Introduction

Insulin-like growth factors (IGFs), including IGF-1 and IGF-2, are evolutionarily conserved peptides that act through a conserved signaling pathway to regulate growth, development, metabolism, and longevity in a wide variety of animals [1]. IGF-1 and -2 are structurally related to insulin and relaxin in vertebrates, and insulin-like peptides (ILPs) in invertebrates [2,3,4,5]. IGFs and insulin share 50% sequence identity in amino acid sequence in their B and A domains. Unlike insulin, however, the C-domains of IGFs are not removed by proteolytic processing. In addition, IGF-1 and IGF-2 contain an E-domain in their carboxy-terminus. The E-domain is removed as a post-translational modification to produce mature IGFs [6]. Activation of the IGF signaling pathway occurs when IGF ligands bind their cognate receptor tyrosine kinases. This leads to activation of a number of downstream signaling cascades, including mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)-Akt pathways [7,8,9].

In mammals and birds, there is a single IGF-1 gene and a single IGF-2 gene, in addition to a single insulin gene. Recent studies have indicated the zebrafish genome contains more than two IGF genes. Chen et al. (2001) reported the presence of a gene encoding an IGF-like peptide [10]. Maures at al. (2002) cloned cDNAs encoding the complete coding sequences for the same IGF-1-like peptide and an IGF-2 peptide [5]. A more recent study by Sang et al. (2008) reported the presence of two distinct IGF-2 genes (igf-2a and -2b) and that these genes exhibited distinct expression patterns [11]. They further showed that knockdown of igf-2a and -2b by antisense morpholinos indicated that they each play a distinct role in early development [12]. Wang et al. (2008) reported the cloning of another IGF peptide that they termed as IGF-3, from tilapia and zebrafish [13].

Despite these new findings, there has been no report on the molecular characterization of any of these zebrafish IGF genes or their full-length transcripts. For instance, little is known about the alternative splicing for any of the previously identified zebrafish IGF genes, although alternative splicing has been shown to be an important way of generating multiple forms of IGF transcripts and prepropeptides in mammals and humans. Moreover, zebrafish, like many teleost fish, are believed to have experienced an additional genome wide duplication event [14,15]. As a result, they often have two co-orthologs in contrast to a single copy gene in humans and other mammals. Indeed, there are two distinct insulin genes, two IGF-1R genes, and two insulin receptor genes in zebrafish [5,16,17,18]. To date, there is no report on the possible presence of the fourth IGF gene in zebrafish. In this study, we have cloned and identified 4 distinct genes encoding 4 IGF peptides (IGF-1a, -1b, -2a, and -2b) from zebrafish. The structures of these 4 zebrafish IGF genes and their transcripts have been determined. Our
molecular and functional analyses suggest that these IGF genes have undergone subfunctionalization partitioning at the levels of gene expression, protein structure, and biological activities. Furthermore, taking advantage of the amenability of the zebrafish model, we unraveled a previously unrecognized role of IGF in regulating midline and notochord development during embryogenesis in vivo.

Results

Identification and characterization of 4 genes encoding distinct IGF peptides in zebrafish

By searching available zebrafish databases and performing 5’ and 3’ RACE experiments, we have identified 4 genes and a number of full-length cDNAs encoding 4 structurally distinct IGF-like peptides (IGF-1a, -1b, -2a, and -2b). The structure of these zebrafish igf genes and their transcripts are shown in Fig. 1. Zebrafish igf-1a has 5 exons and 4 introns and it spans approximately 17 kb. Two distinct igf-1a mRNA transcripts (T1, 1509 bp and T2, 2008 bp) were found (Fig. 1A). These igf1a transcripts have an identical open reading frame of 483 bp encoding a polypeptide of 161 amino acids (a.a.). This peptide can be divided into a 44 a.a. putative signal peptide, a 29 a.a. B domain, a 12 a.a. C domain, a 21 a.a. A domain, a 8 a.a. D domain, and a 47 a.a. E domain. T1 and T2, likely resulted from alternative splicing, have distinct 3’ UTR of 823 and 1322 bp, respectively (Fig. 1A). Zebrafish igf-1b has 4 exons and spans 5.9–70.8 kb DNA (Fig. 1B). Two igf-2a transcripts (1727 and 1723 bp) were identified. igf-2a T1 and T2, likely resulted from alternative splicing sites in the exon 1 and exon 2, differ in the 5’ UTR and part of the signal peptide region. The open reading frame for igf-2a T1 and T2 are 636 bp and 669 bp, respectively. They encode polypeptides of 212 and 223 a.a., which containing a 48/59 a.a. signal peptide, a 29 a.a. B domain, an 11 a.a. C domain, a 21 a.a. A domain, a 7 a.a. D domain, and a 96 a.a. E domain (Fig. 1B). Zebrafish igf-2b has 4 exons and spans about 5.9 kb. Only one transcript was found for this gene. The full-length cDNA is 2066 bp. It contains an open reading frame of 591 bp encoding a polypeptide of 197 a.a., including a 40 a.a. signal peptide, a 28 a.a. B domain, a 9 a.a. C domain, a 21 a.a. A domain, a 7 a.a. D domain, and a 92 a.a. E domain (Fig. 1B).

