Insulin-like growth factor-2 regulates early neural and cardiovascular system development in zebrafish embryos

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ABSTRACT The insulin-like growth factor (IGF) family is essential for normal embryonic growth and development and it is highly conserved through vertebrate evolution. However, the roles that the individual members of the IGF family play in embryonic development have not been fully elucidated. This study focuses on the role of IGF-2 in zebrafish embryonic development. Two igf-2 genes, igf-2a and igf-2b, are present in the zebrafish genome. Antisense morpholinos were designed to knock down both igf-2 genes. The neural and cardiovascular defects in IGF-2 morphant embryos were then examined further using wholemount \textit{in situ} hybridisation, TUNEL analysis and O-dianisidine staining. Knockdown of igf-2a or igf-2b resulted in ventralised embryos with reduced growth, reduced eyes, disrupted brain structures and a disrupted cardiovascular system, with igf-2b playing a more significant role in development. During gastrulation, igf-2a and igf-2b are required for development of anterior neural structures and for regulation of genes critical to dorsal-ventral patterning. As development proceeds, igf-2a and igf-2b play anti-apoptotic roles. Gene expression analysis demonstrates that igf-2a and igf-2b play overlapping roles in angiogenesis and cardiac outflow tract development. IGF-2 is specifically required for cardiac valve development and cardiac looping. Injection of a dominant negative IGF-1 receptor led to similar defects in angiogenesis and cardiac valve development, indicating IGF-2 signals through this receptor to regulate cardiovascular development. This is the first study describing two functional igf-2 genes in zebrafish. This work demonstrates that igf-2a and igf-2b are critical to neural and cardiovascular development in zebrafish embryos. The finding that igf-2a and igf-2b do not act exclusively in a redundant manner may explain why both genes have been stably maintained in the genome.

KEY WORDS: \textit{zebrafish, IGF-2, neural, cardiovascular, development}

Introduction

Insulin-like growth factor-2 (IGF-2) is a single chain polypeptide that acts as a foetal promoter of cell growth, survival and differentiation. In mammals, IGF-2 can bind to two receptors: the IGF-1 receptor (IGF-1R) and the IGF-2 receptor (IGF-2R). The IGF-1R belongs to the tyrosine kinase receptor superfamily. It is a heterotetrameric transmembrane protein, which mediates most of the effects of IGF-1 and IGF-2. The IGF-2R is a single-chain transmembrane protein and there is no evidence of a role for the IGF-2R in transducing IGF-2 signals. The IGF family is made more complex by the presence of IGF binding proteins (IGFBPs).

These act to modulate the actions of IGFs by either inhibiting or augmenting their availability. The majority of IGF signals are transduced by ligand binding to the IGF-1R. This triggers autophosphorylation of the receptor, which ultimately leads to the...
activation of two main signalling pathways: the mitogen activated protein kinase (MAPK) and the phosphatidylinositol-3-kinase/Akt-1 (PI3K/Akt-1) pathways (Wood et al., 2005a). In zebrafish, both of these pathways are required for the mitogenic actions of IGFs (Pozios et al., 2001).

IGF signalling is critical in promoting growth during embryonic development. Loss of igf-1 or igf-2 function in mice results in embryos approximately 60% of their normal body weight at birth. Mice lacking the IGF-1R are 45% the weight of their wildtype mates and die at birth with generalised organ hypoplasia, delayed bone development and abnormal central nervous system development (Liu et al., 2007). Mice lacking a functional IGF-2R are 25-30% larger than their normal siblings and have elevated circulating IGF-2. There is a disproportionate increase in their heart size and the majority of embryos die around birth due to major cardiac abnormalities (Lau et al., 1994).

More recent work on IGF signalling indicates that IGF signals are not only important for growth, but are also critical for organogenesis. Disruption of IGF-1R in Xenopus and zebrafish results in smaller embryos, with disrupted head and central nervous system development. In contrast, overexpression of IGF-1 or IGF-2 results in dorsalised embryos, with an increase in anterior neural structures (Pera et al., 2001; Richard-Parpaillon et al., 2002; Eivers et al., 2004). These studies demonstrate a critical role for IGF signalling in neural induction.

A role for IGF signalling in cardiovascular development has also been suggested. Mice with IGF-1 levels 30% those of wildtype mice are smaller with chronically elevated blood pressure and enhanced cardiac contractility (Embo et al., 1996). Sustained expression of igf-2 in smooth muscle cells leads to mice displaying organomegaly, reduced life span, abnormalities in cardiac structure and hypotension (Zaina et al., 2003). Mice with combined deficiency in the insulin receptor and IGF-1R have decreased heart size (Laustsen et al., 2007). In contrast, overexpression of the IGF-1R in mice induces cardiac hypertrophy (McMullen et al., 2004). In zebrafish, angiomorphosis is compromised in IGFBP-2 morphant embryos (Wood et al., 2005b), while knockdown of igf-1a and igf-1b results in retarded heart morphogenesis (Schlueter et al., 2007). In regenerating zebrafish hearts, the level of igf-2b is significantly upregulated (Lien et al., 2006). These data indicate that the IGF system is necessary for cardiovascular development; however, the roles that individual members of this family play in this process are not clear.

