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Characterization of the insulin-like growth factor binding protein family in *Xenopus tropicalis*

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ABSTRACT The insulin-like growth factor binding protein (Igfbp) family consists of six members designated Igfbp1–6. Igfbps are involved in many vital biological functions. They physically interact with IGFs (IGF1 and IGF2) and act as carriers, thereby protecting IGFs from proteolytic degradation. Thus, they function as modulators of IGF activity. Furthermore, Igfbps have been reported to have IGF-independent activities. They interact with other proteins, including cell surface proteins, extracellular matrix proteins, and potentially intracellular molecules. In *Xenopus tropicalis* (*X. tropicalis*), only four *igfbp* genes (*igfbp1, igfbp2, igfbp4,* and *igfbp5*) have been identified, and their expression is not well characterized. We report that *X. tropicalis* genome lacks the *igfbp3* and *igfbp6* genes based on synteny analyses. We also examined the spatio-temporal expression patterns of *igfbp* genes in early *X. tropicalis* development. Expression analyses indicated that they are differentially expressed during early development. Each *igfbp* gene showed a characteristic spatial expression pattern. Except for *igfbp5,* they demonstrated overlapping expression in the pronephros. The *Xenopus* pronephros is composed of four domains (i.e., the proximal tubule, intermediate tubule, distal tubule, and connecting tubule). Our results showed that at least two *igfbp* genes are co-expressed in all pronephric domains, suggesting that redundant functions of *igfbp* genes are required in early pronephric kidney development.

KEY WORDS: *insulin-like growth factor (IGF), insulin-like growth factor binding protein (IGFBP), Xenopus tropicalis*

Insulin-like growth factors (IGF1 and IGF2) are important in regulating cellular growth and differentiation. Their functions are mediated by the IGF-1 receptor and IGF-2 receptor (mannose-6-phosphate receptor) (reviewed in Nakae *et al*., 2001). The functions of IGFs are modulated by a family of binding proteins termed insulin-like growth factor binding proteins (Igfbps). Igfbps include six members designated Igfbp1 through Igfbp6, and are grouped based on conservation of gene organization, structural similarity, and IGF binding affinity. Igfbps are unusually multifaceted molecules. They distribute IGFs and modulate IGF binding to receptors; therefore, they play a significant role in mediating IGF actions. In addition to their role as IGF carriers, they also regulate IGFs either by inhibiting their binding to receptors or potentiating activities, protecting IGFs from protein degradation (reviewed in Hwa *et al*., 1999).

Igfbps can also function in IGF-independent manners. They interact with the extracellular matrix and cell surface proteins including integrins. Igfbps are transported into the nucleus via nuclear localization signaling and exert IGF-independent effects by transcriptional modulation of genes (reviewed in Hwa *et al*., 1999). Understanding the functions of Igfbps *in vivo* has been difficult, largely because Igfbp knockout mice have no dramatic phenotypes. Examinations of the multiple functions of these proteins and redundancy in their expression in various tissue types will be necessary.

In *Xenopus laevis*, the expression patterns of only two *igfbp* genes, *igfbp4* and *igfbp5,* have been described previously. The *igfbp4* gene is expressed in the anterior part of the liver from stage 38 through 42 (Zhu *et al*., 2008). However, its detailed expres-

Abbreviations used in this paper: IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein.

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sion pattern has not been described. The expression of *igfbp5* is detected maternally, and becomes localized to the floor plate, notochord, and dorsal endoderm during neurulation. At the tailbud stages, additional expression is detected in cranial nerves, ear vesicles, dorsal fins, and somites (Pera *et al.*, 2001). *X. tropicalis* is a useful model animal for the study of early developmental functions of various genes. However, as mentioned above, the specific expression patterns of *igfbp* genes during embryogenesis have not been completely described, and further data are needed. In this study, we showed that the *X. tropicalis* genome lacks the *igfbp3* and *igfbp6* genes. We detected four *igfbp* genes in *X. tropicalis* embryos, and documented their spatial and temporal expression patterns during early embryonic development.

