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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 *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-2b* 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: *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

Abbreviations used in this paper: BMP, bone morphogenetic protein; DN-IGF-1R, dominant negative insulin-like growth factor-1 receptor; eln2, tropoelastin 2; GSK3, glycogen synthase kinase 3; hpf, hours post fertilisation; IGF, insulin-like growth factor; IGFBP, IGF binding protein; IGF-1R, IGF-1 receptor; MAPK, mitogen activated protein kinase; PI3K, phosphatidylinositol-3 kinase; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling.

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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.*, 1993). 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 (Lembo *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, angiogenesis is compromised in IGFBP-2 morphant embryos (Wood *et al.*, 2005b), while knockdown of *igf-1ra* and *igf-1rb* 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 knock down each gene separately (Supplementary Fig. 2) as was 2 ng IGF-2a morpholino plus 2 ng IGF-2b morpholino to knock down *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. Intermediately 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 synergistic 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-2b* RNA 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 uninjected 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 *fgf8* 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-

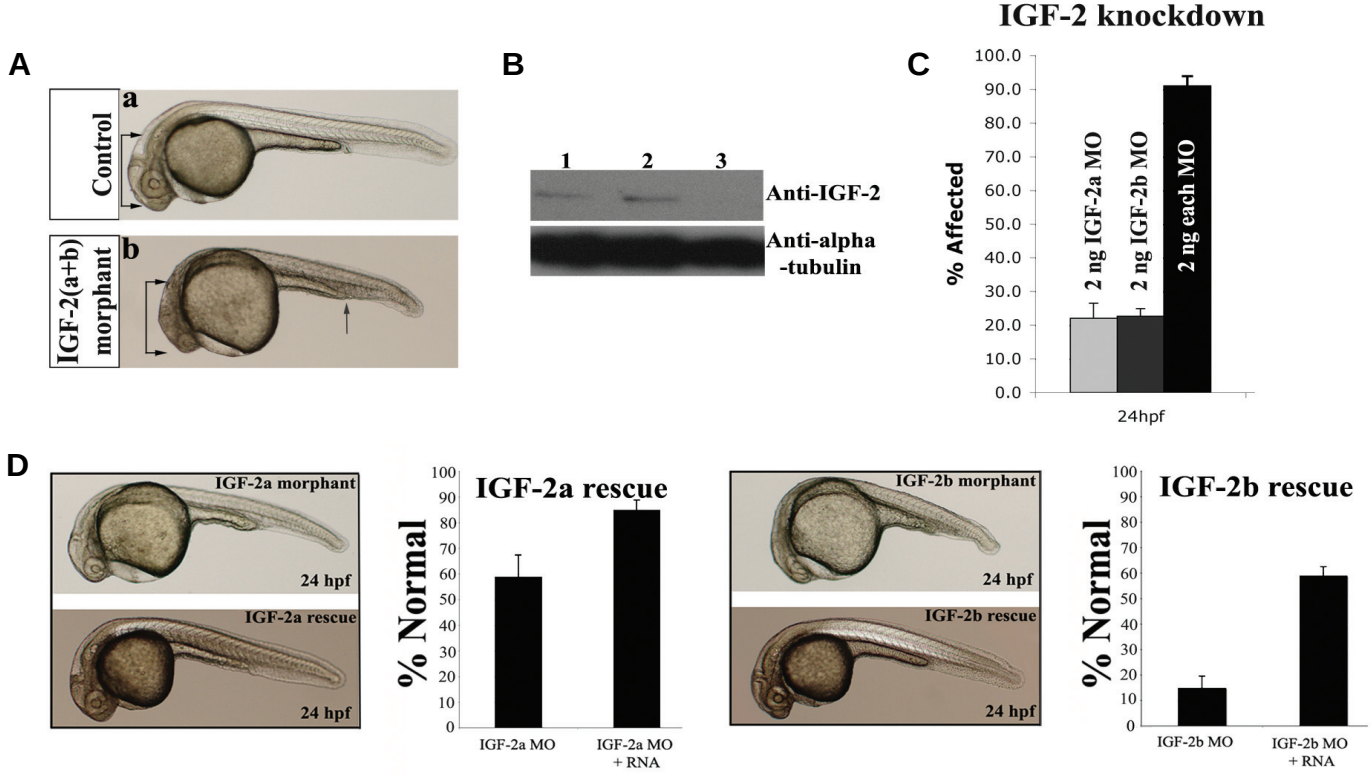

Fig. 1. IGF-2 morpholinos efficiently and specifically knockdown IGF-2 *in vivo*. (A) *Phenotype of (a) control injected and (b) IGF-2(a+b) morphant embryo at 24 hpf. IGF-2(a+b) morphant embryos are ventralised with a shorter body, disrupted brain structures (double arrows), reduced eyes, disrupted somites 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 *or* igf-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.*

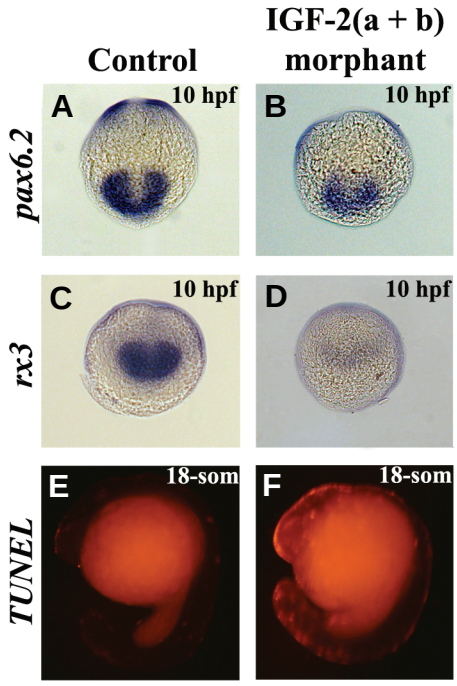

Fig. 2. IGF-2 is required for the development of anterior neural structures during gastrulation and plays an anti-apoptotic role during segmentation. (A,C) *Control morpholino injected embryos show normal* pax6.2 *and* rx3 *expression.* **(B,D)** *IGF-2(a+b) morphant embryos show reductions in* pax6.2 *and* rx3 *expression.* **(E)** *Embryos injected with control morpholinos show low levels of apoptosis during zebrafish segmentation.* **(F)** *Knockdown of* igf-2a *and* igf-2b *results in an increase in apoptosis in the anterior region of the embryo and in the developing spinal cord. Frequency of embryos displaying this staining pattern; B, 14/32; D, 22/40; F, 17/28. (A-D) are views of the future anterior region; (E,F) are lateral views.*

bryos (Supplementary Fig. 3). 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

