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A zebrafish view of the insulin-like growth factor (IGF) signaling pathway*

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Abstract Insulin-like growth factors (IGFs), including IGF-Ⅰ and IGF-Ⅱ, are evolutionarily conserved peptides that are essential for vertebrate growth and development. The biological actions of IGFs are mediated through the IGF-Ⅰ receptor, a receptor tyrosine kinase. Recent studies utilizing cell cultures suggest that the bioactivity of IGFs could also be modulated by several high-affinity IGF-binding proteins (IGFBPs). The developmental roles of various members of the IGF signaling pathway, however, are not well underatood. Research in this area has relied heavily on rodent models, and attempts have been hampered by the inaccessibility of the mammalian fetus which is enclosed in the uterus. In recent years, there has been a remarkable acceleration in our understanding of the IGF signaling system in a model teleost fish, the zebrafish. To date, cDNAs encoding zebrafish IGF-Ⅰ, IGF-Ⅱ, IGF-Ⅰ receptors (IGF-ⅠRs), and several IGFBPs have been cloned and characterized, and their spatial and temporal expression patterns determined using the transparent zebrafish embryos. Using cultured cells and transgenic fish, the actions of IGFs, IGF-ⅠRs and IGFBPs in regulating cell proliferation, differentiation, and apoptosis during early development have been studied. This review will focus on our current understanding of the structure, gene expression, physiological regulation, and biological actions of IGF ligands, receptors, and IGFBPs in zebrafish. The unique value of the free-living and transparent zebrafish embryos for growth physiology will also be discussed [*Acta Zoologica Sinica* **49** (4)：421－431，2003].

Key words Insulin-like growth factor (IGF), IGF receptor, IGF binding protein, Growth and development, Zebrafish, Model organism, Gene expression, Gene knockdown, Transgenic animal

类胰岛素生长因子信号途径及其对脊椎动物生长和发育的调控作用：以斑马鱼为模式动物的新进展*

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摘　要　类胰岛素生长因子（包括 IGF-Ⅰ和 IGF-Ⅱ）是进化上保守性很强的多肽。IGFs 对脊椎动物的生长和早期发育有极其重要的调控作用。IGF 的生理作用是由 IGF 受体中介并受几个分泌性的 IGF 结合蛋白调节。本文主要介绍了以斑马鱼为模式动物，用基因敲除、转基因动物和培养细胞系等现代实验方法对 IGF 信号途径的最新研究进展，综述了 IGF 配体、受体和结合蛋白的结构特点、基因表达和调节和生物学功能。此外，也对斑马鱼作为模式动物的许多优点进行了探讨［动物学报 **49**（4）：421～431，2003］。

关键词　类胰岛素生长因子　IGF 受体　IGF 结合蛋白　生长发育　斑马鱼　模式动物　基因表达　基因敲除　转基因动物

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1 Introduction

The size of an animal or organ is affected primarily by the number and size of its cells. While cell number is determined by the rate of cell proliferation and cell death, changes in cell size usually are the result of cell differentiation. Growth (increase in size) is therefore affected by the proliferative events out balancing cell death, with additional contributions by cell hypertrophy and deposition of extracellular materials. This process begins early in embryogenesis and lasts until a steady state is reached in postnatal stage. Most animals (except for some fish, molluscs and crustaceans) are genetically programmed to grow to a characteristic size as adults. The normal size of most organs is also intrinsically programmed, as are the sizes of the cells that make up these organs. One of the most challenging questions facing the field of regulatory biology is to understand what are the developmental signals that control the body or organ size (Conlon *et al.*, 1999). Current views hold that growth is brought about by the operation of intracellular signaling pathways controlled by a network of growth factors and hormones. Recent studies suggest that the insulin-like growth factor (IGF) signaling system is a central part of this hormonal regulatory network.

There are three components to the IGF signaling system: the IGF ligands, receptors, and binding proteins (Fig. 1). The two ligands, IGF-Ⅰ and IGF-Ⅱ, are polypeptides structurally related to proinsulin. Most, if not all, of the biological actions of IGFs are mediated by the type Ⅰ or IGF-Ⅰ receptor (IGF-IR). In mammals, a second transmembrane protein, the IGF-Ⅱ/cation-independent mannose-6-phosphate (M-6-P) receptor, exists, and it has higher binding affinity for IGF-Ⅱ over IGF-Ⅰ. Binding of IGF-Ⅱ to this receptor has been shown to cause internalization and degradation of IGF-Ⅱ (Le Roith *et al.*, 2001). In addition to the ligands and receptors, there exists another important component of the IGF system, the IGF binding proteins (IGFBPs). Six distinct IGFBPs, designated as IGFBP-1 to -6, have been isolated and cloned from human and other mammals and each represents an individual gene product (Jones *et al.*, 1995; Firth *et al.*, 2001; Duan, 2002). These proteins act as carrier proteins in the bloodstream and control the efflux of IGFs from the vascular space. IGFBPs have been shown to either enhance and/or inhibit IGF actions in cultured mammalian cells. IGF-independent actions have also been demonstrated for some IGFBPs. In addition to the six IGFBPs, a number of proteins, termed IGFBP-related proteins, have been reported to bind IGFs with low affinity (Oh *et al.*, 1998).

It has long been established that IGF-Ⅰ mediates many of the growth-promoting effects of growth hormone (GH) during mammalian postnatal life (Daughaday *et al.*, 1989). Studies utilizing "knockout" mice have demonstrated that both IGF-Ⅰ and IGF-Ⅱ are essential for embryonic growth as well (Liu *et al.*, 1993; Baker *et al.*, 1993). For instance, the birth weight of mice lacking both IGF-Ⅰ and IGF-Ⅱ is approximately 30% of the normal control, and they invariably died shortly after birth. While we know much about the gross effects of the IGF signals in the overall size of the fetus and the clinical manifestations that result from fetal and neonatal deficiency of IGFs (i. e., severe growth retardation - dwarfism), very little is known about the mechanisms by which IGFs, IGF receptors, and in parti-cular IGFBPs act *in vivo* to regulate cell proliferation, differentiation, and apoptosis in a vertebrate embryo. Most research on the developmental role of IGFs has relied on rodent models, and attempts to elucidate the molecular and cellular basis of IGF actions have been difficult due to the inaccessibility of the mammalian fetus enclosed in the uterus.