The 4 zebrafish IGF genes and their various mRNAs encode 4 distinct mature IGF peptides, IGF-1a (70 a.a.), IGF-1b (70 a.a.), IGF-2a (68 a.a.), and IGF-2b (65 a.a.). These peptides are highly similar to human IGFs in their primary structure (Fig. 2A). Like

![Figure 1. Molecular characterization of four zebrafish igf genes and their multiple transcripts.](https://www.plosone.org/article/figures/10.1371/journal.pone.0007026.g001)
human IGFs, all 4 zebrafish IGF peptides consisted of 4 domains (B–C–A–D). They all possess 6 conserved cysteine residues: 2 in the B domain and 4 in the A domain region. Phylogenetic analyses grouped all four zebrafish IGF peptides into the IGF clade, indicating that they are IGF like molecules rather than insulin or relaxin related peptides (Fig. 2B). Zebrafish IGF-2a and IGF-2b are clearly more closely related to human IGF-2 because they share equally high sequence identities with that of human IGF-2 (76% and 75%, respectively) and a 70% sequence identity with each other. Phylogenetic analysis also grouped them into the IGF-2 subgroup. Zebrafish IGF-1a and IGF-1b are more divergent because they are only 50% identical with each other. While zebrafish IGF-1a shares 81% sequence identity to that of human IGF-1, the sequence identity between zebrafish IGF-1b and human IGF-1 is much lower (52%). Phylogenetic analysis also placed IGF-1b in a separate group - together with the recently reported tilapia IGF-3 [13] (Fig. 2B).

The duplicated zebrafish igf-2 genes share strong synteny with the human IGF-2. As shown in Fig. 2C, zebrafish igf-2a and igf-2b are located on LG 25 and 7, respectively. There are a number of genes on LG25 and 7 whose orthologs are located on human chromosome 11, where the human IGF-2 is found. Zebrafish igf-1a is located on LG4. There are five other zebrafish genes (rpl18a, mdm2, impo, idhb, and znf10) in this region whose orthologs are located on human chromosome 12, where the human IGF-1 resides. The zebrafish igf-1b gene, on the other hand, is located on LG8. Although we were able to find 1 gene (COL2a1) in this region whose orthologous gene is located on human chromosome 12, 8 other zebrafish genes (rpl18a, mdm2, impo, idhb, and znf10) in this region whose orthologs are located on human chromosome 12, where the human IGF-1 resides. The zebrafish igf-1b gene, on the other hand, is located on LG8.
Notch2, aad2, sort1, gstm3, atp5f1, mps1, and ghb1) on LG8 have orthologs located on human chromosome 1 (Fig. 2C).

All 4 zebrafish igf genes are functional and they exhibit divergent expression patterns

To determine whether all 4 zebrafish igf genes are functional, capped mRNA encoding each of the zebrafish IGF genes was generated and delivered to zebrafish eggs by microinjection. Their effects on Akt phosphorylation were analyzed by Western blot. Akt is a major downstream effector of the IGF1R in mammals and in zebrafish [19]. Western blot analysis indicated a marked increase in the levels of phosphorylated Akt in all IGF mRNA-injected groups compared to the GFP mRNA-injected controls at both shield and 15 somite stages (Fig. 3A). When quantified and expressed as the ratio of phospho-Akt/total Akt, all of the 4 IGF mRNAs caused significant increases (Fig. 3B), indicating that they are all capable of interacting with IGF-1R and activating the intracellular signaling cascade.

Next, the temporal and spatial expression patterns of the 4 zebrafish igf genes were examined. As shown in Fig. 4A, IGF-1a mRNA was not detectable by RT-PCR in early embryogenesis. IGF-1a mRNA became detectable at the gastrulation stage (around 90%-epiboly). Its level gradually increased after 48 hpf. Likewise, IGF-2b mRNA was detected at 50%-epiboly and was maintained at relatively high levels thereafter (Fig. 4A). In comparison, both IGF-1b and IGF-2a mRNAs were easily detected in all stages of embryogenesis, ranging from the 1-cell stage to 4 dpf larvae (Fig. 4A). These data suggest that IGF-1b and -2a transcripts are maternally deposited, while IGF-1a and -2b mRNAs are the products of the zygotic genes activated after the mid-blastula transition. In the adult stage, IGF-1a mRNA was easily detectable in the liver, brain, and eyes in both male and female fish, while IGF-1b mRNA was only detected in the gonad (Fig. 4B). IGF-2a mRNA was easily detected in all the tissues examined, although its levels in the muscle and gills were very low (Fig. 4B). In contrast, IGF-2b mRNA was exclusively expressed in the liver (Fig. 4B). There was no apparent gender difference.