IGF-2 is more highly expressed than IGF-1 during development, indicating that IGF-2 may be the more important IGF ligand during embryonic development (Sang et al., 2008). Two igf-2 genes are present in the zebrafish genome, igf-2a and igf-2b. They display restricted patterns of expression early in zebrafish development. In particular, the expression of igf-2a at the shield stage, in the notochord and the anterior region of the embryo points to a potential role in neural development (Maures et al., 2002; Eivers et al., 2004; Sang et al., 2008). Studies in other species have revealed expression of igf-2 in embryonic cardiovascular tissue, igf-2 is expressed in the developing mouse heart (Lee et al., 1990), in foetal rat ventricular tissue (Liu et al., 1996) and in the blood vessels and heart of the chick embryo (Holzenberger et al., 2000). Thus, it is likely that IGF-2 plays a role in cardiovascular development in zebrafish. The zebrafish is an excellent model for studying this as the embryos are small and can survive for several days without a functioning cardiovascular system because oxygen can diffuse passively from the medium through the tissues.

In this study, our aim was to investigate the role of IGF-2 in the developing zebrafish embryo. Using gene-specific morpholinos, we provide the first report of two functional igf-2 genes in zebrafish. IGF-2a and IGF-2b play overlapping roles in neural development, angiogenesis and outflow tract development. However, both genes do not act completely in a redundant manner, as igf-2b is specifically required for cardiac valve development and for cardiac looping. In addition, injection of a dominant negative IGF-1R (DN-IGF-1R) construct led to similar defects in angiogenesis and cardiac valve development.

**Results**

**Two functional igf-2 genes in zebrafish**

There are two IGF-2 genes in zebrafish, igf-2a on chromosome 7 and igf-2b on chromosome 25, encoding polypeptides of 197 and 212 amino acids respectively. IGF-2a and IGF-2b amino acid sequences share 65% identity (data not shown). Both genes are maternally deposited and dynamically expressed during embryonic development (Maures et al., 2002; Eivers et al., 2004; Sang et al., 2008). In addition, we show igf-2b expression in the embryonic shield, in anterior neural tissues and the developing heart (Supplementary Fig. 1).

To determine the functions of igf-2a and igf-2b during embryonic development, gene-specific antisense morpholinos targeted to the AUG region (and two control five base-pair mismatch morpholinos) were designed. Sequence analysis indicates that the IGF-2a morpholino does not show sufficient sequence identity to bind to igf-2b to prevent translation and vice versa. 4 ng of IGF-2a or IGF-2b morpholino was found to be an optimal dose to knockdown each gene separately (Supplementary Fig. 2) as was 2 ng IGF-2a morpholino plus 2 ng IGF-2b morpholino to knockdown igf-2a and igf-2b simultaneously. At these doses, embryos injected with control morpholinos were similar to uninjected control embryos (Supplementary Table 1). Injected embryos were analysed by light microscopy and their morphology was recorded (Supplementary Table 2). At 24 hours post fertilisation (hpf), IGF-2 morphant embryos showed varying degrees of ventralisation and were classified as normal, mild, intermediate or severely affected based on their phenotype or recorded as dead. Mildly affected embryos had at least one of the following: reduced head, shorter body or reduced eyes. Intermediate affected embryos displayed a mild phenotype plus at least one of the following: disrupted brain structures, disrupted somites or an expanded intermediate cell mass. Severely affected embryos displayed a mild or intermediate phenotype plus at least one of the following: loss of brain structures or disrupted body plan.

A characteristic ventralised IGF-2(a+b) morphant embryo in the intermediate class is shown (Fig. 1A). Western blot analysis demonstrates that IGF-2 translation was prevented following injection of IGF-2a and IGF-2b morpholinos (Fig. 1B). Knockdown of igf-2a and/or igf-2b shows that both genes act in a synergetic fashion during development, as the number of affected embryos when both morpholinos were injected is greater than the sum of those affected following injection of each morpholino separately (Fig. 1C). To ensure the IGF-2 morphant phenotype was specific,
IGF-2a or igf-2bRNA was co-injected with the corresponding target morpholino increasing the percentage of normal embryos from 59% to 85% for IGF-2a and from 15% to 59% for IGF-2b. Rescued embryos were identical to un.injected controls (Fig. 1D). These data indicate that knockdown of igf-2 results in ventralised embryos and that the morpholinos used in this study efficiently and specifically target their gene products.

**IGF-2 is required for development of anterior neural structures during gastrulation and plays an anti-apoptotic role during segmentation**

In an attempt to understand the molecular basis for the neural defects in IGF-2 morphant embryos, we examined the expression of key genes involved in anterior neural development. **Pax6.2** is expressed in the forebrain and along the midline region at 10 hpf (Nornes et al., 1998). IGF-2(a+b) morphant embryos showed a reduced domain of Pax6.2 expression in both the forebrain and the midline region, compared to control embryos (Fig. 2 A,B). **Rx3**, the retinal homeobox gene, is expressed in the anterior-most neural plate, which gives rise to the forebrain and retinal tissues (Chuang et al., 1999). IGF-2(a+b) morphant embryos showed a reduced expression domain for Rx3 when compared to control embryos (Fig. 2 C,D). Therefore, IGF-2 is required for the normal expression of these key anterior neural marker genes during gastrulation.