Unlike mammalian embryos which live within the uterus and are dependent on maternal contributions through the placenta, fish embryos and larva grow freely in water. Hence, the accessibility and rapid development of teleost fish, make them well-suited for investigating the mechanisms by which IGFs, IGF-IR, and IGFBPs act to regulate cell proliferation, differentiation, and apoptosis in early life stages. The optical transparency of zebrafish embryos provide an additional, immense advantage to their use in developmental studies and makes zebrafish a particularly suitable model system for investigating the mechanisms of IGF actions during early development. Furthermore, recent advances in systematic compilation of information on genomic sequences and expressed sequence tags (EST) have made the cloning of zebrafish genes easier. Outlined below is a synopsis of our current understanding of the teleost IGF signaling system. Since information concerning the fish IGF system has been the topic of several recent reviews (Duan, 1997, 1998; Reinecke *et al.*, 1998; Kelley *et al.*, 2002), this review will focus on the latest progress pertaining to the zebrafish model.

2 The structure, function, and developmental expression of IGFs

To date, the sequences of IGF-Ⅰ and IGF-Ⅱ have been determined in a number of vertebrate species, and their expression has been studied (see above mentioned reviews). Sequence comparison indicates that the structures of fish IGF-Ⅰ and IGF-Ⅱ

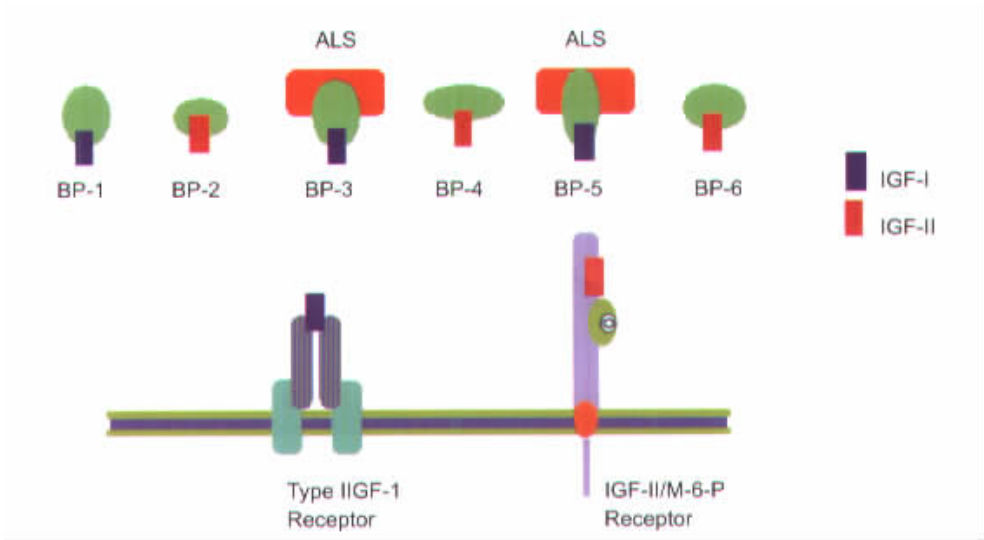

Fig. 1 Schematic diagram showing various components of the IGF signaling pathway

There are two ligands, IGF-Ⅰ and IGF-Ⅱ (indicated by rectangles), two receptors, IGF-Ⅰ receptor and IGF-Ⅱ receptor/manose-6-phosphate (M-6-P) receptor, and six secreted high-affinity IGF binding proteins (BP-1 to BP-6). In the circulation, IGFBP-3 and IGFBP-5 can form ternary complex with IGF and another protein called acid labile subunit (ALS)

are highly similar to those of other vertebrates. For example, zebrafish IGF-Ⅰ and IGF-Ⅱ can be divided into B, C, A, D, and E domains. They both have 6 cysteine residues at B6, B18, A6, A7, A11, and A20 position, which are responsible for maintenance of their tertiary structure. At the amino acid level, the mature zebrafish IGF-Ⅰ is 97%, 94% and 80% to that of carp, salmon and human respectively, and the mature zebrafish IGF-Ⅱ is 76% and 71% identical to that of rainbow trout and human, respectively. Consistent with the high degree of structural conservation, functional analyses indicate that the IGF biological activities are also conserved. For instance, fish, chicken, and human IGFs are equally effective in stimulation of fish and mammalian cell growth and DNA synthesis (Bauchat *et al*., 1997; Upton *et al*., 1998; Pozios *et al*., 2001). These findings suggest that the structure and function of IGFs are highly conserved throughout vertebrate evolution.

The temporal and spatial expression profiles of IGF-Ⅰ and IGF-Ⅱ in zebrafish are similar to those reported for other vertebrates. In the adult fish, IGF-1 mRNA is expressed in various adult tissues with the highest levels in the liver and testes (Maures *et al*., 2002). As in other vertebrate species, the IGF-Ⅰ mRNA level was elevated after growth hormone treatment and reduced after prolonged starvation. High levels of IGF-Ⅱ mRNA were found in all the tissues examined with the exception of ovary. As it was reported in rainbow tout and gilthead seabream (Greene *et al*., 1997, 1999; Funkenstein *et al*., 1996, 1997; Perrot *et al*., 1999), transcripts for IGF-Ⅰ and IGF-Ⅱ are detected throughout the early development, including unfertilized eggs, cleavage, blastulation, gastrulation, segmentation and hatching (Maures *et al*., 2002). Further whole mount *in situ* hybridization analysis indicate that both IGF-Ⅰ and IGF-Ⅱ mRNAs are present in zebrafish embryos in an ubiquitous fashion. Therefore, it appears that IGF-Ⅰ and IGF-Ⅱ are present in most, if not all, cells in developing zebrafish embryos throughout the embryogenesis. This pattern of expression is suggestive of a universal role for IGFs as paracrine and/or autocrine growth regulators during early development.

3 The IGF-Ⅰ receptors and their downstream signaling network

The biological actions of IGFs are mediated through their cell surface receptors. In mammals, two types of IGF receptors, the IGF-Ⅰ receptor and IGF-Ⅱ/M-6-P receptor, have been characterized. As mentioned earlier, the biological actions of IGFs are thought to be mediated through interaction with the IGF-ⅠR. The role of the IGF-Ⅱ/M-6-P receptor or type-Ⅱ receptor remains obscure, but comparative studies indicate that chicken and amphibian M-6-P receptor does not possess the high affinity IGF-binding site (Clairmont *et al*., 1989; Canfield *et al*., 1989; Yang *et al*., 1991). Therefore, the IGF-Ⅱ binding property of this receptor, as well as, any of its physiological functions with regards to IGFs, might have been a later acquisition during evolution.