Result and Discussion

Cloning of four X. tropicalis igfbp *genes*

We identified four *igfbp* genes, *igfbp1*, *igfbp2*, *igfbp4*, and *igfbp5* in *X. tropicalis* (Fig. 1A). In mice and humans, *igfbp2* and *igfbp5* are located on the same chromosome as a tandem repeat,

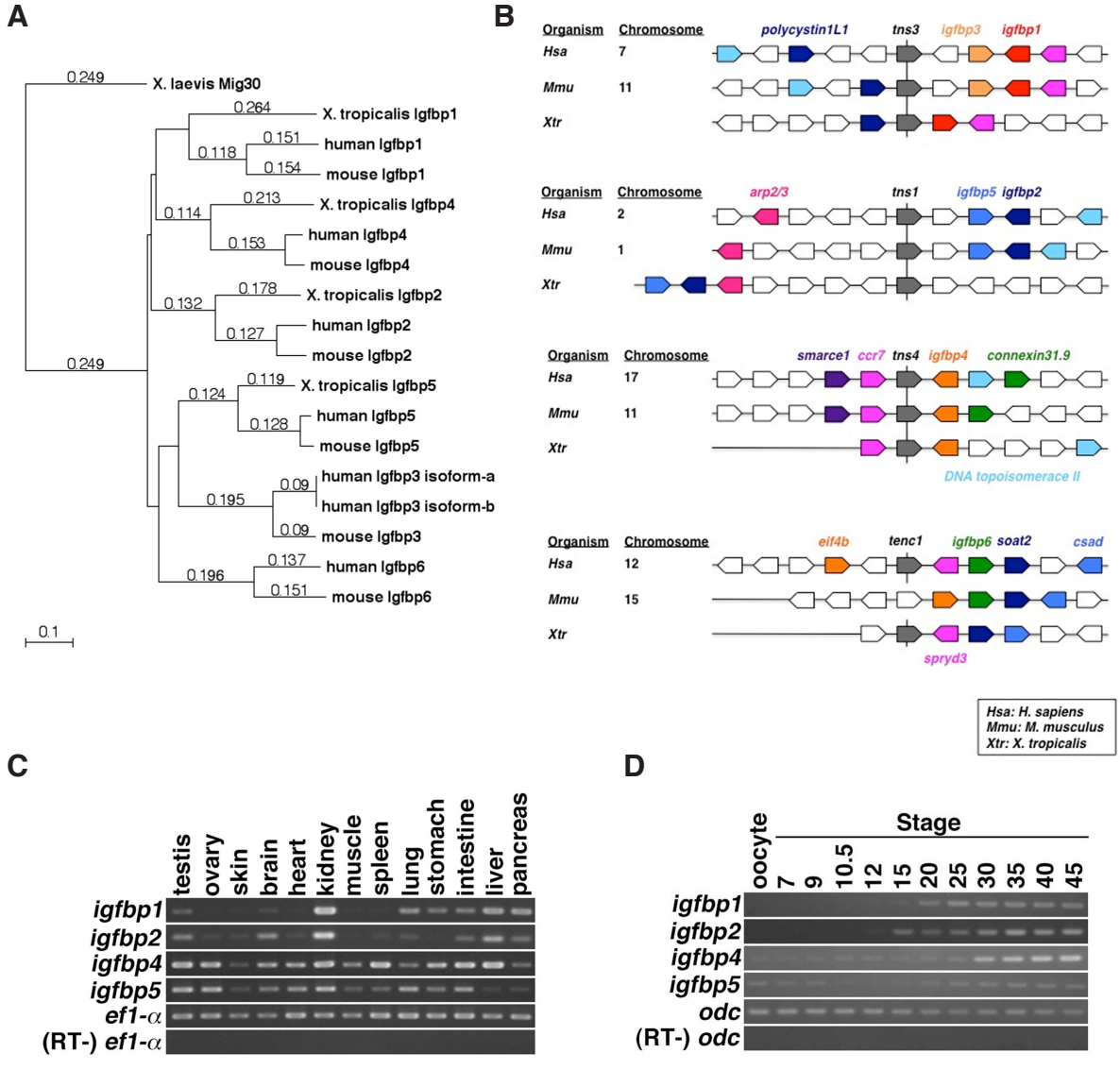
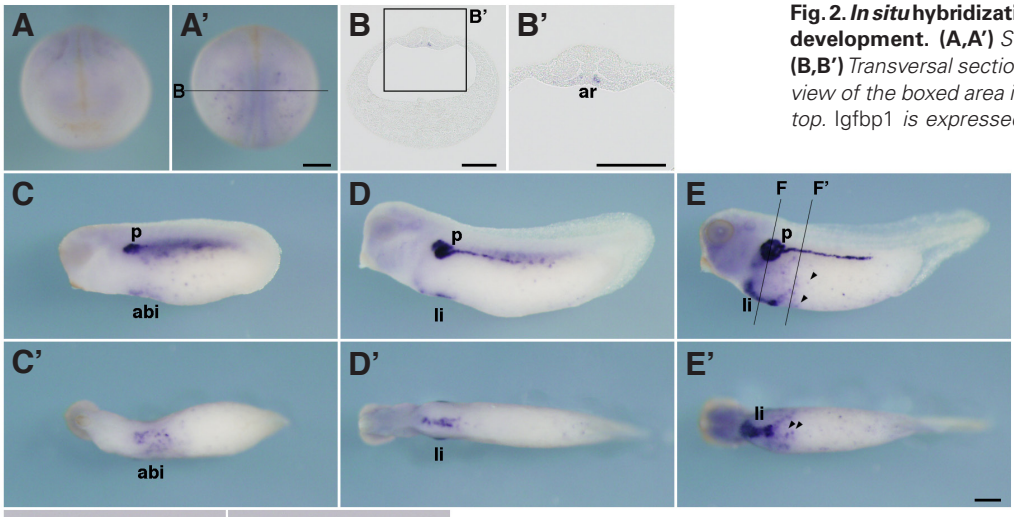