In agreement with the RT-PCR data, whole mount in situ RNA hybridization analysis showed that IGF-1a and IGF-2b mRNA was very low at 10 hpf, became detectable at 24 hpf, and highly expressed at 48 hpf, most abundantly in the anterior part of a embryo (data not shown). In comparison, IGF-1b and IGF-2a mRNA was detected in all stages in a wide range of tissues (data not shown).

Forced expression of zebrafish IGFs causes similar defects in midline formation and notochord development but with different potencies

The phenotypes of the IGF-2a mRNA (500 pg)-injected group are shown in Fig. 5A–L. Injection of IGF-2a mRNA (500 pg)
resulted in delayed mesoderm involution as evident from an open blastopore at the bud stage (Fig. 5B). At the 2-somite stage, there was a notable reduction in the posterior tissues (Fig. 5D). At the 5-somite stage, the IGF-2a mRNA-injected embryos showed poorly formed notochords and widened distance between the two columns of somites (Fig. 5F). This defect became more evident at the 15-somite stage (Fig. 5H). Compared to the control (Fig. 5K), the IGF-2a mRNA-injected embryos showed marked expansion of dorsal neural tissues and axial domain (Fig. 5L). Additionally, these embryos showed a severe shortening body length (Fig. 5J).

To determine whether these IGFs have similar biological activities, each IGF mRNA was injected at the doses of 300, 500, and 800 pg/embryo and percentages of embryos exhibiting the above phenotype were determined at the 2–5 somite stage and at 24 hpf. Zebrafish IGF-1a, -1b, -2a and -2b all caused similar defects in dose-dependent manners (Fig. 5M), but there were considerable differences in their effectiveness. Injection of IGF-2a at 300 pg/embryo resulted in a score value of 33%–35%; a score value of 75%–80% at 500 pg; and over 90% at 800 pg/embryo at the 2–5 somite and 24 hpf stages. Similar results were obtained with the injection of IGF-1a mRNA and to a lesser degree with IGF-2b mRNA (Fig. 5M). In comparison, IGF-1b appeared to have a weaker effect. It only had a score of 20% at the dose of 300 pg/embryo, a score of <40% at 500 pg, and <60% at 800 pg/embryo (Fig. 5M).

**Forced expression of IGF-2a disrupts midline and notochord patterning**

We next analyzed the effect of IGF-2a ectopic expression on embryonic patterning by in situ hybridization using the following markers: 1) _emx1_, a telencephalon marker [20]; 2) _rx1_, a retina marker [21]; 3) _rx2_, a retina marker [21]; 4) _otx2_, an anterior
neural plate marker at the bud stage and midbrain marker at the 13-somite stage [22]; 5) pax6b, an anterior neural plate marker at the bud stage [23]; 6) pax2a, a midbrain hindbrain boundary and otic vesicle marker [24]; and 7) krox20, a third and fifth rhombomere marker in the hindbrain [25]. Ecotropic expression of IGF-2a did not alter the anterior neural plate patterning at the bud stage, as indicated by the similar expression patterns of stc2 (Fig. 6, panels A, B, E, and F) and pax6b (Fig. 6, panels C, D, G, and H) between the GFP mRNA-injected control and IGF-2a mRNA injected embryos. Nor did IGF-2a expression alter the forebrain patterning, as indicated by the similar emx1 expression patterns (Fig. 6, panels M and N). Expression of IGF-2a caused marked changes in pax2a expression patterns. As shown in Fig. 6, panel I, in the control embryos, pax2a mRNA was detected in the midbrain and hindbrain boundary (a V shaped domain) at the bud stage. In the IGF-2a mRNA-injected embryos, however, pax2a mRNA expressing cells remained as two separate domains at both stages (Fig. 6, panels J and V), suggesting a defect in the midline/ notochord development. This effect appeared to be specific to the anterior as the pax2a expression in the otic vesicles was normal (Fig. 6, panel V). There were also notable changes in krox20 mRNA expression at the bud stage. While the krox20 mRNA expression in the third and fifth rhombomere already fused in the midline in the control embryos (Fig. 6, panel K), they remained as two separate domains in the IGF-2a mRNA-injected embryos (Fig. 6, panel L).