Early studies have shown the presence of IGF receptor-like proteins in fish liver, brain, ovaries,

skeletal muscle, and testes (Drakenberg *et al.*, 1993; Loir *et al.*, 1994; Gutierrez *et al.*, 1995; Parrizas *et al.*, 1995a, 1995b). Affinity cross-linking and immunoblotting studies of zebrafish embryonic cells revealed that the fish IGF receptors are receptor tyrosine kinases with similar biochemical characteristics to the mammalian type Ⅰ IGF-Ⅰ receptor (Pozios *et al.*, 2001). Competitive binding assay results indicated that the binding affinities of the zebrafish IGF-Ⅰ receptors to IGF-Ⅰ, IGF-Ⅱ, and insulin are 1.9 nM, 2.6 nM, and > 190 nM (Pozios *et al.*, 2001). This suggests that IGF-Ⅰ and IGF-Ⅱ bind to the fish IGF-Ⅰ receptors with approximately equally high affinity. Since late 1990s, partial sequences of IGF-Ⅰ receptor genes have been reported in several teleost species (Elies *et al.*, 1996; Chan *et al.*, 1996; Green *et al.*, 1999b; Ayaso *et al.*, 2002). Recently, the full-length structure of two distinct IGF-ⅠRs have been identified and characterized in zebrafish (Maures *et al.*, 2002). Structural and phylogenetic analyses suggest that these two IGF-ⅠRs are encoded by two separate genes and both genes are orthologous to the human *igf-1r* gene. They are therefore termed zebrafish *igf-1ra* and *igf-1rb*. The structure of the two zebrafish IGF-ⅠR proteins is shown in Fig. 2. Both of the receptor proteins can be divided into a N-terminal domain, a cysteine-rich domain, a tetrabasic proteolytic cleavage site, a transmembrane domain with juxtamembrane motifs, a tyrosine kinase (TK) domain, and a carboxy-terminal domain. Among these domains, the TK domain is the most highly conserved (96%). Moreover, both TK domains contain structural features with known functional significance, including a potential ATP-binding motif, a triple tyrosine cluster that constitutes the major autophosphorylation site for ma-mmalian IGF-ⅠR. A consensus IRS-1 binding motif, which is known to be critical for IGF-ⅠR signaling, is located at positions 975 – 978 and 965 – 968 in IGF-ⅠRa and IGF-ⅠRb, respectively. The cysteine-rich regions, which are known to be important for ligand binding specificity, contain 26 conserved cysteine residues in a stretch of 196 aa extending from positions 156 – 352 and 151 – 348 in IGF-ⅠRa and IGF-ⅠRb, respectively. With the exception of the last 24 aa at the very C-terminus, the cytoplasmic regions of these IGF-ⅠRs are highly divergent. In addition, an extra 15 aa sequence (APSSQGNTGIQSQNP) is present in this part of the IGF-ⅠRa (Maures *et al.*, 2002). Taking advantage of the free-living and transparent zebrafish embryos, the spatio-temporal expression patterns of the two IGF-ⅠRs were determined using a combination of RT-PCR, whole mount *in situ* hybridization and immunocytochemistry. IGF-ⅠRa and IGF-ⅠRb mRNAs and proteins were expressed in overlapping spatial domains but exhibited distinct temporal expression patterns. In particular, the relative level of IGF-

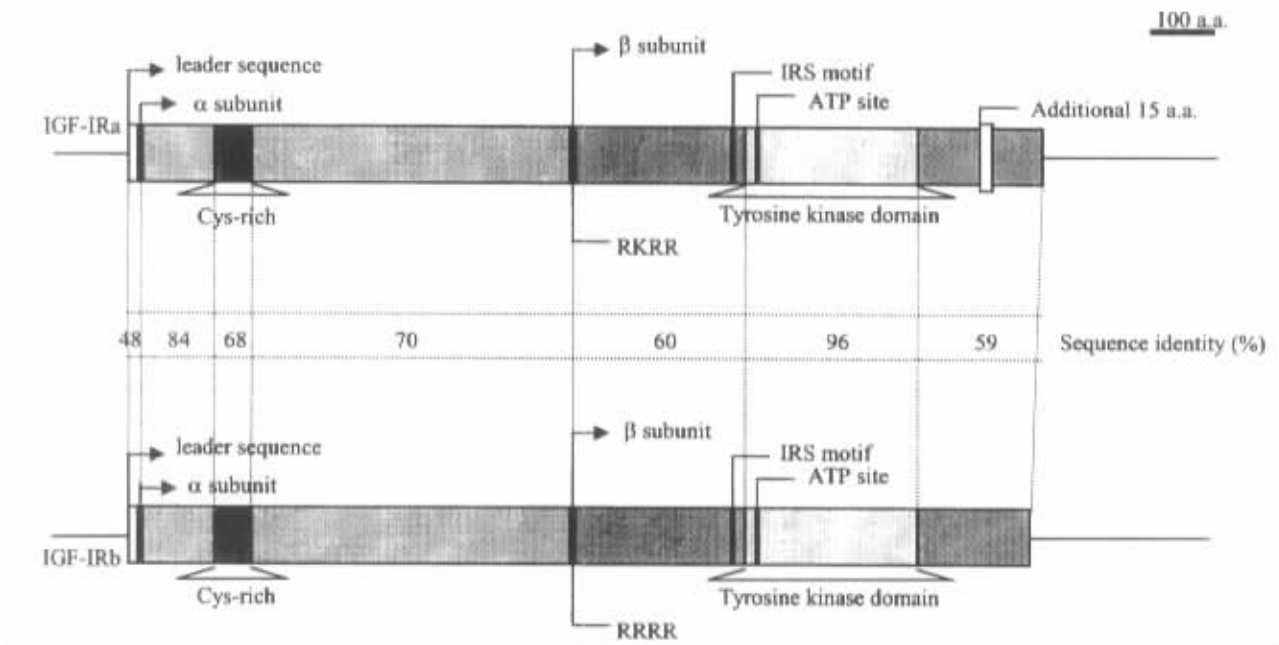

Fig.2 Schematic diagram comparing the domain structures of zebrafish IGF-ⅠRa and IGF-ⅠRb