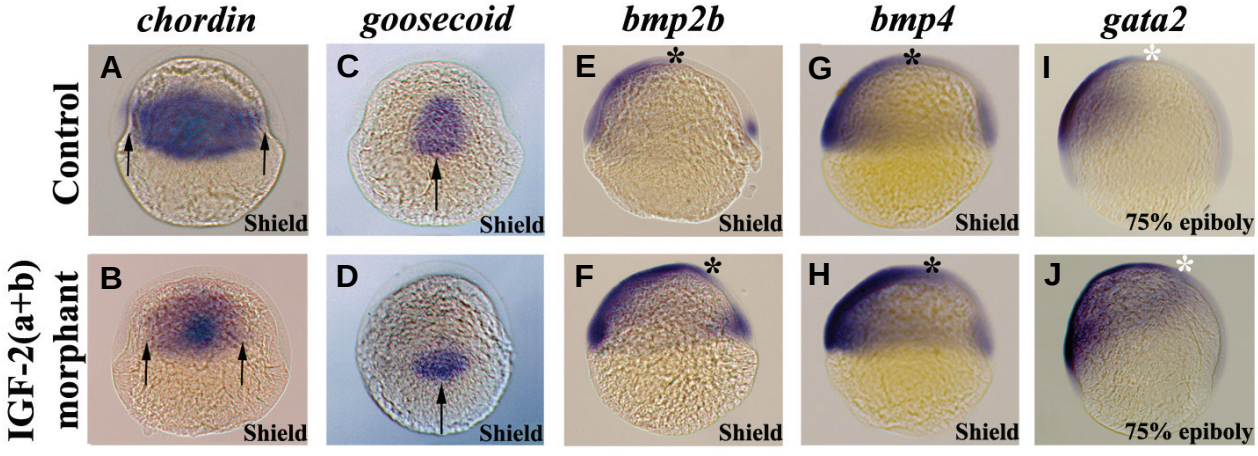

Fig. 3. IGF-2 regulates the expression of genes involved in dorsal-ventral patterning. (A,C) *Embryos injected with control morpholinos show normal* chordin *and* goosecoid *expression.* **(B,D)** *Expression of* chordin *and* goosecoid *is reduced in IGF-2(a+b) morphant embryos (arrows).* **(E,G,I)** *Embryos injected with control morpholinos show normal* bmp2b, bmp4 *and* gata2 *expression.* **(F,H,J)** *Expression of* bmp2b, bmp4 *and* gata2 *is expanded towards the dorsal side in IGF-2(a+b) morphant embryos (asterisks). Frequency of embryos displaying this staining pattern; B, 13/26; D, 17/31; F, 16/25; H, 18/32; J, 26/34. All embryos are shown in a lateral view.*

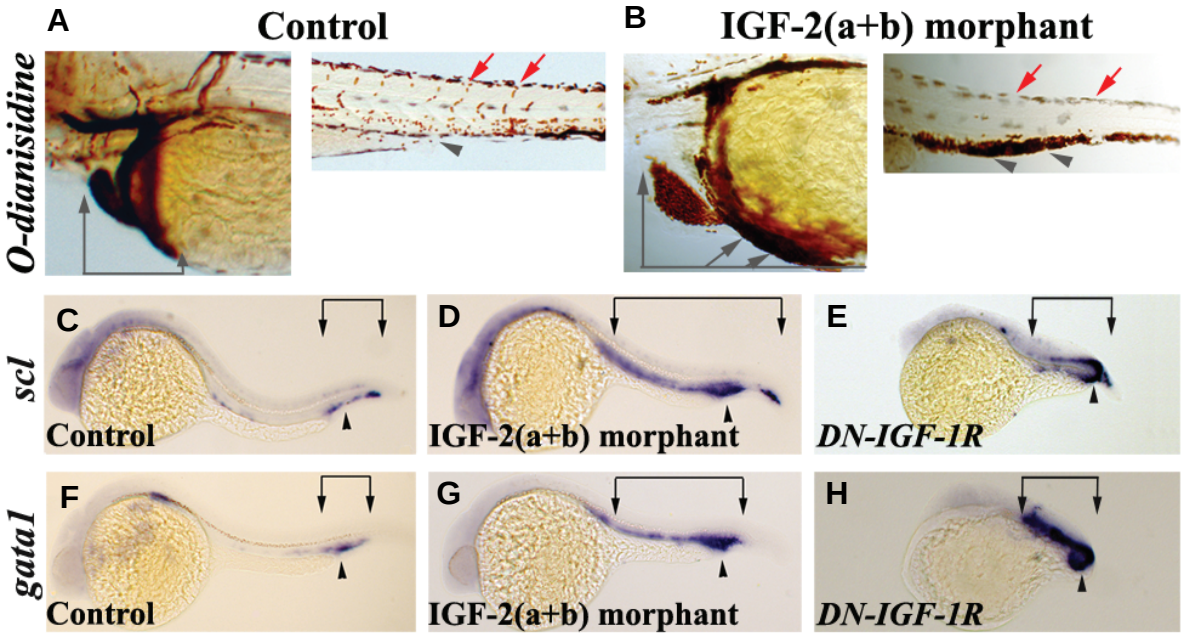

Fig. 4. Blood circulation is disrupted when IGF signalling is reduced. (A) *Control morpholino injected embryos stained with O-dianisidine at 72 hpf show normal blood circulation.* **(B)** *IGF-2(a+b) morphant embryo with reduced circulating blood. Grey arrows indicate blood in the heart region, red arrows blood circulation in the intersomitic vesicles and grey arrowheads the intermediate cell mass.* **(C,F)** *Embryos injected with control morpholinos showing normal* scl *and* gata1 *expression at 26 hpf.* **(D,G)** *Knockdown of* igf-2a *and* igf-2b *results in an increase in* scl *and* gata1 *expression and an expansion of expression outside the intermediate cell mass.* **(E,H)** DN-IGF-1R *injected embryos show an increase in* scl *and* gata1 *expression. Arrowhead indicates position of intermediate cell mass and arrows indicate extent of expression along the embryo. Frequency of embryos displaying this staining pattern; B, 73/86; D, 37/48; E, 30/30; G, 48/58; H, 26/29. All embryos are shown in lateral view.*