At the 15-somite stage, no significant difference was seen with emx1 expression or otx2 mRNA expressing, although the otx2 mRNA expressing domain tended to be smaller (Fig. 6, panels M–P). Likewise, IGF-2a overexpression did not alter the expression patterns of rxl or rx2 mRNA expressing pattern (Fig. 6, panels Q–T). At this stage, pax2a mRNA is expressed in both in the midbrain and hindbrain boundary and the otic vesicles [24 and Fig. 6, panel U]. Interestingly, at this advanced stage, pax2a mRNA expressing cells remained as two separate domains in the IGF-2a mRNA-injected embryos at both stages. This effect appeared to be specific to the brain region as the pax2a expression in the otic vesicles was normal (Fig. 6, panel V). At this stage, the krox20 mRNA expressing cells in the third and fifth rhombomere of the hind brain already fused in the midline in the control embryos (Fig. 6, panel W), while they remained separated domains in the IGF-2a mRNA-injected embryos. Additionally, the distance between the third and fifth rhombomere was shortened in the IGF-2a mRNA-injected embryos (Fig. 6, panel X). These results suggest a possible defect in midline patterning in the IGF-2a mRNA-injected embryos.

To further investigate the possible defects in midline patterning, we performed whole mount in situ hybridization analysis using two midline specific markers, shh [26] and ntl [27], as well as two skeletal muscle markers, myoD [28] and myogenin [29]. As shown in Fig. 7, panels A–D, the expression domain of ntl mRNA at the bud stage was markedly expanded in the IGF-2a mRNA-injected embryos compared to the controls. Among the IGF-2a mRNA-injected embryos, 63% (n = 17) showed marked enlargement in the ntl mRNA expression domain. At the 15-somite stage, a large number of the IGF-2a mRNA-injected embryos showed abnormal ntl mRNA expression patterns (Fig. 7, panel F) and some even had double or partial double notochords (Fig. 7, panel G). Likewise, 71% (n = 21) of the IGF-2a mRNA-injected embryos had enlarged shh mRNA expression domains (Fig. 7, panel M) and 24% had double or partial double notochords (Fig. 7, panel N). In agreement with the enlarged notochord, we found that overexpression of IGF-2a increased the space between the myoD (Fig. 7, panels J and K) and myogenin (Fig. 7, panels O and P) expressing somites at the 15-somite stage. This is most evident in the anterior region of an embryo. Taken together, these results suggest that an excess of IGF signaling causes defects in the midline formation and an expansion of the notochord during early embryogenesis.

**Discussion**

In this study, we have provided evidence suggesting that there are four distinct IGF genes in the zebrafish genome and all four IGF genes are expressed and functional. Although a number of partial or full-length cDNAs encoding several zebrafish IGF peptides have been documented recently, to our knowledge, our study is the first to characterize the structure of these zebrafish IGF genes, map their chromosomal loci, and identify their major transcripts. We show that zebrafish igf-1a has only 1 type of E-domain sequence (Ea like) but two distinct 3’UTRs. All other zebrafish igf genes have one 3’UTR. Both zebrafish igf-2a and igf-1b have two distinct 5’-UTR sequences, which result in two different transcription initiation sites and different signal peptides. Alternative splicing has been previously reported for mammalian IGF-1 and IGF-2 genes [30,31]. For example human IGF-1 has 3 distinct transcripts, which result in 3 distinct types of E-domains (Ea, Eb and Ec) [10,32].

Among the four IGF genes, igf-2a and -2b are clearly the orthologous to human IGF-2 and igf-1a is the IGF-1 ortholog. This conclusion is supported by phylogenetic analysis, detailed structural analysis, and synteny analysis results. The identity of the fourth IGF gene, however, is more enigmatic. This IGF peptide is the same to the so-called IGF-3 recently cloned from Tilapia by Wang et al. [13] recently. Zebrafish IGF-1b/IGF-3 is considerably different from the other 3 zebrafish IGF peptides. Its sequence identity with human IGF-1 is considerably lower (52%) compared to that of zebrafish IGF-1a (82%). Phylogenetic analysis has placed zebrafish IGF-1b in a distinct group, independent from the IGF-1 and IGF-2 subgroups. The synteny relationship of zebrafish igf-1b to the human IGF-1 is also fairly weak. We considered two possible hypothetical scenarios accounting for the origin of this particular zebrafish igf. One is that it represents one of the IGF-1 duplicates but it has diverged significantly from other igf genes after gene duplication due to its restricted expression in the gonads. The other is that it is a novel IGF as proposed by Wang et al. (2008) [13]. We favor the first scenario because it is the simplest way to interpret the data. This would mean that zebrafish igf-1b has diverged at a faster rate than the other IGF paralogs.

Indeed, recent comparative and evolutionary biology studies using large numbers of duplicated genes in *T. nigroviridis*, *D. rerio*, *T. seaerum* and *O. latipes* have shown several cases of asymmetrical acceleration of evolutionary rate among the duplicates after the duplication [33,34,35]. If the second scenario were true, it would mean that one of the IGF-1 duplicates was lost after the genome duplication event and another novel IGF gene was born. Our conclusion is also in agreement with the notion that ray-finned fish have experienced a third whole-genome duplication (3R) approximately 350 million years ago. Therefore, we propose to term this gene as igf-1b and the encoded peptide IGF-1b.