In addition to the leader sequence, α subunit, and β subunit, the cysteine-rich region, the tetrabasic proteolytic cleavage site (RKRR IGF-ⅠRa and RRRR for IGF-ⅠRb), the tyrosine kinase domain, the potential ATP-binding site, and IRS-1 binding site are indicated. The extra 15 aa found in IGF-ⅠRa is indicated by an open box. The percentage of sequence identity for each domain is calculated and presented in the middle column

ⅠRa mRNA was low during early embryogenesis and increased in the hatched larva whereas the situation was different for IGF-ⅠRb mRNA (Maures *et al*., 2002). In adult zebrafish, the overall tissue distribution patterns of the two IGF-ⅠRs were similar but there were differences in their cellular localization and relative abundance in defined cells/regions. The differential expression pattern of IGF-ⅠRa and IGF-ⅠRb suggest that they may play distinct roles in regulating growth and development of zebrafish.

The *in vivo* functions of the *Igf*-1*r* gene in embryonic growth and development was first elucidated by studies using mice homozygous for a targeted disruption of the *Igf*-1*r* gene (Liu *et al*., 1993). These animals die invariably at birth of respiratory failure and exhibit a severe growth deficiency (45% normal size). In addition to generalized organ hypoplasia in these *Igf*-1*r* (-/-) embryos, deviations from normalcy were observed in the central nervous system and epidermis. This and other studies have led to the conclusion that the IGF signal is critical for size control. Recent studies using non-mammalian model organisms, however, have revealed that the IGF signal is not only important for growth control, but also critical for the organogenesis of several vital organs. Pera *et al*. (2001) have shown that introduction of IGF mRNAs to *Xenopus* embryos by microinjection led to the induction of ectopic eyes and ectopic head-like structures containing brain tissue. Expression of a dominant-negative IGFR (DN-IGFR) had the opposite effect. Similarly, knock-down of the *Xenopus* IGF-ⅠR using antisense morpholino oligonucleotides drastically reduces eyes and other nervous tissue (Richard-Parpaillon *et al*., 2002). Thus, the IGF pathway is required for anterior neural induction in *Xenopus*. The potential actions of the IGF signal in other organs were not explored in these studies. *Xenopus* are tetraploidy animals and have two *igf-ir* genes like zebrafish. It was not clear whether both of the IGF-ⅠR proteins or a particular isoform were depleted in these studies. Recently, we have knocked-down the two zebrafish *igf-r* genes using the antisense morpholino oligonucleotide approach (Schlueter *et al*., 2003). The double IGF-ⅠR knocked-down morphants were severely growth retarded and developmentally impaired, and they died invariably before 30 hours post fertilization (hpf). These phenotypic changes are consistent with findings in the mouse model. As in the case of *Xenopus*, the IGF-ⅠR morphants had no morphologically recognizable eye (Fig. 3). Furthermore, the IGF-ⅠR knocked-down zebrafish morphants had no otic vesicle, no myoseptum, greatly diminished heart, and significantly lower cardiac rate. These results suggest that the IGF-ⅠR signaling is not only important for growth but also critical for the organogenesis of eye, inner ear, heart, and perhaps other organs.

There has been very limited information regarding the intracellular signaling cascades acting downstream of the IGF-ⅠRs in fish until recently (Fruchtman *et al*., 2000; Pozios *et al*., 2001). To investigate the cellular mechanism of IGF actions, we have studied the effects of IGFs on two major signal transduction pathways: mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3 kinase) pathways (Pozios *et al*., 2001). IGFs activated MAPK in zebrafish embryonic cells in a dose-dependent manner. This activation occurred within 5 minute of IGF-Ⅰ stimulation and disappeared after 1 hour. IGF-Ⅰ also caused a concentration-dependent activation of PKB/Akt, a downstream target of PI3 kinase, this activation being sustained for several hours. Inhibition of MAPK activation by the MEK1 inhibitor PD98059 inhibited the IGF-Ⅰ-stimulated DNA synthesis. Similarly, use of the PI3 kinase inhibitor LY294002 also inhibited IGF-Ⅰ-stimulated

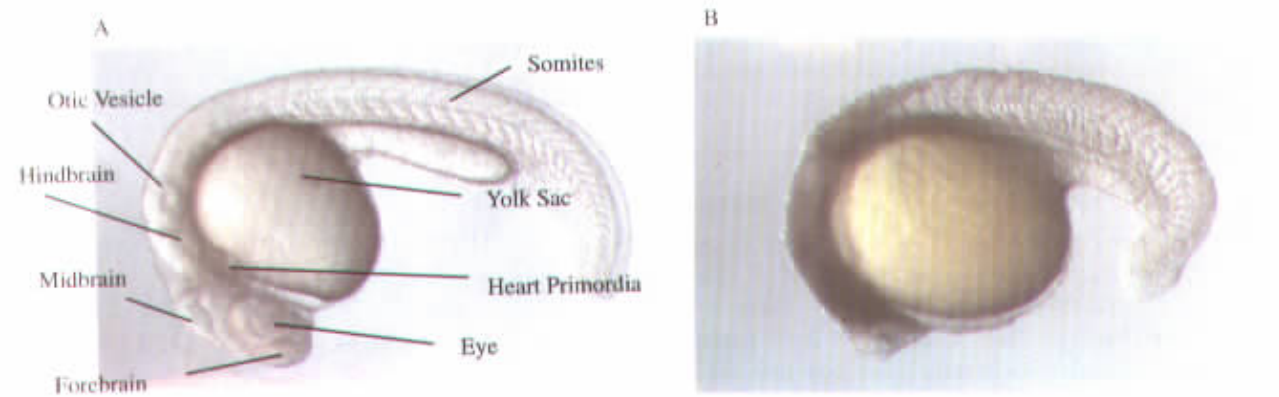

Fig.3 Targeted knockdown of the two zebrafish *igf*-1*r* genes reveals novel functions of the IGF signal in eye, inner ear, and heart development

A. A lateral view of a wild-type 24 hpf zebrafish embryo. Major organs are labeled B. A lateral view of a 24 hpf IGF-ⅠR morphant. The morphant is smaller and had fewer somites. Also note the lack of eyes, otic vesicle, heart, myoseptum in the IGF-ⅠR morphant

