific flk-1 expression in their intersegmental vasculature, suggesting that they do not form. These embryos also possessed a functional DA. Taken together, these results suggest that Vegf-A, but not Radar, is essential for the normal angiogenic assembly of the intersegmental vasculature. The fact that radar morphants possess intersegmental flk-1 expression while vegf-A morphants do not could also be interpreted as meaning that, at least in the context of intersegmental blood vessel development, a reduction in Radar signalling has no direct effect on that of Vegf-A, suggesting that they do not participate in the same signalling pathway for this function.

To further examine a potential interaction between Radar and Vegf-A during vascular development, both signals were simultaneously reduced during embryogenesis. This was performed using sub-optimal doses of each MO that, when injected alone, elicit only mild defects to normal axial vessel development. In doing such an analysis, a synergistic effect on vascular development would provide support for a model in which both signals participate in the same or parallel pathways involved in DA and PCV assembly. Injection of these sub-optimal doses independently resulted in 14% (for both radar- and vegf-A-targeting MOs) of injected embryos lacking trunk circulation through the DA, PCV, CA and CV plexus. However, when injected simultaneously, 96% possessed the same circulatory defect, the majority of which phenocopied the severe vascular defects demonstrated by vegf-A morphants. These defects were characterised by an accumulation of blood in the developing tail and anterior hypochord regions, pericardial oedema and a complete absence of axial circulation. This suggests that both molecules participate either in the same, or parallel pathways, to establish functional trunk vasculature in the zebrafish. Given that Radar does not appear to directly regulate Vegf-A activity during intersegmental
vessel development, and the differences in the morphant phenotypes described above, it would seem more plausible that these molecules signal through parallel pathways. Perhaps with Vegf-A functioning in the assembly of endothelial tubes on which Radar acts to establish structural integrity. Although the synergistic effect of the double knock-down would suggest that, at some level, cross-talk between these two pathways occurs. If such a relationship does exist then it may explain why the double knock-downs possessed the severe vegf-A morphant phenotype, as the integrity function of Radar can only act upon an already assembled blood vessel.

A recently characterised second vegf-A locus, referred to as vegf-Ab, has been knocked-down in the zebrafish using MO technology. These embryos possess an identical phenotype to the Radar loss-of-function model, demonstrating normal DA and PCV circulation and morphology up until 2 to 3 days development, at which point blood cells begin to extravasate (Nathan Bahary, personal communication). In addition, these Vegf-Ab-depleted embryos possess typical vascular patterning and haematopoiesis, as detected by whole mount in situ hybridisation analysis of endothelial- and haematopoietic-specific markers. These strikingly similar phenotypes may be indicative of a shared signalling pathway in establishing vascular integrity of the DA and PCV.

A possible mechanism by which Radar and Vegf interact during vascular development may be through the receptor Neuropilin-1. This interaction may be via Radar indirectly regulating neuropilin-1 expression. Neuropilin-1 has been demonstrated to bind a human VEGF165 isoform and is believed to regulate angiogenic events through a Vegf-dependent pathway (Lee et al., 2002). A number of lines of evidence support this potential scenario: 1) targeted knock-down of Neuropilin-1 in the zebrafish results in circulation defects including short-circuited axial blood flow and a lack of circulation in the intersegmental vessels despite normal patterning of these vessels (Lee et al., 2002) (similar to Radar-depleted embryos); 2) transcripts for neuropilin-1 are located in the lateral mesoderm and later in the hypochord, PGE and tail angioblasts (all domains of radar expression) (Lee et al., 2002); and 3) a double knock-down of Vegf and Neuropilin-1 (Lee
et al., 2002) exhibits an identical phenotype to that displayed by the Vegf/Radar double knock-down characterised in this study. Further research is required to explore such a relationship, in particular, examining neuropilin-I expression in Radar-depleted embryos.

In summary, it appears that Radar does interact with Vegf-mediated assembly of the zebrafish DA and PCV at some level, although it is clear that both molecules also participate in mutually exclusive aspects of vascular development, such as intersegmental vessel assembly. Vegf-A may participate more in the assembly of functional DA and PCV endothelial tubes while Radar and Vegf-Ab act to establish and/or maintain their integrity, perhaps via the recruitment of periendothelial support cells. Vascular development and angiogenic remodelling of the DA, PCV and intersegmental vasculature is clearly a complex and dynamic event, likely to involve the activities of a number of pleiotropic gene products. Indeed, the Vegf signal has been demonstrated to be involved in events ranging from angiogenic growth of the intersegmental vessels and assembly of endothelial tubes to the specification of arterial identity in the zebrafish (Nasevicius et al., 2000; Liang et al., 2001; Lawson et al., 2002). Exactly how the Radar signal interacts with the various Vegf molecules is unclear. However, their expression domains, along with the double knock-down analysis described above, strongly supports an interaction at some level.