Our finding that there are 4 igf genes in zebrafish and that they each encode a functional IGF ligand is interesting when considering the evolution of the insulin/IGF gene family. There are a number of published studies on the evolution of this important family. Chan et al. (1990) has characterized an insulin-like molecule from the protochordate amphioxus [2]. This molecule contains features characteristic of both vertebrate insulin and IGF, and therefore may represent a modern version of the ancestral molecule for the insulin/IGF gene family [2]. In the Atlantic hagfish, an agnathus, 2
Figure 6. Effect of IGF-2a expression. otx2 expression in the anterior neural plate at the bud stage in a control embryo (panels A and E) and an IGF-2a mRNA injected embryo (panels B and F). pax6a expression in the anterior neural plate at the bud stage in a control embryo (panels C and G) and an IGF-2a mRNA injected embryo (panels D and H). pax2a expression in the midbrain hindbrain boundary at the bud stage in a control embryo (panel I) and an IGF-2a mRNA injected embryo (panel J). krox20 expression in the rhombomere 3 and 5 at the bud stage in a control embryo (panel K) and an IGF-2a mRNA injected embryo (panel L). emx1 expression in the forebrain at the 15-somite stage in a control embryo (panel M) and an IGF-2a mRNA injected embryo (panel N). otx2 expression in the midbrain at the 15-somite stage in a control embryo (panel O) and an IGF-2a mRNA injected embryo (panel P). rx1 expression in the optic vesicle at the 15-somite stage in a control embryo (panel Q) and an IGF-2a mRNA injected embryo (panel R). rx2 expression in the optic vesicle at the 15-somite stage in a control embryo (panel S) and an IGF-2a mRNA injected embryo (panel T). pax2a expression in the midbrain hindbrain boundary at the 15-somite stage in a control embryo (panel U) and an IGF-2a mRNA injected embryo (panel V). krox20 expression in the rhombomere 3 and 5 at the 15-somite stage in a control embryo (panel W) and a 500 pg IGF-2a mRNA injected embryo (panel X). Panels A–D, lateral view, head left; panels E–H, dorsal view, head up; panels I–L and U–X, dorsal view, head left; panels M–T, front view. Black line in panels U, V indicates the gap between the midbrain hindbrain boundary and otic vesicle; black line in panels W, X indicates the gap between rhombomere 3 and 5.

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members of the IGF/insulin gene family have been identified - one is an insulin ortholog and the other is IGF-like. The hagfish IGF is equally related to human IGF-1 and IGF-2, and may therefore be a prototypical IGF molecule [3]. Duguay et al. (1995) have shown that the spiny dogfish, an elasmobranch species, has an IGF-1-like molecule and an IGF-2-like molecule, suggesting the prototypical IGF molecule diverged in an ancestor of the extant gnathostomes [4]. A large number of studies have shown that two distinct IGF genes (IGF-1 and IGF-2) and an insulin gene are present in most vertebrates, including birds, and mammals [1]. Based on the previous studies by others and the current study, we speculate that a single ancestral insulin/IGF molecule is present in protochordate animals. The first genome duplication (1R) that presumably occurred 500 million years ago in metazoans, might have resulted in the appearance of a distinct insulin molecule and an IGF molecule in agnathans. The second round whole-genome duplication (2R) that occurred 400 million years ago, might have resulted in two functional IGF/insulin family. The retention of these IGF genes indicates that each of them may have evolved unique and indispensable roles. This notion is supported by a recent study reporting distinct embryonic roles of zebrafish IGF-2a and -2b [12]. In this study, we provide further evidence that these zebrafish IGF genes have evolved overlapping yet distinct biological properties by attaining distinct temporal expression patterns, different peptide structure, and different biological activities (Fig. 8). The duplicated igf-1 and duplicated igf-2 appear to have diversified in several ways. First, each of these IGF ligand genes exhibits distinct spatial and temporal expression patterns. During early development, IGF-1b and IGF-2a mRNAs are detectable throughout the embryogenesis, while IGF-1a and IGF-2b mRNAs are detectable only after the gastrula stage. In the adult, IGF-2a mRNA is found in all tissues examined with the exception of the muscle and gill, while IGF-2b mRNA is only expressed in the liver. In comparison, IGF-1a mRNA is detectable in the liver brain, and eyes. IGF-1b mRNA, on the other hand, is exclusively expressed in the ovary and testes. Furthermore, these 4 IGF peptides have clear differences in their primary structure and biological activities. When tested in zebrafish embryos by mRNA injection experiment, all resulted in the activation of Akt signaling and had biological effects in developing zebrafish embryos. These findings make an important advance in our understanding of the structural and functional evolution of the IGF/insulin family.