change in blood cell differentiation the expression patterns of *scl* and *gata1* were examined. *Scl* is essential for the development of all haematopoietic 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-1R* mRNA, 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-1R* mRNA 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-2* mRNA (Fig. 6 B,D). Therefore, knockdown of *igf-2b* results in more severe defects in heart

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-H,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*

	Increased heart size	Blood Reflux
Controls	**0 ± 0%**	**0 ± 0%**
IGF-2a morphant	19 ± 3.7%	14 ± 1.5%
IGF-2b morphant	52 ± 3%	51 ± 9.7%
IGF-2 (a+b) morphant	48 ± 5.9%	45 ± 2.3%

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

TABLE 2

SUMMARY OF HEART LOOPING DEFECTS IN IGF-2 MORPHANT EMBRYOS

	Normal loop (T)	Normal loop and increased heart size (U)	Incomplete loop to RHS (V)	Incomplete loop at midline (W)	Loop to LHS (X)
Controls	**100%**	**0%**	**0%**	**0%**	**0%**
IGF-2a morphant	73%	27%	0%	0%	0%
IGF-2b morphant	18%	15%	22%	30%	15%
IGF-2(a+b) morphant	23%	13%	27%	27%	10%

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

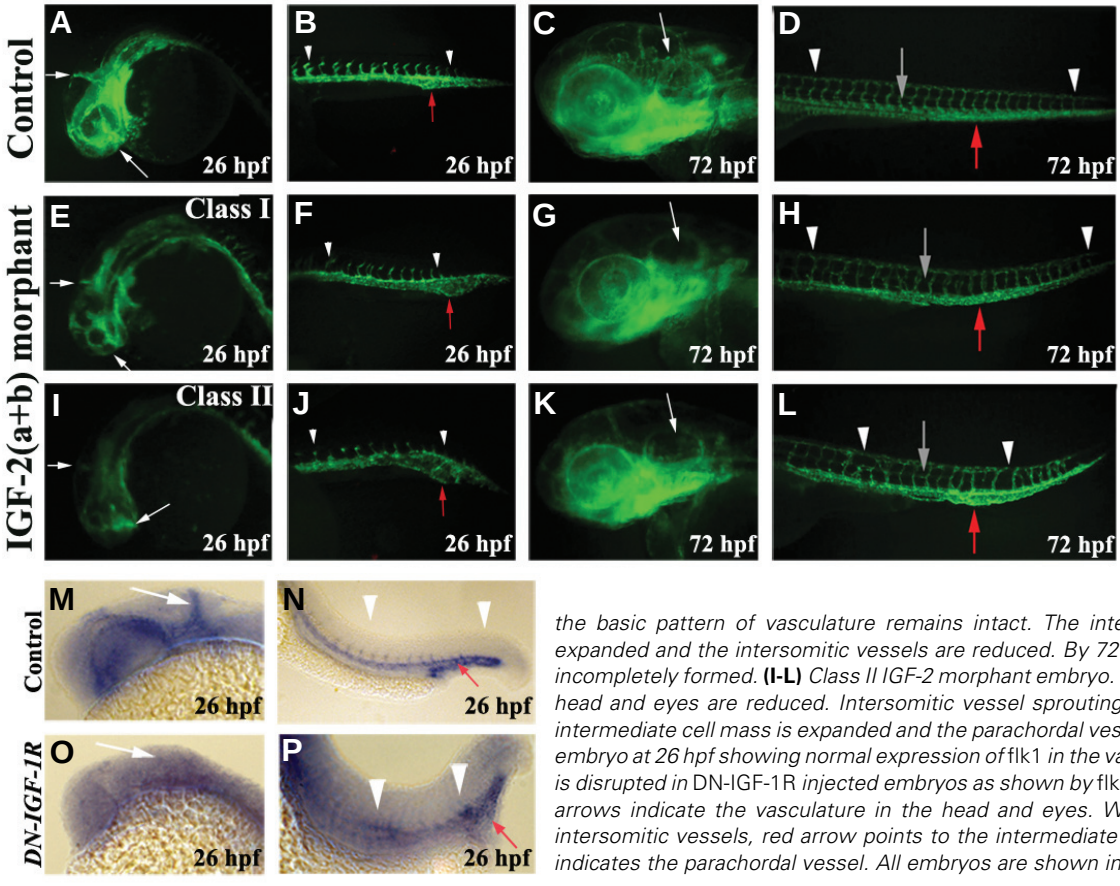

Fig. 5. Angiogenesis is compromised when IGF signalling is reduced. (A-D) fli1:EGFP *transgenic embryos injected with control morpholinos showing normal vascular development.* **(E-H)** *Class I IGF-2 morphant embryo. The intensity of GFP expressing cells in the head and eyes is reduced while the basic 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* flk1 *in the vasculature.* **(O,P)** *Angiogenesis is disrupted in* DN-IGF-1R *injected embryos as shown by* flk1 *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.*