7.8 A model for Radar-mediated establishment of axial vascular integrity

Much of our understanding regarding the cues that modulate angioblast migration, differentiation and vessel assembly in the zebrafish has developed through the investigation of vasculogenic and angiogenic events within zebrafish mutants. From the characterisation of the midline mutants flh, ntl and sqt, we know that signals derived from the notochord mediate the assembly of angioblastic aggregates at the midline into the DA (Sumoy et al., 1997; Brown et al., 2000), and that this function, in part, is likely to be transduced through the hypochord (Lofberg and Collazo, 1997; Cleaver and Krieg, 1998; Cleaver et al., 2000;
Eriksson and Lofberg, 2000; Pham et al., 2001). In addition, analysis of the oep mutant suggests that signals derived from the underlying posterior endoderm modulate the assembly of the immediately dorsal PCV (Brown et al., 2000). In related studies, analysis of the sonic hedgehog mutant sonic-you (syu) suggests that the assembly-signal emanating from the medial notochord and underlying endoderm may be sonic hedgehog (Brown et al., 2000). These and other results, including the morpholino-mediated Vegf-A loss-of-function phenotype (Nasevicius et al., 2000), has lead to a model by which Shh signalling from the notochord may mediate its vascular activities directly, via an intermediate signal generated by the hypochord, or via Vegf secreted from the ventro-medial somites and/or hypochord (Roman and Weinstein, 2000; Lawson et al., 2002). However, before Radar can be placed within such a cascade, a candidate target needs to be identified as well as a potential regulator of Radar activity. Shh appears to be the best candidate for an upstream regulator of Radar activity. Transcripts for shh are located in the notochord, immediately dorsal to the developing hypochord, and in the endoderm (Krauss et al., 1993; Strahle et al., 1996). Embryos deficient for Shh activity fail to form both the DA and PCV (Brown et al., 2000), suggesting its involvement in the genesis of each. The most compelling evidence for an inductive relationship between Shh and Radar is provided by forced expression experiments. In such experiments, transient overexpression of shh during early zebrafish development has been demonstrated to induce ectopic radar expression, as detected by whole mount in situ hybridisation (M. Flores, personal communication).

Two potential candidate targets for the Radar signal are, Alk-1 and Alk-6. Alk-1, a TGF-β type I receptor, is endothelially expressed in the zebrafish. Transcripts are located throughout the trunk in both DA and PCV endothelial cells (Roman et al., 2002). A zebrafish mutant that expresses a defective alk-1 allele, violet beauregarde (vbg), possesses an interesting phenotype with respect to that displayed in Radar-depleted embryos. The vbg mutant is characterised as possessing an increase in endothelial cell number in cranial vessels, pericardial oedema and an absence of axial circulation (Roman et al., 2002). Most significantly, vbg embryos were indistinguishable from wild-type sibling embryos until
around 1.5 dpf when blood flow was reported to increase significantly. This is coincident with the initiation of the Radar-specific vascular defects observed in the *radar* morphant. However, if this integrity establishment function of Radar is mediated by this BMP type I receptor, why is no vascular leakage observed in the trunk? This may be explained by the fact that the *vbg* mutant embryos also possessed extremely dilated cranial vessels that carried the entire embryonic blood flow, precluding an increase in shear stress in the trunk vasculature to test its integrity.

It has been suggested that the *vbg* mutant represents a zebrafish model for the human autosomal dominant disorder, HHT2, that results from a disruption at the human activin receptor-like kinase 1 locus (*ACVRL1*) (Berg *et al.*, 1997; Roman *et al.*, 2002). HHT2 is characterised by vascular malformations that can result in severe nosebleeds, mucocutaneous telangiectases, gastrointestinal haemorrhages and severe vascular malformations in the lung and brain (Guttmacher *et al.*, 1995). A second locus, also responsible for the genetically heterogenous HHT disease (HHT1), has been identified as encoding *endoglin*, the TGF-β-binding auxiliary protein (Bourdeau *et al.*, 2000). The involvement of two genetic loci explains, in part, the phenotypic heterogeneity of HHT, such as the age of onset, severity of the disease and the organs affected. The authors concede that the *vbg* mutant does not possess any haemorrhages, a characteristic feature of our Radar loss-of-function model. This raises the intriguing possibility that the *radar* morphant, if it does indeed signal through the Alk-1 receptor, may represent a functional model of HHT2.

Alk-6, also a TGF-β type I receptor, has been demonstrated to participate during Radar’s early ventralising activity (Goutel *et al.*, 2000). Radar-induced *bmp2b* expression was shown to be blocked by a truncated version of the Alk-6 receptor, suggesting that this receptor can bind, and transduce, the Radar signal (Goutel *et al.*, 2000). During somitogenesis and the assembly of the axial vasculature, transcripts for *alk-6* are located throughout the trunk in the developing somitic tissue, within likely range of the Radar signal produced by the hypochord and PGE (Nikaido *et al.*, 1999). Assuming that Radar
modulates its activity through this receptor, the signal would be transduced via the somites, the major site of *vegf* expression in the zebrafish (Liang *et al.*, 1998; Liang *et al.*, 2001).

How Radar signalling confers vascular integrity during vascular development may be via the recruitment of periendothelial support cells, specifically smooth muscle cells. A number of mice knock-out models that demonstrate fragile vasculature that are prone to leakage, fail to properly recruit periendothelial support cells that provide structural integrity to the endothelial tubes (Soriano, 1994; Suri *et al.*, 1996; Lindahl *et al.*, 1997). However, little is known regarding periendothelial cell development in the zebrafish. No characterised genes with expression restricted to these cell populations have been published to date, preventing an analysis of their differentiation and development within Radar-depleted embryos.