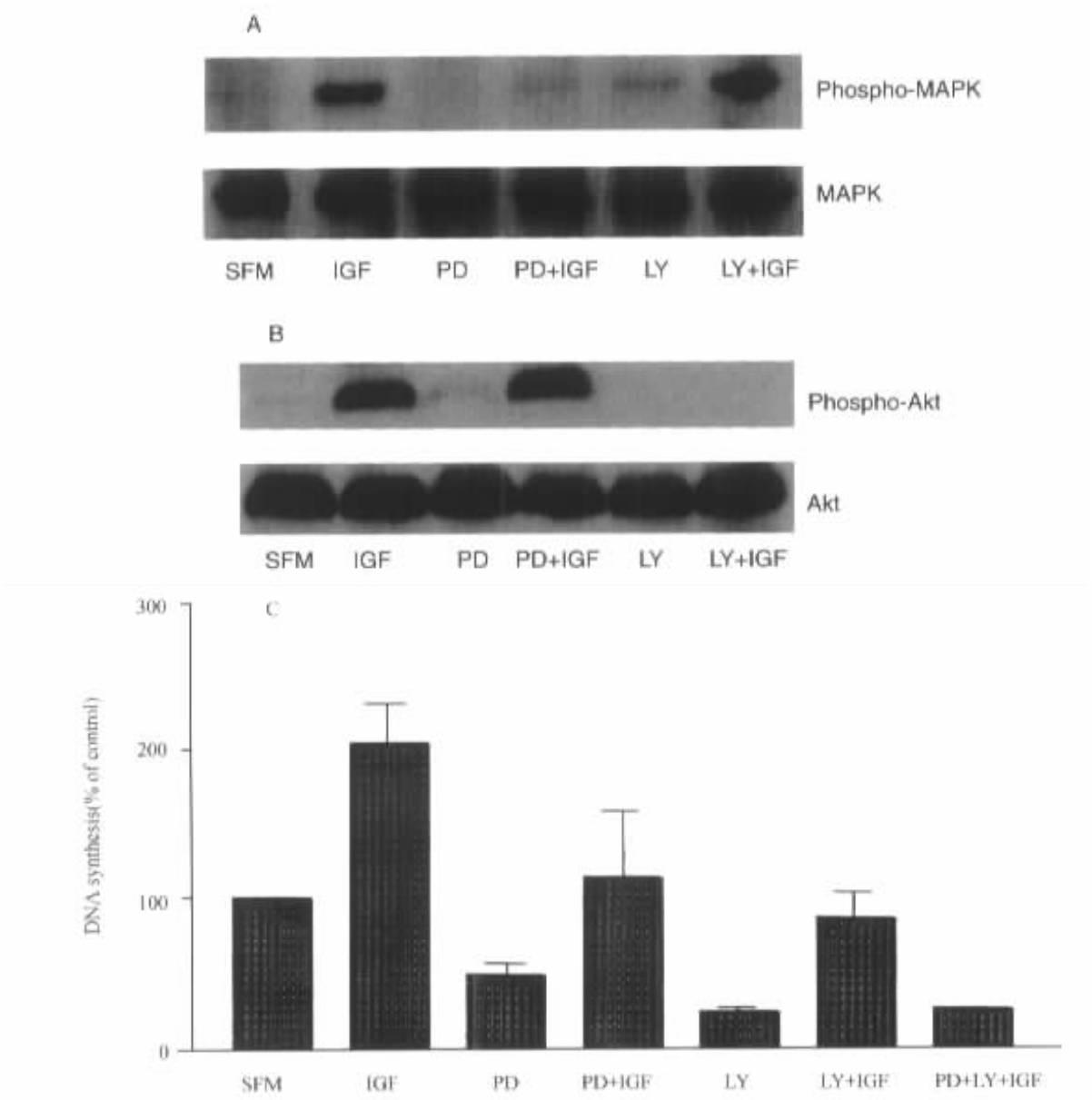

Fig. 4 IGFs stimulate zebrafish cell proliferation by activating the MAPK and PI3-Kinase signaling pathways

A. Specific inhibition of IGF-Ⅰ activation of MAPK by a MEK inhibitor PD98059 (PD) B. Specific inhibition of IGF-Ⅰ activation of PKB/Akt by a PI3 kinase inhibitor LY294002 (LY). Zebrafish embryonic cells were exposed to media with or without PD98059 (40 μM) or LY294002 (20 μM) for 2 h, then treated with or without IGF-Ⅰ (100 ng/ml) for 10 min. Lysates were subjected to Western blotting using phospho-specific and control MAPK and Akt antibodies C. Effects of the MEK inhibitor PD98059 (PD) and PI3 kinase inhibitor LY294002 (LY) on IGF-Ⅰ-stimulated DNA synthesis. Confluent zebrafish embryonic cells were exposed to media containing [H^3] -thymidine and IGF-Ⅰ (100 ng/ml), IGF-Ⅰ + PD98059 (40 μM), PD98059 (40 μM), IGF-Ⅰ + LY294002 (20 μM), LY294002 (20 μM), and IGF-Ⅰ + PD98059 (40 μM) + LY294002 (20 μM). The values are means ± *SE* of two separate experiments, each was performed in triplicate

DNA synthesis. When both the MAPK and PI3 kinase pathways were inhibited using a combination of these compounds, the IGF-Ⅰ-stimulated DNA synthesis was completely negated (Fig. 4). These results indicate that both IGF-Ⅰ and IGF-Ⅱ are potent mitogens for zebrafish embryonic cells and that activation of both the MAPK and PI3 kinase signaling pathways are required for the mitogenic action of IGFs in zebrafish.