Fig. 1. Isolation of *X. tropicalis igfbp* genes and their expression patterns in embryonic and adult tissues. (A) *Phylogenetic tree of* Igfbp *amino acid sequences. The phylogenetic tree was calculated by MacVector 11.1.0 software.* X. laevis *Mig30 (GenBank accession No. NP_001082206) (Hayata* et al., *2002 and Kuerner* et al., *2006) was used as an outgroup. Sequence sources (i.e., GenBank Accession Nos.) are as follows:* X. tropicalis *Igfbp1 (NP_001029118), Igfbp2 (NP_001093707), Igfbp4 (XP_002942630), Igfbp5 (NP_001016042);* Mus musculus *Igfbp1 (NP_032367), Igfbp2 (NP_032368), Igfbp3 (NP_032369), Igfbp4 (NP_034647), Igfbp5 (NP_034648), Igfbp6 (NP_032370);* Homo sapiens *Igfbp1 (NP_000587), Igfbp2 (NP_000588), Igfbp3 isoform-a (NP_001013416), Igfbp3 isoform-b (NP_000589), Igfbp4 (NP_001543), Igfbp5 (NP_000590), and Igfbp6 (NP_002169).* **(B)** *Synteny analysis of* igfbp *genes. Synteny of* igfbp *genes is conserved among human, mouse, and* X. tropicalis. Tensin *genes near* igfbp *loci are shown in grey, and orthologs are connected with a vertical line. Orthologs are shown in the same colors, and genes whose positions are not conserved among human, mouse, and* X. tropicalis *are shown in white. In* X. tropicalis, igfbp3 *and* igfbp6 *seem to be lost.* **(C)** *The expression of* igfbp1, 2, 4, *and* 5 *in adult tissues of* X. tropicalis. **(D)** *Temporal expression of* igfbp1, 2, 4, *and* 5 *during early development of* X. tropicalis. *Numbers indicate developmental stages.*