Further research is required to dissect the precise mechanism underlying the Radar-dependent establishment of vascular integrity. It would appear that this process is related to angiogenic remodelling of the primitive vasculature. Angiogenic remodelling involves a number of integrated and highly regulated steps, requiring the input from a number of pleiotropic signals. Angiogenesis can be divided into two phases (Pepper, 1997): 1) activation, involving increased vascular permeability, basement membrane degradation, cell migration, matrix invasion, cell division and lumen formation; and 2) resolution, involving cessation of cell migration and cell division, basement membrane reconstitution and junctional complex maturation. Interference in a number of these events could result in loss of vascular integrity. From the 24 somite stage, the zebrafish intersegmental vasculature begins to sprout from the DA, as evident by *flk-1* labelling (Fouquet *et al.*, 1997). Therefore, from the commencement of circulation the DA is an area of intense angiogenic remodelling in the zebrafish. Indeed, individual endothelial cells, transiently expressing a *tie2* promoter/enhancer-driven EGFP construct, have been observed to migrate, in real-time, from the DA to constitute the intersegmental vasculature and DLAV (Childs *et al.*, 2002). Zebrafish notochord mutants, such as *flh*, lack this organised angiogenesis of the intersegmental vasculature. This suggests that notochord and/or
hypochord signals participate in their sprouting from the DA (Brown et al., 2000). Intersegmental vasculature clearly formed in the absence of Radar signalling, as evident by whole mount in situ hybridisation analysis with endothelial-specific markers. Hence, Radar does not participate in the migration of angioblasts destined to form the intersegmental vasculature. However, such an analysis does not eliminate a potential role in events such as lumen formation, basement membrane reconstitution and junctional complex maturation, all of which contribute to vessel integrity. This could highlight an alternative mechanism explaining the extravasation of blood cells from the DA in the Radar-depleted background.

In summary, we propose a model by which Shh-induced Radar, secreted from the hypochord and endoderm, elicits its vascular integrity function either via somitic-Alk-6, or directly on the developing axial vasculature via endothelial-Alk-1 (Figure 7.3). Regardless of which path the Radar signal takes to effect its function on the blood vessels, it remains close to domains of vegf expression in the hypochord and developing somites (Figure 7.3). This intimate relationship, in conjunction with the double knock-down results, supports an interaction between these two signals in establishing functional endothelial tubes.

The zebrafish is an ideally suited model in which to investigate vasculogenic and angiogenic events that lead to the assembly of a functional vascular system. Although vasculogenic and angiogenic functions for genes in the zebrafish are just beginning to be unravelled, the genome sequencing initiative, along with MO technology will greatly accelerate the cloning and functional analysis of novel genes involved in these events. Greater insight into these pathways will expand our understanding of vascular development, and in doing so provide a stronger framework in which to develop potential therapeutic strategies.
Figure 7.3 A model for Radar-mediated establishment of vascular integrity throughout the zebrafish trunk. Diagrammatic representation of a cross-section through the zebrafish trunk. The Shh-induced hypochord and endoderm Radar signal may mediate the establishment of vascular integrity, potentially via the recruitment of periendothelial support cells, by: 1) signalling directly to endothelial cells of the DA and PCV where the signal is transduced by the endothelially-expressed BMP type I receptor, Alk-1; or 2) Radar mediates its integrity function via the BMP type I receptor Alk-6, located in the developing somites. Furthermore, Radar may interact with Vegf signalling from the somites and/or hypochord.
IV. Future research

7.9 Mechanism of Radar-dependent establishment of vascular integrity

The precise mechanism by which the Radar signal establishes vascular integrity of the axial blood vessels remains unknown. To investigate a possible role for the VSMC in this integrity function a gene whose expression is restricted to this cell type is required. Although the isolation of such a gene was beyond the scope of this project, the future isolation of such a gene may provide insight regarding perturbed VSMC migration and/or differentiation in the Radar-depleted background. Such an example of a gene is the smooth muscle-specific sm22α, whose expression is reported to be localised to periendothelial regions of the developing zebrafish embryo (this expression was reported as a personal communication by Pham et al., 2001).

Although it appears likely that Radar does not regulate Vegf-dependent angiogenic formation of the intersegmental blood vessels, whether it co-operates with other Vegf activities remains unknown. The combined knock-down of both signals suggests that an interaction occurs at some level. To further investigate the relationship between Radar and Vegf signalling on vascular development, the expression of vegf should be analysed in the absence of Radar function, and vice versa. For example, analysing vegf expression in the Radar-depleted background will help to identify if any of the axial domains of vegf expression are dependent upon Radar activity. One of the vascular events that Vegf is believed to mediate is the specification of arterial identity of the DA (Lawson et al., 2002). Whether part of the radar morphant phenotype is due to an interference in this process could be examined by analysing arterial- and venous-specific markers in the Radar knock-down model. Examples of such markers include the venous-specific gene flt4 and arterial-specific genes ephrin-B2a, notch5 and deltaC (Smithers et al., 2000; Lawson et al., 2002).
Furthermore, examining *neuropilin-1* expression in the *radar* morphant background may reveal whether the Radar signal interacts with the Vegf pathway via modulating *neuropilin-1* expression. Further support for this scenario could result from simultaneous targeting of Radar and Neuropilin-1 activity using MOs.