We have previously reported that zebrafish contain two functional igf-1r genes [5,16,36], two functional igfbp-1, two igfbp-2, and two igfbp-5 genes in zebrafish [37,38]. In this study, we have shown that there are two functional igf-1 and two igf-2 genes in zebrafish. Therefore, it appears that most, if not all, components in the IGF signaling pathway are retained as duplicates in the zebrafish genome. The retention of these IGF genes indicates that each of them may have evolved unique and indispensable roles. One intriguing finding made in this study is that after duplication each pair has evolved very distinct expression profiles. While zebrafish IGF-1a mRNA is easily detected in multiple adult tissues in zebrafish, IGF-1b mRNA is only found in the gonad. Likewise, IGF-2b mRNA is only detected in the liver whereas IGF-2a mRNA is
expressed in most adult tissues. During embryogenesis, IGF-1b and IGF-2a mRNAs are detected from the 1-cell stage and throughout embryogenesis, whereas IGF-1a and IGF-2b mRNAs become detectable after the gastrula stage. In mammals, IGF-1 mRNA is highly expressed in the adult liver under the control of growth hormone (GH). IGF-1 mRNA is also expressed in many non-hepatic tissues and that extra-hepatic sources of IGF-1 can compensate for the absence of hepatic IGF-1 production in mice [39]. In juvenile and young salmon and trout, detectable amounts of IGF-2 mRNA are found in virtually all tissues examined, but the liver has the highest levels, suggesting that the liver is the major site of IGF-I mRNA production, as is the case in mammals and birds [40,41,42]. There is good evidence that GH regulates IGF-1 expression in teleost fish [43,44,45,46]. In mammals, IGF-2 is an important fetal growth factor and the IGF-2 gene is highly expressed in the mammalian fetus [47]. In rodents, the IGF-2 gene is highly expressed in the fetus [47]. Similarly, abundant IGF-2 mRNA is detected in fish embryos and larva [48]. Considerable interspecies variations are found during postnatal or posthatching regulation of IGF-2 gene expression. In the rat, the IGF-II gene expression declines after birth in all tissues except for choroid plexus and leptomenings, whereas in humans and guinea pigs levels of IGF-II mRNA remain high in many adult tissues [47]. Among the 4 zebrafish IGF genes, IGF-1a and IGF-2a are expressed in multiple adult tissues in zebrafish, resembling the profiles of their mammalian orthologs. In contrast, zebrafish IGF-1b mRNA is detected only in the gonad and IGF-2b mRNA in the liver. These data suggest that the duplicated IGF genes have undergone subfunction partitioning in their expression.

The finding that igf-2b is exclusively expressed in the liver of adult zebrafish is intriguing in light of the previous reports that IGF-2 mRNA levels are high in the adult liver and other tissues in rainbow trout and seabream [42,40] and that GH treatment significantly increases the IGF-2 mRNA levels in the liver in rainbow trout [46]. This raises the interesting possibility that IGF-2a may be a major form of IGF-2 mRNA in the circulation in adult zebrafish. The gonad-specific expression of igf-1b in zebrafish is in good agreement with a recent report in tilapia [13]. IGF-1 has been shown to act in a paracrine fashion to regulate ovarian steroidogenesis in humans [49]. IGF-1 plays an important role in mammalian testicular function. Fish ovaries express IGF-1 mRNA [44,50] and have specific binding sites for IGF-1 [51]. IGF-I binding to fish ovaries activates receptor tyrosine kinase activity and increases gonadotropin II-stimulated steroidogenesis in salmon granulosa cells [51]. A role of IGF-1 in inducing final oocyte maturation has also been demonstrated in fish [52]. The discovery of a gonad-specific IGF gene in teleost fish highlights the importance of local IGFs in the gonad and opens new avenues for future studies.