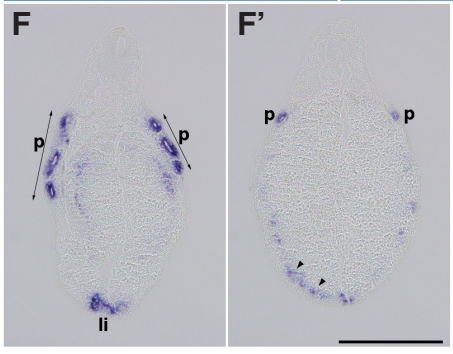


Fig. 2. *In situ* hybridization analysis of *igfbp1* during *X. tropicalis* development. (A,A') *Stage 20,* **(A)** *Anterior view* **(A')** *dorsal view.* **(B,B')** *Transversal section of an embryo at stage 20.* **(B')** *Magnified view of the boxed area in* **(B)**. *Dorsal side is displayed towards the top.* Igfbp1 *is expressed in part of the archenteron roof near the dorsal midline.* **(C,C')** *Stage 25,* **(D,D')** *stage 30,* **(E,E')** *stage 35,* **(C,D,E)** *lateral view,* **(C',D',E')** *ventral view.* **(F,F')** *Transversal section of an embryo at stage 35. Positions of sections are indicated by black lines, and letters mark corresponding panels. ar, archenteron roof; abi, anterior blood islands; li, liver; p, pronephric tubule. Arrowheads indicate scattered blood-like cells. Scale bars indicate 200 μm.*

but are orientated in opposite transcription directions. The *igfbp1* and *igfbp3* genes are linked in the same manner. The *igfbp4* and *igfbp6* genes are located on separate chromosomes (Fig. 1B). All *igfbp* genes are located near tensin-like genes (*tns*) in the mouse and human genomes. However, *X. tropicalis igfbp3* and *igfbp6* could not be identified in the proximity of *tns* loci (Fig. 1B). Furthermore, orthologous *igfbp3* and *igfbp6* sequences were not identified. Zebrafish and medaka genomes contain *igfbp3* and *igfbp6* genes. Our synteny analysis showed that *X. tropicalis* lacks *igfbp3* and *igfbp6* in the genome. Therefore, determining the function of each *igfbp*, and in particular, determining which *Xenopus* genes share the functions of *igfbp3* and *igfbp6* is intriguing.

Expression of igfbp1

Mouse *igfbp1* expression has been detected in the liver after day 12 of gestation (Shuller *et al.*, 1993a, b; Schuller *et al.*, 1994). No expression was detected in other adult mouse tissues (Schuller *et al.*, 1994). In humans, *igfbp1* is most abundantly expressed in the fetal liver (Han *et al.*, 1996). Our results indicated that *X. tropicalis igfbp1* is expressed in various adult tissues, unlike in mouse and human. *X. tropicalis igfbp1* was expressed in the kidney, lung, stomach, intestine, liver, and, pancreas and was detected at low levels in the testis and brain (Fig. 1C). *X. tropicalis igfbp1* was developmentally expressed from the late neurula stage (Fig. 1D, Fig. 2 A–F'). In the neurula (stage 20), *igfbp1* was expressed in part of the archenteron roof near the dorsal midline (Fig. 2 A–B'). At the tailbud and tadpole stages (stage 25–35), *igfbp1* was predominantly expressed in the pronephros (Fig. 2 C–F'). *Igfbp1* was also expressed in anterior blood islands at stage 25 (Fig. 2 C and C'), in a region around the liver at stage 30 (Fig. 2 D and D'), and in the liver and scattered blood-like cells at stage 35 (Fig. 2 E-F').