Despite the relatively normal expression pattern of igf-8 in anterior neural structures, by the pharyngula stage neural structures were disrupted by igf-2 knockdown (Supplementary Fig. 3). A TUNEL assay was completed to determine if apoptosis contributed to this phenotype. In IGF-2(a+b) morphants, an increase in apoptosis was observed in the developing anterior neural structures and the spinal cord in comparison to control embryos (Fig. 2 E,F). Similar results were observed in IGF-2a or IGF-2b morphant embryos (Supplementary Fig. 4). In summary, we find that IGF-2 is required for development of anterior neural structures during gastrulation in zebrafish and as development proceeds, IGF-2 is required as an anti-apoptotic factor.

**IGF-2 regulates expression of BMP and BMP-antagonist genes during gastrulation**

During gastrulation the dorsal-ventral axis is established by the competing actions of bone morphogenetic proteins (BMPs) and BMP antagonists, which act to promote either ventral or dorsal cell fates (De Robertis and Kuroda, 2004). Expression of IGF-2 in the organiser/embryonic shield led us to examine whether IGF-2 is involved in the regulation of early patterning genes which could explain the ventralised IGF-2 morphant phenotype. **Bozozok** is required at blastula stages for the formation of the shield and specification of dorso-anterior structures (Fekany et al., 1999). Expression of bozozok was unaffected in IGF-2 morphant em-

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**Fig. 1. IGF-2 morpholinos efficiently and specifically knockdown IGF-2 in vivo.** (A) Phenotype of (a) control injected and (b) IGF-2(a+b) morphant embryo at 24 hpf. IGF-2(a+b) morphant embryos are ventralised with a shorter body, disrupted brain structures (double arrows), reduced eyes, disrupted somites and an expanded intermediate cell mass (arrow). (B) Western blot showing IGF-2 and alpha-tubulin at 24 hpf in (1) uninjected control embryos, (2) embryos injected with control morpholinos or (3) embryos injected with IGF-2(a+b) morpholinos. (C) Igf-2a and igf-2b mediate their effects on development in a synergistic fashion. (D) Co-injection of igf-2a origf-2b RNA with the corresponding morpholino rescues the IGF-2 morphant phenotype. Rescued embryos display normal body size, eyes and brain structures at 24 hpf. Results shown are a summary of three independent experiments (n ≥ 80). Error bars indicate the standard deviation of the mean.
As bozozok and chordin function synergistically in the negative regulation of bmp4 expression and chordin is a critical component of the shield (Gonzalez et al., 2000), the expression pattern of chordin was examined. In IGF-2(a+b) morphant embryos the chordin expression domain was reduced in comparison to control injected embryos (Fig. 3 A,B). Goosecoid is also critical to shield function and is expressed in the shield along the anterior-posterior axis (Thisse et al., 1994). In IGF-2(a+b) morphant embryos, the goosecoid expression domain was reduced in the shield and did not extend towards the animal pole as in controls (Fig. 3 C,D). Bmp2b and bmp4 expression are normally restricted to the ventral region of the embryo (Martinez-Barbera et al., 1997). In IGF-2(a+b) morphant embryos, expression of bmp2b and bmp4 were expanded towards the dorsal region of the embryo in comparison to control injected embryos (Fig 3 E-H). Gata2 acts downstream of the bmp family members and is expressed in the ventral ectoderm (Detrich et al., 1995). Expression of gata2 was expanded towards the dorsal region of IGF-2(a+b) morphants in comparison to controls (Fig. 3 I,J). Similar effects were seen with either igf-2a or igf-2b knockdown (Supplementary Fig. 5). These data indicate that removal of IGF-2 influences the balance between BMPs and their antagonists at the shield stage, which likely contributes to the ventralised IGF-2 morphant phenotype thus indicating a role for IGF-2 in embryonic patterning.

Characterisation of blood and vascular defects in IGF-2 morphant embryos

IGF-2 morphant embryos had defective blood circulation. In zebrafish, blood and vessel formation is thought to arise from a common haemangioblast that is specified early in development from the ventral mesoderm (Crosier et al., 2002). As IGF-2 regulates key genes involved in organiser function we suspected that these circulatory defects may have been specified during gastrulation when patterning occurs. O-dianisidine was used to examine blood circulation. In IGF-2(a+b) morphants circulation was reduced in the anterior region and blood pooling was evident below the heart, while in the intersomitic vessels circulation was reduced or absent in comparison to control embryos (Fig. 4 A,B). To determine if the defects in blood circulation were due to a
change in blood cell differentiation the expression patterns of scl and gata1 were examined. Scl is essential for the development of all hematopoietic lineages (Gering et al., 1998) and gata1 is a marker for erythroid differentiation (Detrich et al., 1995). At 26 hpf, both scl and gata1 were expressed in the intermediate cell mass of control embryos (Fig. 4 C,F). IGF-2(a+b) morphant embryos displayed an increase in scl and gata1 at the intermediate cell mass and ectopic expression of these genes along the trunk was also evident (Fig. 4 D,G). A similar phenotype was observed when DN-IGF-1RmRNA, which acts as an inhibitor of endogenous IGF-1R signalling (Eivers et al., 2004), was injected into zebrafish embryos (Fig. 4 E,H). The expression of these genes was unaffected before the onset of circulation in IGF-2 morphant embryos (Supplementary Fig. 6). As the expression pattern of scl and gata1 was normal before circulation commences, the defective blood circulation in IGF-2 morphant embryos is unlikely to be due to the effects on bmps and their antagonists during gastrulation.