Recently, the engineering of a transgenic zebrafish line in which the angioblastic lineage is labelled with EGFP under control of the zebrafish *tie2* promoter/enhancer element has enabled real-time observation of blood vessel formation (Motoike *et al.*, 2000). A similar line has also been described using the *fli1* promoter/enhancer element (Roman *et al.*, 2002). Vascular morphogenesis, from angioblast migration and ICM formation to tubulogenesis of the DA and PCV, and subsequent angiogenic sprouting of the intersegmental vasculature have been described at the single-cell level (Motoike *et al.*, 2000; Childs *et al.*, 2002). An analysis of the Radar-specific vascular defects in this context may reveal insight into Radar's integrity function. This would require the early delivery of *radar*-targeting MOs into *tie2* or *fli1:EGFP* transgenic embryos and analysis of the vascular architecture throughout embryogenesis. Such an approach has the advantage over microangiography in that both patent and forming blood vessels are highlighted, as well as migrating angioblasts.

Currently, a number of mutagenesis screens are underway in the zebrafish with the specific aim of identifying genes required for vascular development. Once the genes underlying the resulting vascular mutations have been identified and functionally characterised, a more complete picture regarding vertebrate vascular development will emerge. This will provide a dynamic working model for vertebrate vessel assembly in which the role of the Radar signal will become progressively clearer.
7.10 Further characterisation of inducible *radar* transgenic line

To further characterise the inducible *radar* transgenic line, a genomic Southern analysis should be conducted to determine how many copies of the transgene have integrated into the genome. Genomic DNA isolated from wild-type and transgenic zebrafish should be isolated, digested with an appropriate restriction endonuclease(s) (*BamHI* and *PstI* will release the 1.5 kb *hsp70* promoter/enhancer element from the transgene) and probed. Bands representing a wild-type gene and the transgene can then be identified and their respective intensities compared to determine the copy number of the transgene. To visualise ectopic *radar* expressed within the transgenic embryos, whole mount *in situ* hybridisation using an antisense RNA probe to detect EGFP could be employed. Alternatively, the EGFP peptide can be detected by immunostaining using an alkaline phosphatase-conjugated anti-EGFP antibody (Clontech).

To investigate and confirm the circulatory defect observed in the transgenic line as being specific to elevated levels of ectopic *radar*, a phenotype rescue could be attempted using *radar*-targeting MOs. Early delivery of these MOs will act to deplete both wild-type Radar and Radar translated from transgenic transcripts, resulting in the dose-dependent restoration of wild-type levels of the Radar signal. Such an experiment will help differentiate the phenotype as being specific to elevated levels of the Radar signal and not due to the integration, and potential interference, of the transgene into a locus whose expression is required for angiogenic events. Whole mount *in situ* hybridisation analysis, using a battery of markers to highlight the expression of genes required for vascular development, will facilitate the functional dissection of the defect displayed by the transgenic embryos. Such genes would include *flk-1*, *tie1*, *tie2*, *fli1*, *ang1*, *vegf* and *neuropilin-1*. Furthermore, microangiography conducted on transgenic embryos will facilitate a more detailed description of this perturbed axial circulation phenotype. In addition, observation of this circulation defect within an endothelial-specific...
promoter/enhancer reporter background (such as the *tie2* or *fli1:EGFP* lines) may also shed light on the mechanism underlying the defect.

Further characterisation of the 318 line should also be undertaken to try and reproduce the lack of circulation phenotype in homozygous embryos. This will require the genotyping of sexually mature F₂ hemizygous fish that can be crossed to generate the homozygotes. Such experiments will help classify the defect as being specific to the *radar* transgene.

Although the hemizygous transgenic embryos failed to generate any phenotypes following whole animal heat-induction treatments, studies involving an *hsp70:EGFP* transgenic line have demonstrated efficient and robust transgene expression following a laser-mediated induction treatment (Halloran *et al*., 2000). Using this elegant technique to induce *sema3a1* expression within individual muscle fibres of an *hsp70:sema3a1* transgenic embryo, motor axons were demonstrated to be retarded, confirming the repulsive activity of Sema3a1 for growth cones (Halloran *et al*., 2000). By focussing a sublethal laser microbeam onto specific populations of cells, forced expression of *radar* could be controlled both spatially and temporally with exquisite precision. This would enable the function of Radar to be studied at the single-cell level. This high resolution gain-of-function analysis may reveal subtle effects of temporally- and spatially-restricted *radar* overexpression during vascular development. An alternative to this laser induction technique is the use of photo-mediated gene activation employing caged RNA or DNA. By using the caging agent 6-bromo-4-diazomethyl-7-hydroxycoumarin (Bhc-diazo), which is highly stable in the zebrafish embryo, overexpression can be temporally and spatially controlled with a similar high degree of accuracy. In brief, Bhc-caged synthetic transcripts, injected into early stage zebrafish embryos, are exposed to long wavelength UV light (350 to 365 nm) to promote photolysis, liberating the mRNA for translation (Ando *et al*., 2001).
Appendix 1