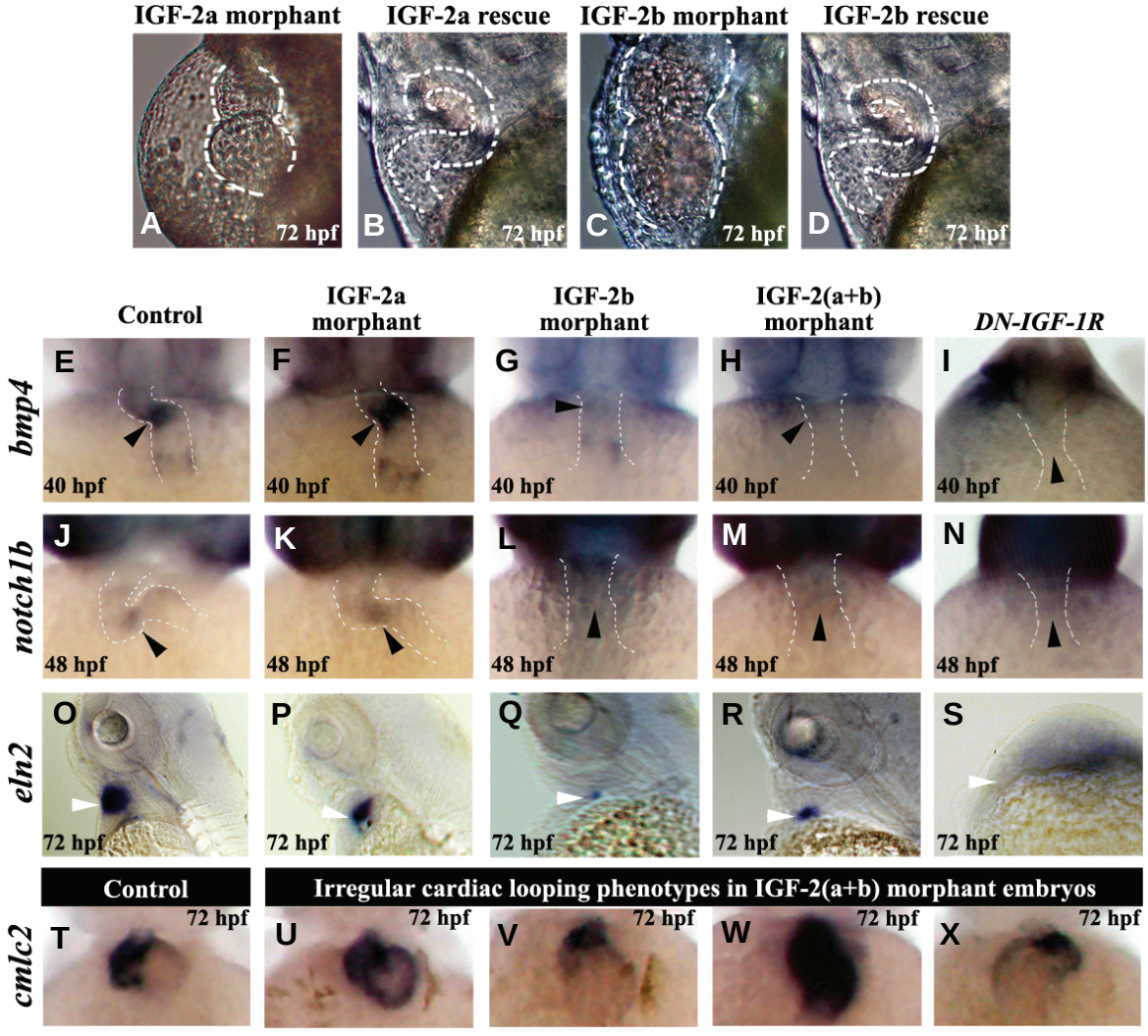

Fig. 6. IGF signalling is critical for cardiac valve development in zebrafish. (A,C) *Heart region of IGF-2a or IGF-2b morphant embryos at 72 hpf. Hearts are enlarged and incompletely looped (white outline) with blood reflux between the two chambers.* **(B,D)** *Co-injection of* igf-2a *or* igf-2b *RNA with the corresponding morpholino rescues the IGF-2 morphant phenotype. Hearts in rescued embryos are identical to hearts in uninjected embryos.* **(E,J)** *Expression of* bmp4 *and* notch1b *in embryos injected with control morpholinos. Note the restriction of expression at the atrioventricular boundary (black arrowheads). The white outline indicates the shape of the heart.* **(F,K)** Bmp4 *and* notch1b *are restricted to the atrioventricular boundary in IGF-2a morphant embryos.* **(G,H,L,M)** *IGF-2b and IGF-2(a+b) morphant embryos show a loss of* bmp4 *and* notch1b *expression at the atrioventricular boundary (black arrowheads). The heart is also incompletely looped at this stage (white outline).* **(I,N)** DN-IGF-1R *injected embryos show a loss of* bmp4 *and* notch1b *at the atrioventricular boundary.* **(O)** *Expression of* eln2 *at the outflow tract of the heart in embryos injected with control morpholinos (white arrowhead).* **(P,Q,R)** *IGF-2a, IGF-2b and IGF-2(a+b) morphant embryos show a decrease of* eln2 *expression at the outflow tract.* **(S)** DN-IGF-1R *injected embryos show a loss of* eln2 *at the outflow tract. Frequency of embryos displaying this expression pattern: F, 30/30; G, 20/40; H, 15/27; I, 29/44; K, 33/33; L, 14/32; M, 12/30; N, 49/56; P, 5/24; Q, 31/36; R, 24/26; S, 31/39.* ***(T)*** *Normal cardiac looping in control injected embryos at 72 hpf as shown by the expression of* cmlc2 *(heart loops to the right in this view).