To determine if IGF-2 plays a role in vascular development, we knocked down igf-2a and/or igf-2b in fli1:EGFP zebrafish embryos. No defects in fli1 expression were observed in IGF-2 morphant embryos during somitogenesis indicating that vasculogenesis was normal (data not shown), however, by the pharyngula period defects in angiogenesis were evident. Affected embryos were classified based on the severity of disruption to angiogenesis (Supplementary Tables 3 and 4). At 26 hpf, IGF-2(a+b) morphant embryos showed defective angiogenesis, including reduced sprouting of blood vessels in the head and of intersomitic vessels. By 72 hpf, affected embryos showed reduced sprouting of vessels in the head, sprouting of the intersomitic vessels was irregular and the parachordal vessel was disrupted or absent (Fig. 5 E-L). Even in the minority of morphant embryos in class III, vasculogenesis was patterned despite being highly disorganised, however angiogenesis was severely affected (Supplementary Fig. 7). Furthermore, reducing IGF signalling by injection of DN-IGF-1FmRNA also led to defects in angiogenesis (Fig. 5 M-P). Therefore, IGF signalling is critical to angiogenesis in zebrafish.

Igf-2b is critical for atrioventricular boundary specification and cardiac looping in zebrafish

Our data so far demonstrates that knockdown of igf-2a or igf-2b results in similar defects in development, which is likely due to both genes playing overlapping roles in these processes. However, defects in heart development differed in IGF-2a or IGF-2b morphants. At 72 hpf, the atrium and ventricle of IGF-2a or IGF-2b morphant embryos were increased in size with blood reflux. In IGF-2b and IGF-2(a+b) morphant embryos the heart was extremely enlarged, incompletely looped and was full of blood with very little blood entering or leaving the heart. In contrast, in IGF-2a morphant embryos there was a slight increase in heart size and a reduced amount of blood could enter and leave the heart (Figure 6 A,C, Supplementary Movies 1, 2 and 3). Consistent with this, 51% of IGF-2b and 45% of IGF-2(a+b) morphant embryos displayed blood reflux in the heart in comparison to 14% of IGF-2a morphants (Table 1) and the heart rates in IGF-2b and IGF-2(a+b) morphants were also significantly lower than control embryos (Supplementary Table 5). These effects on heart development were specific as the morphant phenotype was rescued by coinjection with the appropriate igf-2mRNA (Fig. 6 B,D). Therefore, knockdown of igf-2b results in more severe defects in heart development.
development than knockdown of *igf-2a* which is consistent with *igf-2b* expression in the zebrafish heart (Supplementary Fig. 1).

To determine the primary cause of these defects, the expression patterns of cardiac-specific markers were examined. The size of the heart field was unaffected in IGF-2 morphant embryos at the 16-18 somite stage and at 26 hpf (data not shown). Therefore, early cardiac development proceeds normally in IGF-2 morphant embryos. This, combined with the blood reflux phenotype observed suggested that cardiac valve development may be disrupted. In zebrafish, atrioventricular boundary specification occurs at 37 hpf, with the restriction of *bmp4* expression to the atrioventricular myocardium which is required for the proper formation of the myocardial layer. The restriction of *notch1b* expression is required at 45 hpf for proper formation of the endocardial layer of the atrioventricular boundary (Armstrong and Bischoff, 2004). Expression of these markers was similar in control and IGF-2a morphant embryos. However, knockdown of *igf-2b* resulted in the loss of both *bmp4* and *notch1b* expression at the atrioventricular boundary. IGF-2(a+b) morphant embryos also showed a loss of expression of these genes to this boundary (Fig. 6 E-J,M). Therefore, IGF-2b functions early in valve formation and is critical for patterning of both the myocardium and endocardium at the atrioventricular boundary. It is likely that *igf-2b* signals through the *igf-1r* to elicit these effects as expression of *bmp4* and *notch1b* were also lost at the atrioventricular boundary in DN-IGF-1R mRNA injected embryos (Fig. 6 I,N). These defects in patterning at the atrioventricular boundary may disrupt cell-cell communication in the developing valve leading to alterations in cell fate decisions in this region, thereby explaining the prominent blood reflux phenotype observed in IGF-2b and IGF-2(a+b) morphant embryos.