Expression of igfbp2

Expression of mouse *igfbp2* has been detected in neural tissues as early as day 11 of gestation. The mouse *igfbp2* transcript was detected in differentiating sclerotomes, the esophagus, nasal placode, lung, and liver starting on day 13. After day 14, the expression of mouse *igfbp2* was also found in other tissues such as the eye, meninges, vertebrae, kidney, and intestine (Schul-

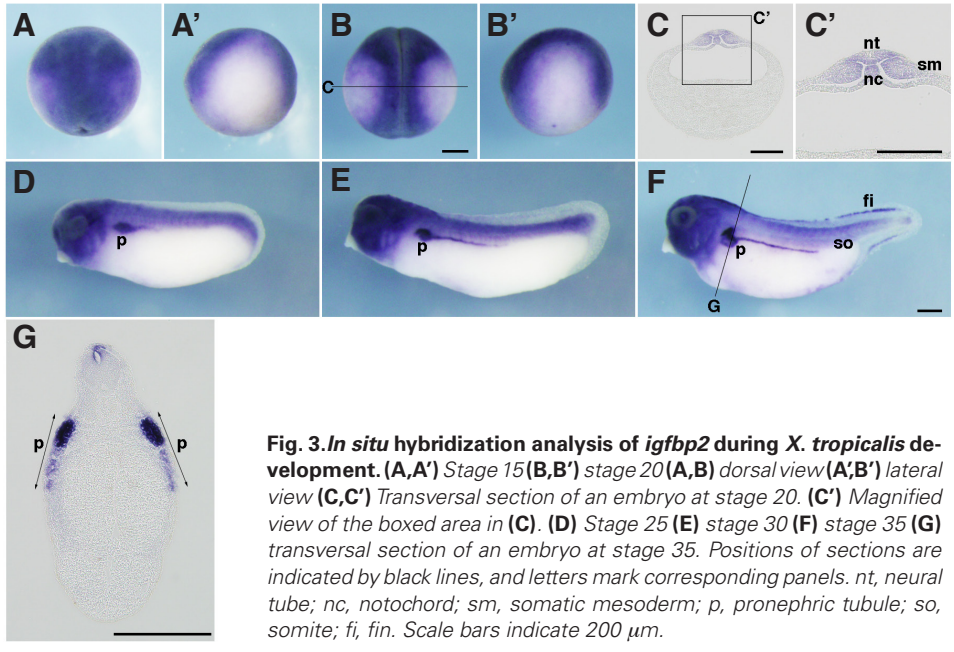


Fig. 3. *In situ* hybridization analysis of *igfbp2* during *X. tropicalis* development. (A,A') *Stage 15* **(B,B')** *stage 20* **(A,B)** *dorsal view* **(A',B')** *lateral view* **(C,C')** *Transversal section of an embryo at stage 20.* **(C')** *Magnified view of the boxed area in* **(C)**. **(D)** *Stage 25* **(E)** *stage 30* **(F)** *stage 35* **(G)** *transversal section of an embryo at stage 35. Positions of sections are indicated by black lines, and letters mark corresponding panels. nt, neural tube; nc, notochord; sm, somatic mesoderm; p, pronephric tubule; so, somite; fi, fin. Scale bars indicate 200 μm.*