* **(U-X)** *Variety of abnormal cardiac looping phenotypes in IGF-2(a+b) morphant embryos. (The frequencies of the irregular looping phenotypes are given in Table 2.) A-D and O-S are lateral views, E-N and T-X are ventral views.*

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 *bmp4* and *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 28ºC (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 Snab 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 nuclease 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-1R* mRNA 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 4ºC, 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 4ºC. 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 PAAA1) 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 (Pierce).

Rescue experiment

Mutated *igf-2a* and *igf-2b* pCS2+ constructs were linearised with Not I and transcribed with SP6 polymerase using mMessage mMachine kit (Ambion). 10 pg *igf-2a* or 40 pg *igf-2b* RNA 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) *cmlc2* (Yelon *et al.*, 1999), *eln2* (Miao *et al.*, 2007), *fgf8* (Furthauer *et al.*, 1997), *flk1* (Thompson *et al.*, 1998), *gata1* and *gata2* (Detrich *et al.*, 1995), *goosecoid* (Thisse *et al.*, 1994) *igf-2b* (Sang *et al.*, 2008), *notch1b* (Westin and Lardelli, 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 permeabilized 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 (Iuchi and Yamamoto, 1983). Briefly, 72 hpf embryos were incubated in O-dianisidine (0.6 mg/ml), 0.01 M sodium acetate (pH 4.5), 0.65% H_2O_2 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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