In addition to the atrioventricular boundary defects, cardiac

**TABLE 1**

INCIDENCE OF INCREASED HEART SIZE AND BLOOD REFLUX PHENOTYPES IN IGF-2 MORPHANTS AT 72 HPF

<table>
<thead>
<tr>
<th></th>
<th>Increased heart size</th>
<th>Blood Reflux</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>0 ± 0%</td>
<td>0 ± 0%</td>
</tr>
<tr>
<td>IGF-2a morphant</td>
<td>19 ± 3.7%</td>
<td>14 ± 1.5%</td>
</tr>
<tr>
<td>IGF-2b morphant</td>
<td>52 ± 3%</td>
<td>51 ± 9.7%</td>
</tr>
<tr>
<td>IGF-2 (a+b) morphant</td>
<td>48 ± 5.9%</td>
<td>45 ± 2.3%</td>
</tr>
</tbody>
</table>

*(n ≥ 100; numbers are shown as mean ± SD)*

**TABLE 2**

SUMMARY OF HEART LOOPING DEFECTS IN IGF-2 MORPHANT EMBRYOS

<table>
<thead>
<tr>
<th></th>
<th>Normal loop (T)</th>
<th>Normal loop and increased heart size (U)</th>
<th>Incomplete loop to RHS (V)</th>
<th>Incomplete loop at midline (W)</th>
<th>Loop to LHS (X)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>100%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>IGF-2a morphant</td>
<td>73%</td>
<td>27%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>IGF-2b morphant</td>
<td>18%</td>
<td>15%</td>
<td>22%</td>
<td>27%</td>
<td>15%</td>
</tr>
<tr>
<td>IGF-2(a+b) morphant</td>
<td>23%</td>
<td>13%</td>
<td>27%</td>
<td>27%</td>
<td>10%</td>
</tr>
</tbody>
</table>

T, U, V, W, X refer to images in Fig. 6.

Fig. 5. Angiogenesis is compromised when IGF signalling is reduced. (A-D) *fltl1:EGFP* transgenic embryos injected with control morpholinos showing normal vascular development. (E-H) Class I IGF-2 morphant embryo. The intensity of GFP expressing cells in the head and eyes is reduced while the basic pattern of vasculature remains intact. The intermediate cell mass is mildly expanded and the intersomitic vessels are reduced. By 72 hpf, the parachordal vessel is incompletely formed. (I-L) Class II IGF-2 morphant embryo. The sprouting of vessels in the head and eyes are reduced. Intersomitic vessel sprouting is irregular and reduced, the intermediate cell mass is expanded and the parachordal vessel is disrupted. (M,N) Control embryo at 26 hpf showing normal expression of *fltl1* in the vasculature. (O,P) Angiogenesis is disrupted in DN-IGF-1R injected embryos as shown by *fltl1* expression (n=14/29). White arrows indicate the vasculature in the head and eyes. White arrowheads point to the intersomitic vessels, red arrow points to the intermediate cell mass and the grey arrow indicates the parachordal vessel. All embryos are shown in a lateral view.
IGF-2 in zebrafish embryonic development

Looping was irregular in IGF-2b and IGF-2(a+b) morphant embryos (Fig. 6 T-X and Table 2), indicating a requirement for IGF-2b in normal cardiac looping.

*Igf-2a* and *igf-2b* are required for patterning of the outflow tract

A mild blood reflux phenotype was observed in IGF-2a morphant embryos, however, expression of early cardiac valve markers were normal. *Tropoelastin 2* (*eln2*) is restricted to the outflow tract of zebrafish hearts at 72 hpf (Miao *et al.*, 2007). IGF-2 morphant embryos showed a reduction in expression of *eln2* at the outflow tract (Fig. 6 O-R), indicating that the outflow tract in IGF-2 morphant hearts may not have been patterned correctly and therefore prevents blood from leaving the heart. This is the first cardiac-specific defect observed in IGF-2a morphant embryos and so it may be the cause of the IGF-2a morphant phenotype. In the case of IGF-2b and IGF-2(a+b) morphants, this effect is likely to represent the combined effects of abnormal atrioventricular boundary development and of compromised outflow tract development. Consistent with a role for IGF-2 in outflow tract development, injection of *DN-IGF-1R* mRNA resulted in a loss of *eln2* expression (Fig. 6S).

**Discussion**

This is the first report describing two functional *igf-2* genes in zebrafish. Using gene-specific antisense morpholinos, we show that *igf-2a* and *igf-2b* act in a synergistic fashion during zebrafish development. Both genes play overlapping roles in anterior neural development, angiogenesis and outflow tract development, while *igf-2b* is critical to cardiac valve development and cardiac looping. Injection of *DN-IGF-1R* resulted in similar defects in
blood development, angiogenesis and cardiac valve development. This suggests that IGF-2a and IGF-2b signal through the IGF-1R to regulate cardiovascular development.

**Requirement for IGF signals in neural development**

Heterozygous mice with a disrupted *igf-2* gene exhibit a growth-deficiency phenotype but are apparently normal otherwise (DeChiara *et al.*, 1990). In contrast, IGF signalling is essential for anterior neural development in both *Xenopus* and zebrafish (Pera *et al.*, 2001; Richard-Parpaillon *et al.*, 2002; Eivers *et al.*, 2004). More recently, knockdown of the zebrafish IGF-1Rs resulted in embryos with defects in the retina, inner ear and motoneurons as a result of increased neuronal apoptosis (Schlueter *et al.*, 2007). Therefore, multiple reports exist for IGF involvement in regulating growth, neural development and apoptosis. In this study, knockdown of *igf-2* results in ventralised embryos with aberrant early neural marker expression and increased apoptosis leading to defective anterior neural development. The defects in early neural patterning are likely to be mediated during gastrulation when communication between the dorsal organiser and the ventral region of the vertebrate embryo specifies dorsal-ventral patterning (De Robertis and Kuroda, 2004). Evidence for this is that both *igf-2* genes are expressed at the shield stage (Eivers *et al.*, 2004; this study) and IGF-2 knockdown expands the expression of bmp2b and bmp4 while reducing the expression domains of their antagonists chordin and goosecoid. Therefore, this indicates that IGF-2 acts to regulate key genes involved in establishing the dorsal-ventral axis.