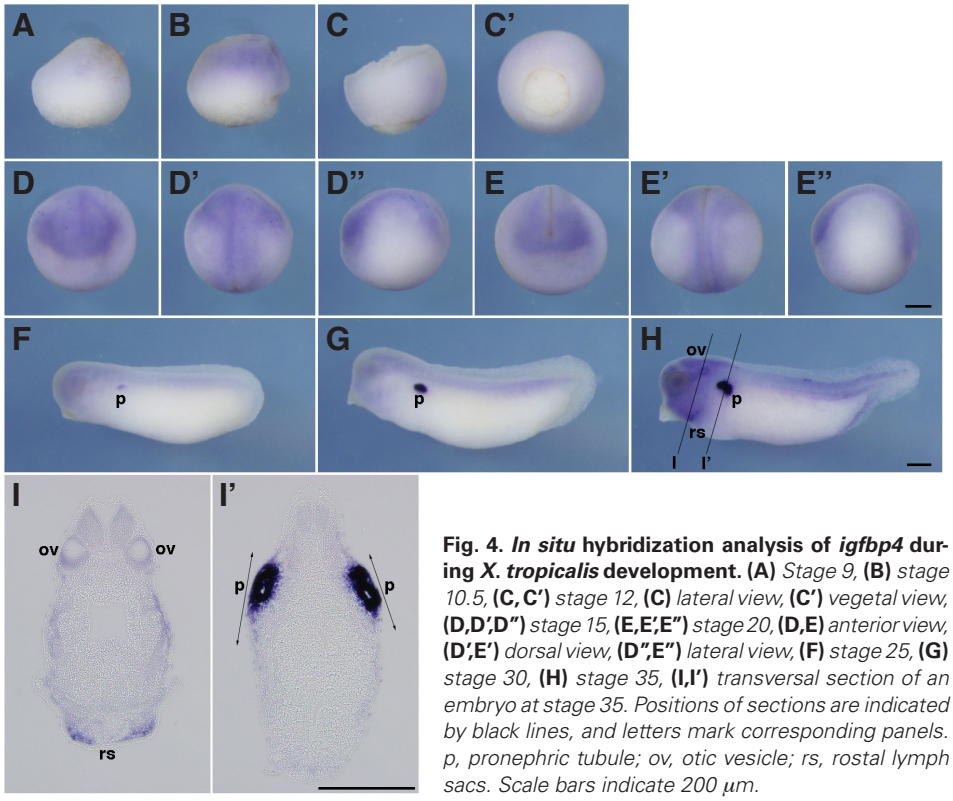


Fig. 4. *In situ* hybridization analysis of *igfbp4* during *X. tropicalis* development. (A) *Stage 9,* **(B)** *stage 10.5,* **(C, C')** *stage 12,* **(C)** *lateral view,* **(C')** *vegetal view,* **(D,D',D")** *stage 15,* **(E,E',E")** *stage 20,* **(D,E)** *anterior view,* **(D',E')** *dorsal view,* **(D",E")** *lateral view,* **(F)** *stage 25,* **(G)** *stage 30,* **(H)** *stage 35,* **(I,I')** *transversal section of an embryo at stage 35. Positions of sections are indicated by black lines, and letters mark corresponding panels. p, pronephric tubule; ov, otic vesicle; rs, rostal lymph sacs. Scale bars indicate 200 μm.*