IGF signaling is widely considered a key growth regulator in fetal and postnatal stages [1,53]. This dogma is primarily derived from mouse genetic studies. Overexpression of IGF-1 in mice increases the body weight by 30% [54]. IGF-1 or IGF-II knockout mice had birth weights about 60% that of their littersmates, while IGF-1/IGF-II double knockout mice were even smaller, weighing only 30% of their littersmates [55,56]. The IGF-IR knockout mice had a birth weight 45% of their littersmates [55,56]. The importance of IGFs in growth is also supported by the growth-retarded phenotype resulting from mutations in human IGF-I or IGF-IR genes. A homozygous partial deletion in the human IGF-I gene is associated with fetal and postnatal growth retardation [57]. Patients with point mutations in the IGF-IR gene exhibited severe intrauterine growth restriction and poor postnatal growth [58]. Recent studies in lower vertebrates suggested that IGF signaling may play additional roles during early development. In Xenopus, ectopic expression of IGF-2 was shown to induce anterior neural tissues [59]. Inhibition of IGF signaling using a dominant-negative IGF-IR or antisense morpholino reduced anterior tissues in Xenopus [59,60]. Contradictory evidence has been reported on this issue in zebrafish. Evers et al. (2004) reported that overexpression of a secreted form of IGFIR (a truncated IGF-IR lacking both the transmembrane and intracellular domains) in zebrafish resulted in defects in anterior neural structure and overexpression of IGF-1 resulted in expansion in anterior structures at the expense of trunk and tail [61]. Loss-of-function studies in zebrafish, however, showed that suppression of IGF signaling by a dominant-negative IGF-IR or antisense morpholinos did not result in any anterior/neural patterning defects [16,36]. Likewise, knockdown of zebrafish igf-2a and/or igf-2b did not cause defects in anterior neural patterning [12]. In this study, we have shown that an excess of IGF signaling did not cause notable anterior defects in zebrafish. These mRNAs encoding various IGF ligands were clearly functional because they induced Akt phosphorylation and caused morphological changes in developing zebrafish embryos. The lack of notable anterior/neural patterning defects in zebrafish revealed by previous loss-of-function studies [12,16,36] and the current gain-of-function study strongly argue against a critical role for IGF signaling in anterior/neural induction in zebrafish.

A major finding made in this study is that IGF signaling plays a critical role in midline and notochord development. When tested in zebrafish embryos by mRNA injection experiments, all 4 zebrafish IGFs resulted in delayed mesoderm involution, poorly formed notochords and widening distance between somites. Our results indicate that ectopic expression of zebrafish IGF-2a or any IGF ligands, resulted in defects in the midline formation and an
expansion of the notochord. These morphological changes are associated with a marked expansion in the null mRNA expression domain and myoD mRNA expression domain. Overexpression of IGF-2a also caused a widening in the space between the myoD and myogenin in the anterior region of an embryo. Many of these embryos had duplicated or partial duplicated notochords, suggesting that an excess of IGF signaling causes defects in the midline formation and an expansion of the notochord. Brown et al. (2009) reported that knockdown of either ifg-2a or ifg-2b led to defects in dorsal midline development, although knockdown of ifg-2b also induced ectopic fusion of the neophron primordia [12]. Collectively, these studies suggest that IGF signaling plays a critical role in midline formation and notochord development. These results not only provide new insights into the functional conservation and divergence of the multiple ifg genes but also reveal a novel role of IGF signaling in midline formation and notochord development in a vertebrate model. Given the central role of IGF signaling in growth regulation, it will be important to investigate whether this high rate of gene retention in genes belonging to the IGF signaling pathway in the ray-finned fish plays any role in the continuous growth pattern of the teleosts.

Materials and Methods

Chemicals and reagents

All chemicals and reagents were purchased from Fisher Scientific (Pittsburgh, PA) unless otherwise noted. Restriction endonucleases were purchased from New England BioLabs (Beverly, MA). Superscript II reverse transcriptase (RT) and Oligonucleotide primers were purchased from Invitrogen Life Technologies, Inc. (Carlsbad, CA). RNase-free DNase was obtained from Promega (Madison, WI). The anti-Akt and anti-Phospho-Akt antibodies (Ser473) were purchased from Cell Signaling (Danvers, MA).

Experimental animals

Zebrafish (Danio rerio) were maintained at 28 C on a 14 h:10 h (light:dark) cycle, and fed twice daily. Embryos were generated by natural crosses. Fertilized eggs were raised in embryo medium at 28.5 C and staged according to the standard criteria of Kimmel et al. [62]. For in situ hybridization analysis, embryo medium was supplemented with 0.003% (w/v) 2-phenylthiourea to inhibit embryo pigment formation. All experiments were conducted following guidelines approved by the University of Michigan Committee on the Use and Care of Animals.

Molecular cloning

Searching the zebrafish EST and genome databases (Ensembl Zebrafish: http://www.ensembl.org/Danio_rerio/blastview) and the NCBI database (http://www.ncbi.nlm.nih.gov/) identified two distinct IGF-2 mRNAs (GenBank Accession No. XM_001383006, 3404 bp; NM_131433, 2065 bp, respectively) and one IGF-1 mRNA (GenBank Accession No. NM_131825, 1521 bp). We further searched these databases by BLAST searches using the IGF-1 sequence as a query. Two IGF-1-like EST sequences (GenBank Accession No. XM_684982, 636 bp; CO53655, 600 bp, respectively) were obtained. Based on these sequences, the following gene-specific primers were designed to clone and confirm the predicted IGF cDNA sequences.

ifg-1b: 5’ RACE primer 5’-AAAGCCYCTGTCCTCCRCACAACAATCTSCAG-3’, 5’ nest RACE primer 5’-TCTGCGCCCTGTCAGGTGTGCTGGACG-3’; 3’ RACE primer 5’-CTGAGTGTGTTGCTGGACG-3’; 5’ RACE primer 5’-ATAGCCACTCTTCTGTAAGG-3’ and 3’ nest RACE primer 5’-TGAGGACACGCGGGAGGATAAACTGAGTA-3’.