Gene products implicated in blood vessel development

A list of genes implicated during embryonic vascular development along with their mammalian and zebrafish expression domains is provided in Table A1.1.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Embryonic expression</th>
<th>Reference</th>
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<tr>
<td><strong>Receptors:</strong></td>
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<tr>
<td>VEGFR1/Fli1</td>
<td>Endothelial cells</td>
<td>(Fong et al., 1995)</td>
</tr>
<tr>
<td>VEGFR2/Flik1</td>
<td>Endothelial cells</td>
<td>(Liao et al., 1997)</td>
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<tr>
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<td>Venous endothelium</td>
<td>(Thompson et al., 1998)</td>
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<tr>
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<td>Endothelial cells</td>
<td>(Lyons et al., 1998)</td>
</tr>
<tr>
<td>Tie2</td>
<td>Endothelial cells</td>
<td>(Lyons et al., 1998)</td>
</tr>
<tr>
<td>Notch1</td>
<td>Endothelial cells</td>
<td>(Westin and Lardelli, 1997)</td>
</tr>
<tr>
<td>PDGFR-β</td>
<td>VSMC/pericytes</td>
<td>(Lindahl et al., 1998)</td>
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<td>TGF-βRII</td>
<td>VSMC/pericytes</td>
<td>(Oshima et al., 1996)</td>
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<tr>
<td>EphB4</td>
<td>Venous endothelium</td>
<td>(Zhong et al., 2001)</td>
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<tr>
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<td>neuropilin-1</td>
<td>Endothelial cells</td>
<td>(Lee et al., 2002)</td>
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<tr>
<td>endoglin</td>
<td>Endothelial cells</td>
<td>(Jonker et al., 2002)</td>
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<tr>
<td>Jagged-1</td>
<td>Endothelial cells</td>
<td>(Loomes et al., 1999)</td>
</tr>
<tr>
<td>ephrin-B2</td>
<td>Arterial endothelium</td>
<td>(Zhong et al., 2001)</td>
</tr>
<tr>
<td>endothelin-1</td>
<td>Artery endothelium</td>
<td>(Miller et al., 2000)</td>
</tr>
<tr>
<td>VEGF</td>
<td>Endoderm, somites</td>
<td>(Liang et al., 2001)</td>
</tr>
<tr>
<td>angiopoietin-1</td>
<td>Mesenchyme, mesenchyme, hypochond</td>
<td>(Pham et al., 2001)</td>
</tr>
<tr>
<td>angiopoietin-2</td>
<td>Mesenchyme, pronephric cells</td>
<td>(Pham et al., 2001)</td>
</tr>
</tbody>
</table>

**Transcription factors:**

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Expression Domain</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCL</td>
<td>Endothelial/blood precursors</td>
<td>(Liao et al., 1998)</td>
</tr>
<tr>
<td>Fli1</td>
<td>Endothelium</td>
<td>(Brown et al., 2000)</td>
</tr>
<tr>
<td>Ets-1</td>
<td>Endothelium</td>
<td>(Lelievre et al., 2001)</td>
</tr>
<tr>
<td>Hex</td>
<td>Endothelium</td>
<td>(Liao et al., 2000b)</td>
</tr>
<tr>
<td>gridlock</td>
<td>Arterial endothelium</td>
<td>(Zhong et al., 2000)</td>
</tr>
<tr>
<td>Smad5</td>
<td>Mesenchyme, somites</td>
<td>(Hild et al., 1999)</td>
</tr>
</tbody>
</table>

Where an entry for the expression domain of a given gene has not been made, either that gene has not been isolated from that organism, or its expression profile has not yet been characterised.

**Table A1.1** Key molecules involved in vasculogenesis and angiogenesis, together with their embryonic expression domains in the mouse and zebrafish.