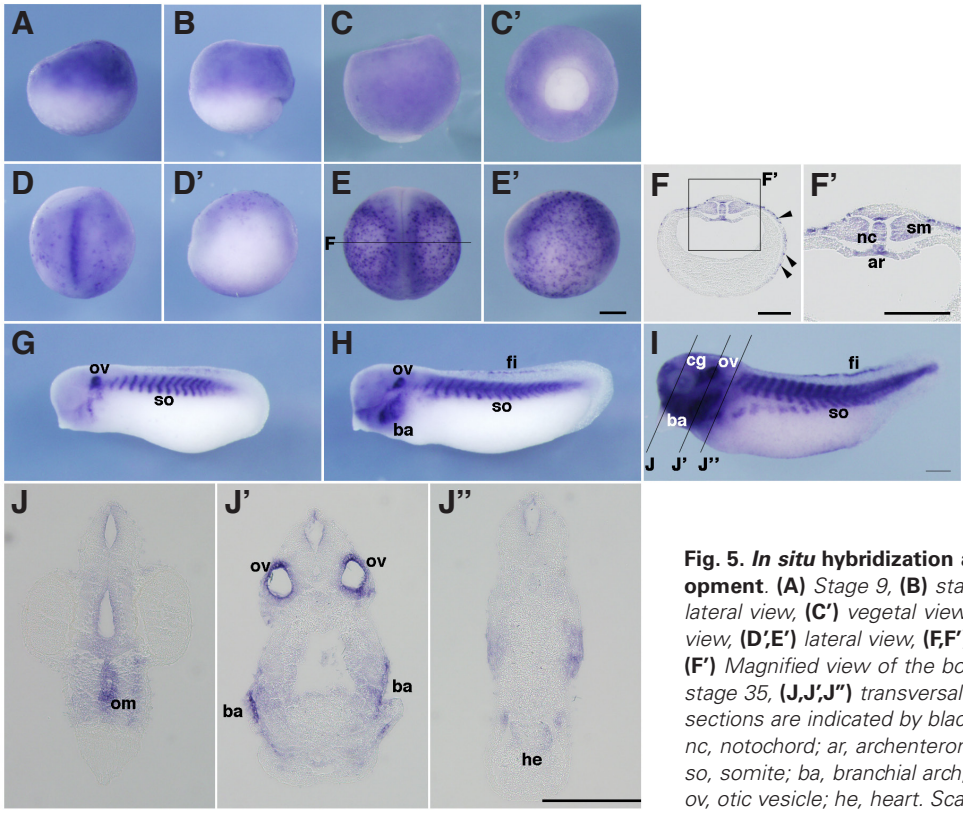


Fig. 5. *In situ* hybridization analysis of *igfbp5* during *X. tropicalis* development. (A) *Stage 9,* **(B)** *stage 10.5,* **(A,B)** *lateral view,* **(C,C')** *stage 12,* **(C)** *lateral view,* **(C')** *vegetal view,* **(D,D')** *stage 15,* **(E,E')** *stage 20,* **(D,E)** *dorsal view,* **(D',E')** *lateral view,* **(F,F')** *transversal section of an embryo at stage 20.* **(F')** *Magnified view of the boxed area in* **(F)**. **(G)** *Stage 25,* **(H)** *stage 30,* **(I)** *stage 35,* **(J,J',J")** *transversal section of an embryo at stage 35. Positions of sections are indicated by black lines, and letters mark corresponding panels. nc, notochord; ar, archenteron roof; sm, somatic mesoderm; ov, otic vesicle; so, somite; ba, branchial arch; fi, fin; cg, cranial ganglia; om, oral membrane; ov, otic vesicle; he, heart. Scale bars indicate 200 μm.*

ler *et al.*, 1993a, b). High expression of mouse *igfbp2* has been detected in the adult liver and kidney, and is also detectable in the lung, spleen, brain, testis, and ovary (Schuller *et al.*, 1994). No expression was detected in muscle tissues. In humans, *igfbp2* is expressed at moderate levels in every tissue, and is highest in the liver during gestational ages 10–16 weeks (Han *et al.*, 1996). *X. tropicalis igfbp2* was expressed in the testis, brain, kidney, intestine, liver, and pancreas, and was detected at low levels in the ovary, skin, heart, spleen, and lung (Fig. 1C). The expression of *X. tropicalis igfbp2* was developmentally detected from the neurula stage (Fig. 1D, Fig. 3 A–G). At the neurula stage (stages 15–20), *igfbp2* was broadly expressed around the dorsal region (Fig. 3 A–C'), i.e., in the neural tube, somite, and notochord (Fig. 3 C and C'). At the tailbud and tadpole stages (stages 25–35), *igfbp2* was predominantly expressed in the pronephros and neural region (Fig. 3D–G). At stage 35, *igfbp2* was also detectable in part of the fin (Fig. 3F). There were no remarkable differences between the expression of *igfbp2* in *X. tropicalis*, mouse, and human.

Expression of igfbp4

Transcripts of mouse *igfbp4* have been detected as early as day 11 in neural tissues and differentiating sclerotomes. After day 14, mouse *igfbp4* expression is decreased in the brain, and is clearly detected in other tissues such as the lung, liver, kidney, intestine, and vertebrae (Schuller *et al.*, 1993a, b). High expression of mouse *igfbp4* is detected in the adult liver, kidney, and spleen, and is also detectable in the lung, heart, spleen, and muscle. No expression has been detected in the testis or ovary (Schuller *et al.*, 1994). In humans, *igfbp4* is widely expressed at moderate levels in all tissues during gestational ages 10–16 weeks (Han *et*