4 Structure, expression, and biological actions of IGFBPs

Using Western ligand blot with [^{125}I] -labeled human IGF-Ⅰ as a ligand, previous studies by several groups have detected three IGFBPs with the molecular sizes of 24 – 25, 29 – 32, and 40 – 50 kDa in a number of teleost fish species (Kelley *et al.*, 1992; Niu *et al.*, 1993; Anderson *et al.*, 1993; Siharath *et al.*, 1995a, b; Fukuzawa *et al.*, 1995; Shimizu

et al., 1999；Park *et al*., 2000). The levels of these fish IGFBPs have been shown to be regulated by multiple hormonal and nutritional factors. Local production of these IGFBPs has also been demonstrated by organ cultures of various fish tissues (Fukuzawa *et al*., 1995；Siharath *et al*., 1995a). In striped bass, an additional protein with a molecular size of 80 – 85 kDa has been found to bind IGFs (Siharath *et al*., 1995b). This protein is similar in size to a 70 – 75 kDa IGFBP identified in chicken serum (McMurtry *et al*., 1996), which has been identified as vitronectin (Upton *et al*., 1999). In addition, a 20 kDa IGFBP, which may represent either the proteolytic fragment of a known IGFBP or a novel IGFBP, has been detected in hypophysectomized tilapia (Park *et al*., 2000). This "big" IGFBP or the 20 kDa IGFBP, however, was not detected in coho salmon or rainbow trout serum (Shimizu *et al*., 1999；Bauchat *et al*., 2001). Of the 3 major IGFBPs, the 40 – 50 kDa IGFBP has been postulated to be a candidate of fish IGFBP-3, because it has a similar molecular weight to human IGFBP-3 and showed a similar regulation pattern as mammalian IGFBP-3 (i.e., increased by GH treatment and decreased by fasting) (Kelley *et al*., 1992；Niu *et al*., 1993；Shimizu *et al*., 1999). In the longjawed mudsucker, the levels of the 24 – 29 and 31 – 33 kDa IGFBPs changed with the levels of insulin (Kelley *et al*., 1992). These two fish IGFBPs are also found in many peripheral tissues. Incubation of various nonhepatic organs has revealed production, mainly of the 30 – 31 kDa IGFBP, whereas the liver secretes two forms of IGFBPs with the molecular sizes of 30 and 29 kDa (Siharath *et al*., 1995a). Secretion of these two forms of IGFBPs by primary cultures of hepatocytes has also been shown in coho salmon (Moriyama *et al*., 1997). When cultured liver slices from striped bass are exposed to various hormones and growth factors, prolactin, insulin, and IGF-Ⅰ all reduce medium content of the 29 kDa IGFBP. Addition of estradiol *in vitro*, however, increases production of this binding protein. The 30 kDa IGFBP level, on the other hand, is decreased by glucagon, thyroxine and human IGF-Ⅰ and increased by GH, estradiol and epidermal growth factor (Fukuzawa *et al*., 1995). Thus, these two IGFBPs are produced by multiple organs and their levels are regulated by hormonal and nutritional factors. These studies clearly indicate that there are multiple forms IGFBPs present in teleost fish, and these IGFBPs are produced by various tissues and their levels are tightly regulated by hormonal and nutritional factors. The identities of these fish IGFBPs, however, were not determined until recently.

Using a combination of RT-PCR, cDNA library screening, EST database searching, and RACE techniques, the structures of several zebrafish IGFBPs, including IGFBP-1, IGFBP-2, IGFBP-3, and IGFBP-5, have been characterized (Duan *et al*., 1999；Maures *et al*., 2002；Ding *et al*., 2003). Partial sequences of zebrafish IGFBP-4 and IGFBP-6 are also found in the EST database. In addition, the structure of tilapia IGFBP-3 has been reported (Cheng *et al*., 2002). Therefore, all six IGFBPs appear to exist in the teleost group. Among these fish IGFBPs, IGFBP-2 is the first whose full-length structure, gene expression, and biological actions have been determined (Duan *et al*., 1999). The mature fish IGFBP-2 has a predicted molecular size of 28 kDa and shows high sequence identity with human IGFBP-2 (50%). The

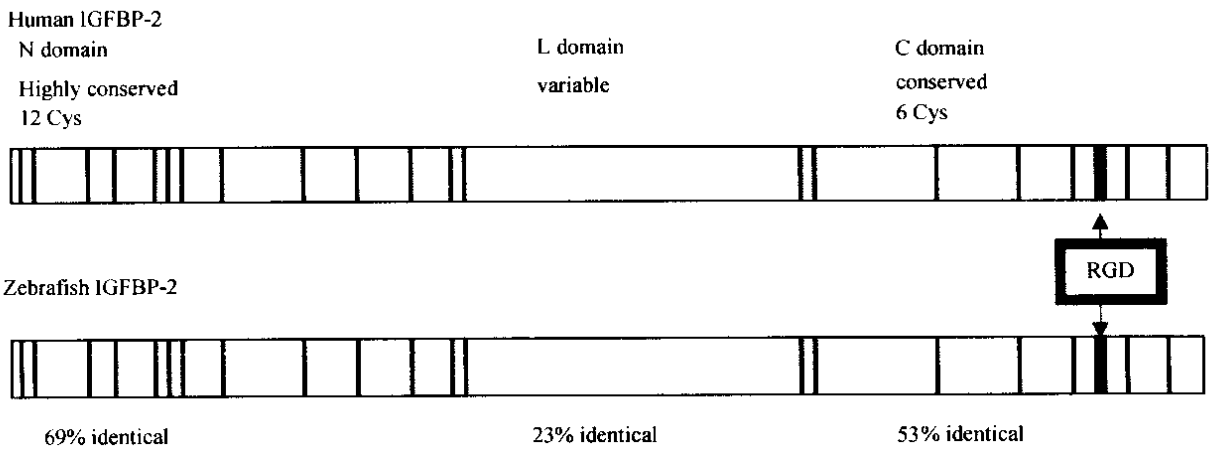

Fig.5 Comparison of the fish IGFBP-2 primary structure with that of human IGFBP-2

These two IGFBPs share a common domain organization：a highly conserved N-terminal domain containing 12 cysteine residues (69%), a conserved C-terminal domain that has 6 cysteines (53%), and a highly variable central L domain (23%). In addition, both proteins contain a RGD motif in the C-domain. Each vertical bar represents a cysteine residue