Consistent with this observation is the phenotypical similarity between the ventralised IGF-2 morphant embryos, zebrafish embryos overexpressing bmps (Nikaido *et al.*, 1997) and the zebrafish mutants, *dino* and *ogon*, which encode the BMP antagonists chordin and sizzled (Hammerschmidt *et al.*, 1996). While the ventralised phenotype of IGF-2 morphant embryos is milder, these embryos all share the common feature of expansion of ventral cell fates at the expense of dorsal cell fates. Therefore, reducing IGF signals or increasing BMP signals in the zebrafish embryo leads to similar phenotypes suggesting integration of these two pathways during embryonic development. The mechanism for this is unclear in zebrafish, however previous studies in *Xenopus* demonstrated integration of IGF and BMP pathways through Smad1, an intracellular BMP effector gene, in regulating neural development. BMP receptors phosphorylate Smad1 at the C-terminus ultimately resulting in the promotion of ventral cell fates. Activation of IGF/MAPK can antagonise Smad1 activity by phosphorylating MAPK sites in the linker region of Smad1, which inhibits the BMP signal. This results in the inhibition of ventral cell fates and the promotion of dorsal/neural cell fates. This integration of MAPK and BMP signalling on Smad1 is well established (Kretzschmar *et al.*, 1997; Pera *et al.*, 2003). Therefore, it is likely that this mechanism may also occur in zebrafish neural induction. In this way, in the ventralised IGF-2 morphant embryo the anti-neural effects of Smad1 could manifest themselves when IGF-2/ MAPK is reduced, BMP antagonists are reduced and BMP signals are increased resulting in the promotion of ventral cell fates at the expense of dorsal cell fates. More recently, it has been shown that phosphorylation of both MAPK and glycogen synthase kinase 3 (GSK3) sites in the linker region of Smad1 terminates the BMP signal by targeting Smad1 for degradation (Fuentealba *et al.*, 2007; Sapkota *et al.*, 2007). The phosphorylation of GSK3 sites on Smad1 introduces a role for Wnt signalling in regulating neural development through Smad1, as Wnt acts to inhibit GSK3 and thus stabilise BMP signals. In this way, the intensity of the Smad1 signal is determined by the dose of BMP and its duration is regulated by the phosphorylations mediated by MAPK and GSK3. Therefore in *Xenopus*, neural induction occurs by integrating multiple signalling pathways at the level of Smad1 phosphorylation (Fuentealba *et al.*, 2007). It will be interesting to determine if a similar mechanism is used in zebrafish to regulate neural development.

**IGF-2 is required for angiogenesis in zebrafish**

Blood circulation was compromised in IGF-2 morphant embryos. After circulation commenced, *scl* and *gata1* expression were increased in the intermediate cell mass. We concluded that this was not due to the patterning defects observed during gastrulation as the expression patterns of *scl* and *gata1* which act downstream of bmps were unaffected before the onset of circulation. Therefore, IGF-2 does not change early blood progenitor numbers but it may affect erythropoiesis later in development. Alternatively, the defects in blood circulation may be secondary to vascular or cardiac defects which could result in a build-up of differentiated blood in the site where it is produced.

The process of vascular development is highly conserved through evolution. Primary embryonic vasculature is formed by vasculogenesis and it is subsequently completed and remodelled by angiogenesis (Risau, 1997). Our demonstration of a role for IGF-2 and the IGF-1R in zebrafish angiogenesis indicates that IGF involvement in angiogenesis is conserved through evolution (Herr *et al.*, 2003; Wood *et al.*, 2005b). This is consistent with the conservation of MAPK sites in mammalian and zebrafish *fli1* genes, which indicates a role for receptor tyrosine kinase signal transduction pathways, such as IGF, in regulating *fli1* activity (Brown *et al.*, 2000).

**Igf-2b is required for specification of the atrioventricular boundary and heart looping**

At 72 hpf, IGF-2 morphant embryos had enlarged hearts and blood reflux between the heart chambers. Cardiac valves form at chamber boundaries and act to prevent retrograde blood flow through the heart. In mice and chick, the extracellular matrix of the atrioventricular canal swells and its composition changes. The atrioventricular endothelial cells undergo an endothelial-to-mesenchymal transition, migrate into the cardiac jelly and proliferate to form the endocardial cushions, which are then remodelled into mature valve leaflets (Armstrong and Bischoff, 2004). Recently it has been reported that the zebrafish atrioventricular valve does not form through an intermediate stage of endocardial cushions but directly forms leaflets by a process of invagination (Scherz *et al.*, 2008). In both cases, the signalling pathways leading to atrioventricular valve formation are the same. Therefore, the loss of *bmp4* and *notch1b* signals at the atrioventricular canal in IGF-2b morphants, which likely leads to the blood reflux phenotype, demonstrates a critical role for *igf-2b* in the cell signalling events involved in atrioventricular cardiac valve formation. Furthermore, *DN-IGF-1R*-injected embryos also showed a loss of restriction of *bmp4* and *notch1b* to the atrioventricular boundary. Although this discovery represents a novel role for IGF-2 in zebrafish, previous
work has demonstrated a role for IGF signalling in endothelial-to-mesenchymal and epithelium-to-mesenchymal transitions, emphasising the importance of IGF signals in the molecular mechanisms underlying this process (Morali et al., 2001; Arciniegas et al., 2006).