A list of knock-out phenotypes following the targeted inactivation of loci required for normal blood vessel development is provided in Table A1.2.
<table>
<thead>
<tr>
<th>Targeted gene</th>
<th>Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>Heterozygote embryonic lethal between day E11 and E12. Defects in embryonic vasculogenesis and angiogenesis.</td>
<td>(Carmeliet et al., 1996; Ferrara et al., 1996)</td>
</tr>
<tr>
<td>Flt1</td>
<td>Embryonic lethal between day E8.5 and E9.5. Grossly abnormal vasculature resulting from disrupted organisation of endothelial cells into vascular tubes.</td>
<td>(Fong et al., 1995)</td>
</tr>
<tr>
<td>Flt4</td>
<td>Embryonic lethal by day E9.5 due to disorganised vasculature displaying large lumens.</td>
<td>(Taipale et al., 1999)</td>
</tr>
<tr>
<td>Flk1</td>
<td>Embryonic lethal between day E8.5 and E9.5. Almost complete lack of vascular structures.</td>
<td>(Shalaby et al., 1995)</td>
</tr>
<tr>
<td>Tie1</td>
<td>Failure to establish structural integrity of vasculature resulting in localised haemorrhages.</td>
<td>(Sato et al., 1995)</td>
</tr>
<tr>
<td>Tie2</td>
<td>Display defects including dilated vasculature, absence of capillary sprouts and a disrupted vascular network.</td>
<td>(Sato et al., 1995)</td>
</tr>
<tr>
<td>Ang1</td>
<td>Lethal by day E12.5 with defects in vascular complexity and angiogenic remodelling.</td>
<td>(Suri et al., 1996)</td>
</tr>
<tr>
<td>ephrin-B2</td>
<td>Defects in angiogenesis of both the arteries and veins in the capillary networks of the head and yolk sac.</td>
<td>(Wang et al., 1998b)</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Die at midgestation exhibiting defects in vascular development of the placenta and yolk sac and an absence of circulating red blood cells.</td>
<td>(Larsson et al., 2001)</td>
</tr>
<tr>
<td>TGF-βRII</td>
<td>Die at midgestation due to defects in yolk sac vasculogenesis and haematopoiesis.</td>
<td>(Oshima et al., 1996)</td>
</tr>
<tr>
<td>Smad5</td>
<td>Lethal between day E9.5 and E11.5 displaying disrupted yolk sac vasculogenesis.</td>
<td>(Chang et al., 1999)</td>
</tr>
<tr>
<td>PDGF-B</td>
<td>Mice die perinatally due to severe haemorrhaging.</td>
<td>(Leveen et al., 1994)</td>
</tr>
<tr>
<td>Gene</td>
<td>Phenotype Description</td>
<td>Reference</td>
</tr>
<tr>
<td>------------</td>
<td>---------------------------------------------------------------------------------------</td>
<td>--------------------</td>
</tr>
<tr>
<td>PDGFR-β</td>
<td>Embryonic lethal with severe haemorrhaging.</td>
<td>(Soriano, 1994)</td>
</tr>
<tr>
<td>Jagged-1</td>
<td>Embryonic lethal due to severe haemorrhages and exhibit defects in angiogenic remodelling of the embryonic and yolk sac vasculature.</td>
<td>(Xue et al., 1999)</td>
</tr>
<tr>
<td>Notch1</td>
<td>Severe defects in angiogenic vascular remodelling.</td>
<td>(Krebs et al., 2000)</td>
</tr>
<tr>
<td>Endoglin</td>
<td>Lethal by day E11.5 due to vascular defects including poor vascular smooth muscle development and arrested endothelial remodelling.</td>
<td>(Li et al., 1999)</td>
</tr>
<tr>
<td>VE-cadherin</td>
<td>Lethal by day E9.5 to E10 due to a lack of assembly and angiogenic remodelling of the vasculature.</td>
<td>(Carmeliet et al., 1999a)</td>
</tr>
<tr>
<td>Neuropilin-1</td>
<td>Impairment of neural vascularisation and disorganised yolk sac vasculature.</td>
<td>(Kawasaki et al., 1999)</td>
</tr>
<tr>
<td>Fli1</td>
<td>Lethal by day E12.5, displaying haemorrhaging from the dorsal aorta to the lumen of the neural tube and ventricles of the brain.</td>
<td>(Spyropoulos et al., 2000)</td>
</tr>
</tbody>
</table>

**Table A1.2**  A compilation of knock-out phenotypes in the mouse of genes involved in vasculogenesis and angiogenesis.
**Appendix 2**