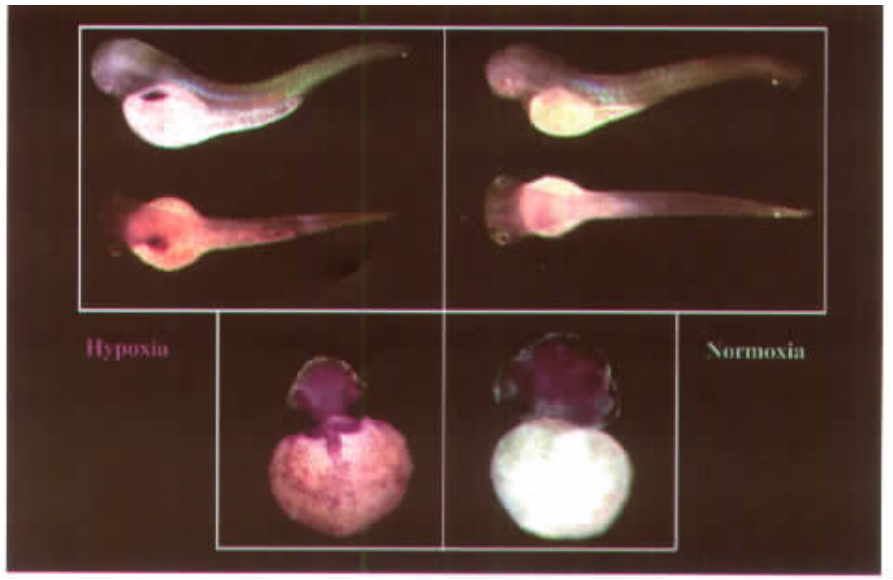

Fig.6 Regulation of zebrafish IGFBP-1 gene expression by changing environmental cues

Lower panels: hypoxia (low oxygen) induces IGFBP-1 mRNA expression in the brain, heart, and pharyngeal arches in early embryonic stages. In this study, zebrafish embryos at the stage of 48 hours post fertilization (hpf) were exposed to low oxygen (hypoxia, lower left panel) or kept in normal oxygen level (normoxia, lower right panel) for 24 h. The embryos were fixed and subjected to whole mount *in situ* RNA hybridization. Front views are shown. The puruple color represents the IGFBP-1 transcripts. Note the elevated IGFBP-1 mRNA signal in the brain, heart, and pharyngeal arches in the hypoxia group. Upper panels: the hypoxia-induced IGFBP-1 mRNA expression is restricted in the liver after hatching. Hatched embryos of 72 hpf were exposed to hypoxia (upper left panel) or normoxia (upper right panel) for 24 h. The embryos were fixed and subjected to whole mount *in situ* RNA hybridization. Lateral and dorsal views are shown. The puruple color represents the IGFBP-1 transcripts. Note the elevated IGFBP-1 mRNA signal in the liver in the hypoxia group

sequence identities with other human IGFBPs are < 37%. Sequence analysis suggest that fish IGFBP-2 is similar to their mammalian homologues in structure (Fig.5). Other fish IGFBPs also share high structural similarity with their perspective mammalian homologues (Maures *et al*., 2002; Funkenstein *et al*., 2002; Cheng *et al*., 2002; Ding *et al*., 2003). All these fish IGFBPs share a common domain organization: a highly conserved N-terminal domain containing 12 cysteine residues, a conserved C-terminal domain that has 6 cysteines, and a highly variable central domain (Fig.5).

When the spatial and temporal expression patterns of these IGFBP genes throughout embryogenesis were elucidated, it was found that all these IGFBPs are expressed in a tissue-specific and developmental stage-specific manner. For example, IGFBP-1 mRNA is initially expressed in multiple embryonic tissues but became restricted to the liver shortly after hatching. When adult fish or embryos were subjected to hypoxic conditions, the IGFBP-1 mRNA expression increased dramatically. Intriguingly, the hypoxia-induced IGFBP-1 expression operated in different embryonic tissues in a developmental-stage dependent manner. In early embryos, hypoxia stimulated IGFBP-1 mRNA expression in the pharyngeal arches, ventricle, atrium, and brain. After hatching, the hypoxia-induced IGFBP-1 expression became liver-specific (Fig.6). Likewise, IGFBP-2 mRNA is expressed in a restricted fashion during embryogenesis. In the adult stage, IGFBP-1 mRNA was only found in the liver at low levels. Prolonged food deprivation caused a significant increase in the hepatic IGFBP-1 mRNA levels, and re-feeding restored the IGFBP-1 mRNA to the basal levels. IGFBP-2 mRNA levels were greatly reduced by growth hormone (GH) treatment, but strongly increased by fasting (Duan *et al*., 1999). These results suggest that IGFBP-2 may act downstream in the GH-IGF-Ⅰ axis in zebrafish. The IGFBP-5 mRNA expression, on the other hand, began in somites and branchial mesenchyme at 24 hours post fertilization (hpf). By 48 hpf, its expression became restricted in the newly forming craniofacial cartilage tissues. This craniofacial skeletal tissue-specific expression pattern remained until juvenile stage (Ding *et al*., 2003).

To date, the biological actions of two fish IGFBPs have been studied *in vitro* and/or *in vivo*. The physiological function of IGFBP-5 *in vivo* has been investigated by injecting morpholino-based antisense oligonucleotides into fertilized eggs (Ding *et al*., 2003). The IGFBP-5 knocked-down morphants had curved body shape and little craniofacial skeletal tissues. The lack of cartilage tissue was further confirmed by Alcian blue staining. To determine whether the lack of the craniofacial skeletal tissues was due to