In zebrafish, the looping of the heart tube is one of the first morphological manifestations of left-right asymmetry during embryogenesis (Stainier, 2001). A number of genes involved in left-right asymmetry have recently been identified, and in zebrafish, members of the nodal or BMP family are expressed asymmetrically before looping occurs to direct cardiac looping. Despite the identification of these genes, little is known about the early left-right patterning steps in zebrafish and whether the initial steps occur prior to or after the start of gastrulation (Levin, 2005). Therefore, the mechanisms underlying the cardiac looping defect in IGF-2(a+b) morphants identified in this study are unknown. Future work will aim to determine if knockdown of igf-2 disrupts the asymmetric expression of genes involved in establishing this asymmetry.

**IGF-2 plays a role in outflow tract development**

Interestingly, we found a reduction of eln2 expression at the outflow tract of the heart in IGF-2 morphant embryos. In IGF-2a morphant embryos, atrioventricular boundary specification was normal yet the expression of notch1b by 72 hpf at this boundary was disrupted (data not shown). This indicates that the reduced domain of eln2 may lead to a loss of restriction of atrioventricular boundary markers at later stages of development with the myocardial signal more affected than the endocardial signal. This may be due to the defects in neural development in these embryos. The process of migration of cardiac neural crest cells to the heart has been conserved through evolution and, in zebrafish, cardiac neural crest cells migrate to the outflow tract and take on a myocardial cell lineage (Li et al., 2003). Therefore, we postulate that cardiac neural crest cell migration to both the outflow tract and myocardium may be disrupted in IGF-2a morphants and this may contribute to the cardiovascular defects observed in these embryos.

**Evolution of two zebrafish IGF-2 genes**

The IGF signalling system in zebrafish is highly similar to that of mammals but there is only one IGF-2 gene in mammals. Analysis of the zebrafish Hox gene cluster led to the hypothesis that zebrafish and other ray-finned fishes experienced an additional gene duplication event during evolution (Amores et al., 1998). The presence of two IGF-2 genes in zebrafish adds further weight to this hypothesis and raises questions about their functional relationship and how both genes have been stably maintained. Two common hypotheses for duplicated genes to be stably maintained in a species are (i) that one of the daughter genes acquires a novel function while the other daughter gene maintains the initial function (neofunctionalisation) or (ii) that both daughter genes share the functions of their parental gene (subfunctionalisation). More recently it has been proposed that many duplicated genes initially undergo subfunctionalisation followed by neofunctionalisation (He and Zhang, 2005). It is clear from our study that both igf-2a and igf-2b are functional. However, they are not entirely redundant as igf-2b is specifically required for cardiac valve development and cardiac looping. These differences in function may explain why both copies of igf-2 have been maintained in the genome.

As IGF-2 plays multiple roles in the developing embryo, it is likely that it acts in concert with other factors during development that provide instructive signals. This is seen in the case of igf-2 and chordin, which cooperate in the dorsalisation of Xenopus embryos (Pera et al., 2003). In this way, IGF-2 may mediate different effects on development in different tissues depending on the developmental stage and this may change due to cross-talk with other signalling pathways. This may explain why IGF-2 signals are required separately during gastrulation and later in angiogenesis and cardiac valve development. Another reason for the multiple roles for IGF signals in development is that depending on availability of substrate, activation of IGF signalling can elicit different outcomes. For example, the IGF-1R has been shown to promote proliferation or differentiation in a haematopoietic cell line depending on the availability of substrate (Valentinis et al., 1999). Therefore the potential outcome of IGF signalling is regulated at multiple levels.

In future experiments, it will be important to knock down the IGF-2 genes in a tissue-specific and temporally controlled manner and to examine if it is activation of MAPK, PI3K or both that results in IGF-2 playing multiple roles in development. Once this is established, the possibility of IGF signals cooperating with other signalling pathways will be critical to understanding IGF contributions to development. Finally, as the molecular mechanisms underlying cardiovascular development are being determined, it has emerged that many of these processes are conserved in zebrafish and other vertebrates (Stainier, 2001). This is the first study to provide evidence for the role of IGF-2 in angiogenesis and cardiac valve development in zebrafish, an excellent model in which to gain a greater understanding of the genetic factors involved in cardiovascular development, which will ultimately be critical for the treatment of cardiovascular diseases.

**Materials and Methods**

**Zebrafish maintenance**

Zebrafish, AB strain and the transgenic line (fli1:EGFP) (Lawson and Weinstein, 2002) were maintained under standard conditions at 28°C (Westerfield, 1995). Embryos were obtained from natural crosses.