**Zebrafish vascular nomenclature**

A list of zebrafish vascular nomenclature along with their abbreviations is provided in Table A2.1.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full name of vessel</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA1</td>
<td>Mandibular arch</td>
</tr>
<tr>
<td>ACeV</td>
<td>Anterior (rostral) cerebral vein</td>
</tr>
<tr>
<td>ACV</td>
<td>Anterior (rostral) cardinal vein</td>
</tr>
<tr>
<td>AMA</td>
<td>Anterior (rostral) mesenteric artery</td>
</tr>
<tr>
<td>BA</td>
<td>Basilar artery</td>
</tr>
<tr>
<td>BCA</td>
<td>Basal communicating artery</td>
</tr>
<tr>
<td>CA</td>
<td>Caudal artery</td>
</tr>
<tr>
<td>CaDI</td>
<td>Caudal division of the internal carotid artery</td>
</tr>
<tr>
<td>CCV</td>
<td>Common cardinal vein</td>
</tr>
<tr>
<td>CrDI</td>
<td>Cranial division of the internal carotid artery</td>
</tr>
<tr>
<td>CtA</td>
<td>Central artery</td>
</tr>
<tr>
<td>CV</td>
<td>Caudal vein</td>
</tr>
<tr>
<td>DA</td>
<td>Dorsal aorta</td>
</tr>
<tr>
<td>DCV</td>
<td>Dorsal ciliary vein</td>
</tr>
<tr>
<td>DLAV</td>
<td>Dorsal longitudinal anastomotic vessel</td>
</tr>
<tr>
<td>DLV</td>
<td>Dorsal longitudinal vein</td>
</tr>
<tr>
<td>H</td>
<td>Heart</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>IOC</td>
<td>Inner optic circle</td>
</tr>
<tr>
<td>LDA</td>
<td>Lateral dorsal aorta</td>
</tr>
<tr>
<td>MCeV</td>
<td>Middle cerebral vein</td>
</tr>
<tr>
<td>MsV</td>
<td>Mesencephalic vein</td>
</tr>
<tr>
<td>MtA</td>
<td>Metencephalic artery</td>
</tr>
<tr>
<td>NCA</td>
<td>Nasal ciliary artery</td>
</tr>
<tr>
<td>OV</td>
<td>Optic vein</td>
</tr>
<tr>
<td>P</td>
<td>Pronephric glomus</td>
</tr>
<tr>
<td>PAV</td>
<td>Parachordal vessel</td>
</tr>
<tr>
<td>PCeV</td>
<td>Posterior (caudal) cerebral vein</td>
</tr>
<tr>
<td>PCV</td>
<td>Posterior (caudal) cardinal vein</td>
</tr>
<tr>
<td>PHBC</td>
<td>Primordial hindbrain channel</td>
</tr>
<tr>
<td>PHS</td>
<td>Primary head sinus</td>
</tr>
<tr>
<td>PICA</td>
<td>Primitive internal carotid artery</td>
</tr>
<tr>
<td>PMBC</td>
<td>Primordial midbrain channel</td>
</tr>
<tr>
<td>PPrA</td>
<td>Primitive prosencephalic artery</td>
</tr>
<tr>
<td>PrA</td>
<td>Prosencephalic artery</td>
</tr>
<tr>
<td>Se</td>
<td>Intersegmental vessel</td>
</tr>
<tr>
<td>SIA</td>
<td>Supraintestinal artery</td>
</tr>
<tr>
<td>SIV</td>
<td>Subintestinal vein</td>
</tr>
<tr>
<td>VA</td>
<td>Ventral aorta</td>
</tr>
<tr>
<td>VTA</td>
<td>Vertebral artery</td>
</tr>
</tbody>
</table>

* Adapted from Isogai et al., 2001.

**Table A2.1** Zebrafish vascular nomenclature.
Appendix 3

Transgenesis strategies in the zebrafish

A list of transgenesis strategies and their success rates in generating germ-line transgenic founders is provided in Table A3.1.

<table>
<thead>
<tr>
<th>Delivery method</th>
<th>Germ line transmission efficiency/% germ-line transgenic founders</th>
<th>Construct/expression cassette</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microinjection</td>
<td>30.5</td>
<td>α-actin promoter-driven GFP flanked by I-SceI recognition sites</td>
<td>(Thermes et al., 2002)</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>α-actin promoter/enhancer-driven GFP</td>
<td>(Higashijima et al., 1997)</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>RSV LTR driven lacZ</td>
<td>(Culp et al., 1991)</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>Pα-actin promoter-driven EGFP flanked with AAV-ITRs</td>
<td>(Hsiao et al., 2001)</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>krt8 promoter-driven EGFP</td>
<td>(Gong et al., 2002)</td>
</tr>
<tr>
<td></td>
<td>2.1</td>
<td>hsp70 heat shock promoter-driven EGFP</td>
<td>(Halloran et al., 2000)</td>
</tr>
<tr>
<td></td>
<td>1.3</td>
<td>gata-1 promoter-driven GFP</td>
<td>(Long et al., 1997)</td>
</tr>
<tr>
<td>Retroviral infection</td>
<td>16</td>
<td>LacZ viral construct</td>
<td>(Lin et al., 1994)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>Xenopus efi-α promoter/enhancer-driven EGFP</td>
<td>(Linney et al., 1999)</td>
</tr>
<tr>
<td>Method</td>
<td>Promoter/Enhancer</td>
<td>Gene(s)</td>
<td>Reference</td>
</tr>
<tr>
<td>----------------------------------------------------------</td>
<td>-------------------</td>
<td>-------------------------------------------------------------------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>Electroporation</td>
<td></td>
<td>CMV IE1 promoter-driven lacZ and firefly luciferase</td>
<td>Muller <em>et al.</em>, 1993</td>
</tr>
<tr>
<td>Microprojectile bombardment</td>
<td></td>
<td>Plasmids containing beta-galactosidase and neomycin phosphotransferase genes</td>
<td>Zelenin <em>et al.</em>, 1991</td>
</tr>
<tr>
<td>Sperm nuclear transplantation</td>
<td></td>
<td>Xenopus elf1-α promoter/enhancer-driven EGFP</td>
<td>Jesuthasan <em>et al.</em>, 2002</td>
</tr>
<tr>
<td>Direct DNA delivery using tissue culture transfection reagents</td>
<td></td>
<td>CMV promoter-driven GFP and luciferase</td>
<td>Sussman <em>et al.</em>, 2001</td>
</tr>
</tbody>
</table>

**Table A3.1** Transgenesis strategies in the zebrafish.
Appendix 4

Nucleotide sequences of PCR products for *EGFP-radar* fusion construct

A4.1 Amplification product from primers SP6RAD5' and RADKpnI

AAGCTTTAGTTAGGTGACACTATAGAATACAAGCTACTTTGTCTTTTTGCAGGA
TCCCCATCGATTCAATTCCGACACGAGGCCGATCTCGCGCTGTCCCGCTCGTCA
AACAAACACAAACACAAAGCTCCGGCTTCACACTGGAAGACACGGCTCCACTCCACTTTAC
TCTTTTGGAGATAGTAAACACATGGGTTCCCTTGAAGAGCTCGCCTTTTTACGCC
CTCTTTGTTTCTCTGTGGATTACGGTGACTGGCCAGCTCGGTACC