Phylogenetic analysis and physical mapping

The genomic structure and chromosomal location of each zebrafish ifg gene were determined by searching the zebrafish genome (http://www.ensembl.org/Danio_rerio/index.html). Amino acid sequences of IGFs were aligned by CLUSTAL W. Phylogenetic analysis was done using B and A domain sequences by Neighbor-joining method in the MEGA3 program. Gap sites in the alignment were not used in the phylogenetic reconstruction. The reliability of the estimated tree was evaluated by the bootstrap method with 1000 pseudo-replicates. Synten analysis was carried out based on Danio rerio Zv6 and Homo sapiens Build 36.3 (http://www.ensembl.org/Homo_sapiens/index.html) and data from zebrafish and human synten map. Genomic structure was determined by searching the zebrafish genome (http://www.ensembl.org/Danio_rerio/index.html).

Plasmid construction

The open reading frame of each zebrafish IGF gene was amplified by PCR using Pfu DNA polymerase (Stratagene, La jolla, CA, USA). The following primers were used: ifg-1a 5’-GGTGAATTCCAGTCTCGAGTGGTCTCACATCC-3’ and 5’-GGTCTACGAGCTGAGATCTAGG-3’; ifg-1b 5’-GGTGAATTCCAGTCTCGAGTGGTCTCACATCC-3’ and 5’-GGTCTACGAGCTGAGATCTAGG-3’; ifg-2a 5’-GGTGAATTCCAGTCTCGAGTGGTCTCACATCC-3’ and 5’-GGTCTACGAGCTGAGATCTAGG-3’. The resulting DNA was cloned into the pGEM-T Easy vector (Promega, USA). To gene-recombine the IGF peptide expression construct, the above PCR product was digested with BamHI and XhoI and subcloned into BamHI and XhoI sites of pG82+.

Reverse transcription (RT)-PCR and whole mount in situ hybridization

Total RNA was isolated from embryo or adult tissues using the TRIzol reagent (Invitrogen). One µg of RNA was reverse-transcribed to single strand cDNA using SuperScript II reverse
transcriptase according to the following protocol. The following primers were used for the PCR amplifications: 5'-gggagt-cgtttgctcag-3' and 5'-ccctcaggaaggagttgccatt-tat-3'; 5'-tggtggctttctcctct-3' and 5'-ttcacccttgctggttctcagag-3'; 5'-tggtggctttctcctct-3' and 5'-tggtggctttctcctct-3'; 5'-tggtggctttctcctct-3' and 5'-tggtggctttctcctct-3'.

Bromide staining and a no-template control was the negative control. The products were analyzed by electrophoresis followed by ethidium bromide staining and a no-template control was the negative control.

Whole mount in situ hybridization using digoxigenin (DIG)-labeled RNA riboprobe was carried out essentially as reported previously [63]. Each template plasmid was linearized by restriction enzyme digestion, followed by in vitro transcription reactions with T7 or Sp6 RNA polymerase to generate the antisense or sense RNA riboprobes. Embryos were hybridized with the appropriate riboprobe at 65°C for 3 min, 34 cycles of 94°C for 30 s, 53°C (igf-1a and igf-1b) or 56°C (igf-2a, igf-2b, and igf-2c) for 30 s, and 72°C for 1 min. PCR products were analyzed by electrophoresis followed by ethidium bromide staining and a no-template control was the negative control.

Microinjection experiments
Capped mRNA was synthesized using a commercial kit and linearized plasmid DNA as template (mMESSAGE mMACHINE kit; Ambion, Inc.). mRNA (500 pg per embryo) was microinjected into zebrafish embryos at the 1-2 cell stage as reported previously [36]. GFP mRNA injected and wild type embryos were used as controls. After injection, embryos were placed in embryo rearing medium and kept at 28.5°C. Western immunoblot
Twenty embryos from each treatment group were dechorionated, deyolked, and homogenized in 40 μl of RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 0.1% Triton X-100, pH 7.5) containing 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 100 mM PMSF. The homogenates were briefly centrifuged to pellet cellular debris and the supernatant was retained. Protein levels of each sample were quantified using a protein assay kit (Pierce Biotechnology, Rockford, IL). Equal amounts of protein were analyzed by SDS-PAGE and Western immunoblot as previously described [36]. Antibodies against total Akt or the phospho-Akt (Ser473) were used at a 1:1000 dilution.

Statistics
Values are means±S.E. Differences among groups were analyzed by one way ANOVA followed by Fisher's Post Hoc tests or unpaired t-test. Significance was accepted at p<0.01.

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Author Contributions
Conceived and designed the experiments: CD. Performed the experiments: SZ HK CD. Wrote the paper: SZ CD.

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