**cDNA cloning and plasmid constructs**

The open reading frames of igf-2a (AF194333) and igf-2b (AF250289) were amplified from cDNA from sphere stage and 72 hpf embryos, respectively, using IGF-2a forward, IGF-2a reverse, IGF-2b forward and IGF-2b reverse oligonucleotide primers (Supplementary Table 6). PCR products were subcloned into the pGEMTeasy vector (Promega) and sequenced by Agowa Sequencing Service, Germany. To prepare the zebrafish igf-2a and igf-2b expression vectors, the open reading frame of each gene was subcloned into Cla I and Sna I restriction sites of the pCS2+ expression vector. Five point mutations were introduced at the morpholino-binding region of each gene using the Gene Tailor site directed mutagenesis kit (Invitrogen) and the following oligonucleotide primers: IGF-2aFSDM, IGF-2aRSDM, IGF-2bFSDM and IGF-2b RSDM (Supplementary Table 6).

**Morpholino design and microinjection of zebrafish embryos**

Morpholinos (Genetools, Oregon) were designed to hybridise to the AUG region of igf-2a and igf-2b as were five base-pair mismatch control morpholinos (Supplementary Table 6). Alignment of the IGF-2a mor-
pholino sequence to the AUG region of IGF-2b, and the IGF-2b morpholino sequence to the AUG region of IGF-2a, indicates only 11/25 matches in each case. Morpholinos were resuspended in nuclelease free water at a stock concentration of 1 mM. IGF-2 morpholinos and control morpholinos (2 nl) were injected at the 1-cell stage. IGF-1R signalling was inhibited by injection of 500 pg of DN/IGF-1RmRNA at the 1-cell stage (Eivers et al., 2004). Western blot

Embryos at 24 hpf were dechorionated, deyolked and rinsed three times in phosphate buffered saline (PBS). Whole embryo extracts were centrifuged at 12,000 x g for 2 min at 4°C, the supernatant was removed and 50 µl of lysis buffer (400 mM NaCl, 20 mM Tris pH 8.0, 20% glycerol, 2 mM DTT and 10% protease inhibitor cocktail from Sigma) was added to the pellet from 100 embryos. Lysates were sonicated, vortexed and centrifuged at 12,000 x g for 15 min at 4°C. The supernatant was removed and protein content was estimated using the Bradford method. Protein (25 µg) was resolved by 15% SDS-PAGE and transferred onto nitrocellulose membrane. The membrane was incubated with primary antibodies rabbit anti-IGF-2 (1:1000, Gropep PAAAI) or mouse anti-α-tubulin (1:2000, Sigma) and then with the secondary antibodies rabbit peroxidase-conjugated (1:140,000, Sigma) or mouse peroxidase-conjugated (1:8000, Sigma). The membranes were washed and bound antibodies were visualised using SuperSignal West Pico chemiluminescent substrate

Rescue experiment

Mutated igf-2a and igf-2bpcS2+ constructs were linearised with Not I and transcribed with SP6 polymerase using mMessage mMachine kit (Ambion). 10 pg igf-2aor 40 pg igf-2bRNA were resuspended in nuclease free water and diluted in phenol red prior to injection.

Whole mount in situ hybridisation

Whole mount in situ hybridisation was performed as previously described (Eivers et al., 2004). The following probes were used: bmp2b and bmp4 (Martinez-Barbera et al., 1997), bozozok (Fekany et al., 1999), chordin (Miller-Bertoglio et al., 1997) cmic2 (Yelon et al., 1999), eln2 (Miao et al., 2007), fgf8 (Furthauer et al., 1997), fibrin (Thompson et al., 1998), gata1 and gata2 (Detrich et al., 1995), goosecoid (Thiisse et al., 1994) igf-2b (Sang et al., 2008), notch1b (Westin and Lordelli, 1997), pax6.2(’Nornes et al., 1998), rx3 (Chuang et al., 1999) and scl (Gering et al., 1998). Probes were transcribed in vitro using T3, T7 or SP6 RNA polymerases and labelled using a digoxigenin-labelling kit (Roche). Embryos were fixed in 4% paraformaldehyde, dehydrated and hydrated through a methanol series and hybridised with the probe. The bound probe was detected with AP conjugated anti-digoxigenin antibody and BM-purple AP substrate (Roche). Embryos were visualised using a Nikon Eclipse E600 microscope with Nomarski optics and photographs were taken using a Nikon DXM1200F camera. Numbers for control injected embryos are summarised in Supplementary Table 7.

TUNEL assay

Embryos were fixed overnight in 4% paraformaldehyde and dehydrated in a PBS/methanol series, followed by incubation in 100% acetone at –20°C. Embryos were washed in PBS with 0.1% Tween-20 (PBST) and permeabilised by incubation in fresh 0.1% sodium citrate in PBST, followed by three rinses in PBST. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed using the In situ Cell Death Detection Kit, TMR Red (Roche).

O-dianisidine staining

O-dianisidine staining of zebrafish embryos was carried out as previously described (luchi and Yamamoto, 1983). Briefly, 72 hpf embryos were incubated in O-dianisidine (0.6 mg/ml), 0.01 M sodium acetate (pH 4.5), 0.85% H2O2 and 40% ethanol in the dark. Embryos were washed in PBS, and then dehydrated through a methanol series and cleared with benzyl benzoate/benzyl alcohol (2:1) for examination by microscopy.

Statistics

Results were expressed as mean ± standard deviation. For statistical analysis of heart rates, one-way ANOVA and Tukey post-hoc analysis was performed using SPSS software. A P-value of less than 0.05 was considered statistically significant.

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