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAGCTTTAGTTAGGTGACACTATAGAATACAAGCTACTTTGTCTTTTTGCAGGA</td>
<td>54</td>
</tr>
<tr>
<td>TCCCCATCGATTCAATTCCGACACGAGGCCGATCTCGCGCTGTCCCGCTCGTCA</td>
<td>108</td>
</tr>
<tr>
<td>AACAAACACAAACACAAAGCTCCGGCTTCACACTGGAAGACACGGCTCCACTCCACTTTAC</td>
<td>162</td>
</tr>
<tr>
<td>TCTTTTGGAGATAGTAAACACATGGGTTCCCTTGAAGAGCTCGCCTTTTTACGCC</td>
<td>216</td>
</tr>
<tr>
<td>CTCTTTGTTTCTCTGTGGATTACGGTGACTGGCCAGCTCGGTACC</td>
<td>266</td>
</tr>
</tbody>
</table>

A4.2 Amplification product from primers RADBgIII and RADSmaI

AGATCTCAGAAAGGAGCAAGGGTGGCCAGGAGCGGTCTTTGATGGACAAAGGTCA
CATTTTCAAGACCCGTTTGCTCAGCTCATCACCAGGGCGAGTCGTCGGAT
GATTTTAAAGACCCGTTTGCTCAGCTCATCACCAGGGCGAGTCGTCGGAT
TACTCCGCGCCCTGAGAAACTCTGGGCTCATCGACTCATGATCTCCATATACAGGACT
TCTGCAAACACCATAACGAGTCTTCGTTGACAAAGGAAAAGAGATCTCAGGCTC
TCTCTTTGCGGACACACGGTGATTTTGGATTTTCAACTCTCTCAGACAAA
GAGGAGCTGGTCGGTGCTGAATTAAGGATATTTCGCAAATCGCCCGGG

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGATCTCAGAAAGGAGCAAGGGTGGCCAGGAGCGGTCTTTGATGGACAAAGGTCA</td>
<td>54</td>
</tr>
<tr>
<td>CATTTTCAAGACCCGTTTGCTCAGCTCATCACCAGGGCGAGTCGTCGGAT</td>
<td>108</td>
</tr>
<tr>
<td>GATTTTAAAGACCCGTTTGCTCAGCTCATCACCAGGGCGAGTCGTCGGAT</td>
<td>162</td>
</tr>
<tr>
<td>TACTCCGCGCCCTGAGAAACTCTGGGCTCATCGACTCATGATCTCCATATACAGGACT</td>
<td>216</td>
</tr>
<tr>
<td>TCTGCAAACACCATAACGAGTCTTCGTTGACAAAGGAAAAGAGATCTCAGGCTC</td>
<td>270</td>
</tr>
<tr>
<td>TCTCTTTGCGGACACACGGTGATTTTGGATTTTCAACTCTCTCAGACAAA</td>
<td>324</td>
</tr>
<tr>
<td>GAGGAGCTGGTCGGTGCTGAATTAAGGATATTTCGCAAATCGCCCGGG</td>
<td>372</td>
</tr>
</tbody>
</table>
A4.3 Amplification product from primers EGFP5′KpnI and EGFP3′BglII

GGTACC
GGACGGCGACGTAAACGCCACAAAGTTTACGTGCTCCGGCGAGGGCGAGGC
TGCCACCTAGGGGAAGCTGACCCTGAGTTTCATGTGACCACCGGCAAGCTG
CGTGCCCTGCCCACCCCTCGTGACACCCTGACCTACGGCGTGAGTCCTTGAG
CCGCTACCCGACCACATGAAGCGACGCAGCTCTTCTTCAAAGGACGAGGCCAACATACGAAGAC
AGGCTACGTCCAGGACGAGCGACCACATCTTTCTTTCAAGGACGACGCCAAACTACGAAGAC
CCGCGCCAGGTGAAGTTCGAGGGGACACCCTGTGAAACCACATCGAGCTGAA
GGGCACTCGACTTCAAGGGGACGGAACATCCTTGGGGCAAGCTGGGATGATCA
CTACAACAGCCACAACGTCTATATCATGGCCGACAAAGCAGAAGACGCCATCAA
GGTGAACTTCAAGATCGGCCACAAACATCGAGGACGAGCGACGCTGACGCTGCGCGA
CCACTACCGAGCAACACCCCATCGCGAGCGCCCGCTGCTGCTGCCCGACAA
CCACTACCTGACCCAGCTCGCCCTGAGGACAAGACCCCAACGAGAAGGCACG
TCACATGGTCTCCGAGTTTGGTAGCTGGACGCCGCGGATCATCTCTCGCATG
CGAGCTGACGATCCT

Underlined sequences denote restriction endonuclease recognition sites, bold sequence highlights pCS2+ vector sequence.
Bibliography