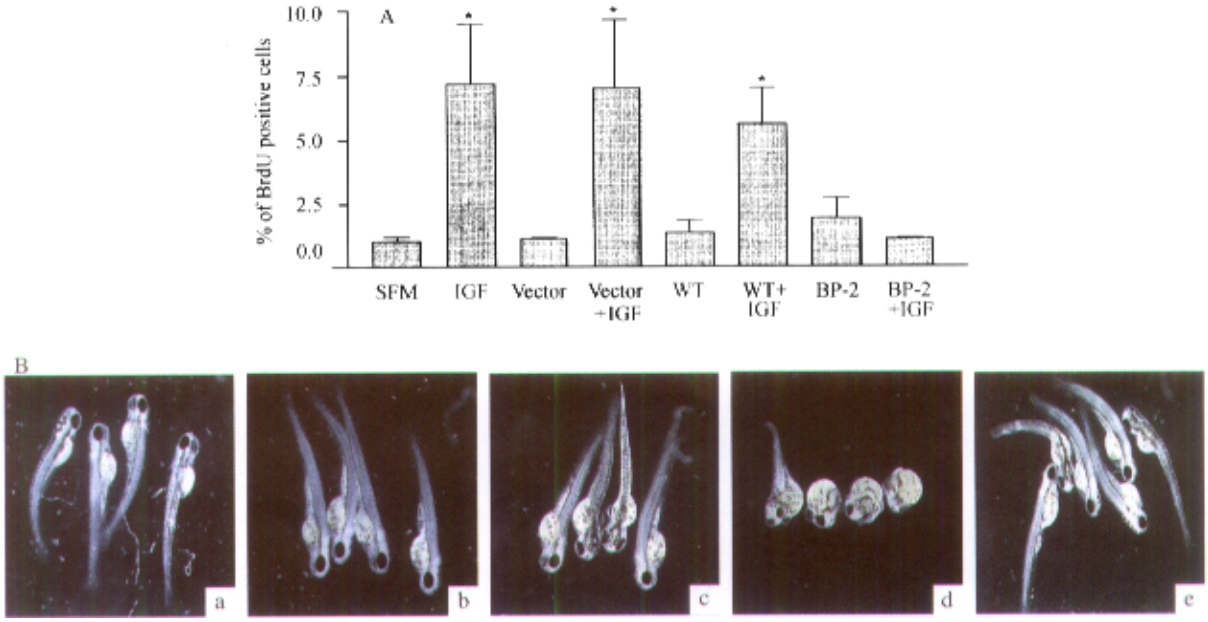

Fig.7 IGFBP-2 inhibits IGF actions in zebrafish *in vitro* and *in vivo*

A. Effects of IGFBP-2 on IGF-Ⅰ induced cell proliferation. Serum starved, confluent zebrafish embryonic cells were exposed to media with or without IGF-Ⅰ (100 ng/ml) in the presence of wild type, empty vector-transfected, or IGFBP-2 transfected cell media (0.5% volume) and BrdU (20 *M*) for 22 h. Cells were fixed and immunostained for BrdU and DAPI. The percentage of brdU positive cells were shown. * : $P < 0.05$ compared with the serum free medium (SFM) control group

B. Ectopic expression of IGFBP-2 results in growth retardation and abnormal development in zebrafish. Zebrafish embryos of 1 – 2 cell stage were injected with pCMV vector (b), pCMV-IGF-Ⅰ (c), pCMV-IGFBP-2 (d), pCMV-IGF-Ⅰ plus pCMV-IGFBP-2 (e). In addition, equal amount of pCMV-EGFP was injected to each of the groups. The expression of transgene was comfirmed by examining EGFP expression in living embryos. The transgenic fish were raised and photographed at 72 hpf. (a) is the non-injection control

defects in tissue formation and/or altered tissue growth/survival, we analyzed the expression of dlx-2, a key transcriptional factor expressed in the precursor cells of branchial arches, at earlier developmental stages. Dlx-2 expression was greatly diminished in the IGFBP-5 morphants, suggesting that ablation of IGFBP-5 disrupts the formation of craniofacial skeletal tissues. This effect was specific to craniofacial skeletal tissues because ablation of IGFBP-5 had little effect on the expression of brain and neuronal marker genes such as Emx-1 and Rx-1. Likewise, no change in the expression patterns of muscle specific genes such as MyoD and myogenin, was observed in IGFBP-5 morphants. This study provides the first compelling *in vivo* evidence that IGFBP-5 plays a critical role in the formation of craniofacial skeletal tissues in a vertebrate model organism.

The cellular actions of zebrafish IGFBP-2 were investigated *in vitro* by 1) directly adding the pure recombinant protein to cultured cells, and 2) by stably transfecting cells (Duan *et al*., 1999). When added exogenously to or expressed in cultured fish and mammalian cells, the zebrafish IGFBP-2 inhibited IGF-Ⅰ-stimulated DNA synthesis and cell proliferation in a concentration-dependent manner (Fig. 7: A). The inhibitory effects of the fish IGFBP was comparable to those of human IGFBP-2. These results indicate that IGFBP-2 has a inhibitory effect on IGF action at the cellular level. To directly test the importance of IGFBP-2 in zebrafish embryos *in vivo*, we took a genetic approach to ectopically express IGFBP-2 in zebrafish. When IGFBP-2 was ectopically expressed in zebrafish embryos, the transgenic embryos had a significantly higher rate of mortality compared to the vector-injected or non-injected control groups. The survived IGFBP-2 transgenic embryos were severely growth retarded and had abnormal morphology (Fig.7 :B-d). This effect is specific, because the IGFBP-2 caused growth retardation and had abnormal morphology could be "rescued" by co-expression of IGF-Ⅰ (Fig.7 :B-e). These studies indicate that IGFBP-2 can inhibit growth of developing zebrafish embryos by binding to endogenous IGFs and preventing them from binding to the IGF receptors.

5 Conclusion remarks

The last several years has witnessed a remarkable acceleration in the accumulation of knowledge about the fish IGF signaling system. The structures and functions of IGF-Ⅰ, IGF-Ⅱ, IGF-ⅠR have been determined in a number of teleost species and major intracellular signaling pathways of IGF actions have been elucidated. The presence of several fish IGFBPs have been demonstrated and their structures have

been determined.

Despite these recent advances, many important questions remain. For instance, while the IGF-ⅠR depletion and knocked-out studies have revealed the critical importance of the IGF signal in vertebrate developmental and growth, the individual role (s) of the duplicated *igf-ir* genes in zebrafish remains to be elucidated. Although the ectopic expression of IGFBP-2 can affect embryonic growth and development in zebrafish, they do not necessarily provide insights on the physiological functions of the endogenous IGFBP-2. For a full understanding of the physiological function of IGFBP-2 *in vivo*, a targeted gene ablation approach is needed. The developmental and hormonal regulation mechanisms that govern the spatial and temporal expression of the IGFBP genes have not been studied. Furthermore, with the exception of IGFBP-5, the physiological functions of IGFBPs are undefined in zebrafish or any other species. Thus, research activity is still lacking in this area even though the zebrafish model can contribute immensely to our understanding of the role of the IGF signaling system in vertebrate growth and development.

Fish represent the largest, most diverse group of vertebrate animals and occupy an important position in vertebrate evolution. Their diversity, adaptability to different environments, and continuing growth make fish ideal for studying the evolution of growth regulatory mechanisms in vertebrates. An understanding of the underlying mechanisms of growth regulation in fish will undoubtedly contribute to the basic physiology of vertebrates in general. Knowledge gained from these studies on fish growth physiology may prove to be valuable to aquaculture for efficient production of animal protein to meet the needs of a continually growing human population.

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