al., 1996) and is most abundant in the kidney, stomach, intestine, and lung and least abundant in the liver during gestational ages 14–18 weeks (Delhanty *et al.*, 1993). *X. tropicalis igfbp4* was expressed in almost all tissues tested including the testis, ovary, brain, heart, kidney, muscle, spleen, lung, stomach, intestine, liver, and pancreas, and was detected at low levels in the skin (Fig. 1C). *X. tropicalis igfbp4* expression increased gradually during development (Fig. 1D, Fig. 4 A–I'). At the blastula and gastrula stages (stages 9–12), *igfbp4* was expressed in animal region, and gradually diminished thereafter (Fig. 4A–C'). At the neurula stage (stages 15–20), *igfbp4* was expressed in the neural region (Fig. 4 D–E''). At the tailbud and tadpole stages (stages 25–35), *igfbp4* was predominantly expressed in the pronephros. At stage 35, *igfbp4* was also detectable in the otic vesicle and rostral lymph sacs (Fig. 4 H–I'). *X. tropicalis igfbp4* was expressed in the testis and ovary, unlike mouse *igfbp4*.

Expression of igfbp5

Expression of mouse *igfbp5* is detectable as early as day 11 of gestation in differentiating sclerotomes. Expression of mouse *igfbp5* in 14-day-old embryos has been found in the nasal placodes, pharynx, and esophagus. After day 14 of gestation, expression of mouse *igfbp5* has been found in tissues such as the cornea and sclera of the eye, meninges, lung, kidney, intestine, and vertebrae (Schuller *et al.*, 1993a, b). High expression of mouse *igfbp5* is detected in the adult kidney, muscle, and ovary, and is also detectable in the lung, heart, brain, and testis (Schuller *et al.*, 1994). In humans, *igfbp5* is expressed most abundantly in the skin, muscle, and stomach during gestational ages 10–16 weeks (Han *et al.*, 1996) and is most abundant in the muscle, skin, stomach, and intestine during gestational ages 14–18 weeks (Delhanty *et al.*, 1993). *X. tropicalis igfbp5* was expressed in almost all tissues tested, including the testis, ovary, brain, heart, kidney, muscle, spleen, lung, stomach, and intestine, and was detected at low levels in the skin, liver, and pancreas (Fig. 1C). *X. tropicalis igfbp5* was maternally expressed. The expression of *igfbp5* gradually decreased until the neurula stage (stage 15), and then increased from the late neurula stage (stage 20) (Fig. 1D, Fig. 5 A–J''). At the blastula and gastrula stages (stages 9–12), *igfbp5* was broadly expressed in the animal hemisphere (Fig. 5 A–C'). At the neurula stage (stages 15–20), *igfbp5* was expressed in the notochord, somite, the dorsal part of the archenteron roof, and in scattered cells in the ectodermal epithelium (Fig. 5 D–F'). At the tailbud and tadpole stages (stages 25–35), *igfbp5* was predominantly expressed in the somite, otic vesicle, branchial arch, and part of the fin (Fig. 5 G–J''). At stage 35, *igfbp5* was also detectable in the oral membrane, cranial ganglia, and heart (Fig. 5 I–J'').

Expression of igfbps *in the pronephric kidney*

In this study, we focused on the expression of *igfbps* in the pronephros, because all *igfbp* genes except for *igfbp5* were expressed in the pronephric tubule or duct. Synteny analysis indicated that the *X. tropicalis* genome lacks *igfbp3* and *igfbp6*. Therefore, functional validation of the lack of these genes in *X. tropicalis* development is of interest. High expression of mouse *igfbp3* has been detected in the adult kidney, and is detectable in the liver, lung, heart, spleen, and muscle (Schuller *et al.*, 1994). Importantly, *igfbp1,* which is not detected in the kidney in the mouse or human, was highly expressed in the pronephric tubule and duct and the adult kidney of *X. tropicalis.* This expression of *igfbp1* might compensate for the lack of *igfbp3,* which is nearby in the mice and human genomes. High expression of *X. tropicalis igfbp1* was detected in region 3 of the proximal tubule (PT), all regions of the intermediate tubule (IT), and the connecting tubule (CT), and was detectable in all regions of the distal tubule (DT) (Fig. 6 A–B' and G). *Igfbp1* was also expressed in the nephrostome, and probably in PT1 (Fig. 6 A–A' and G). High expression of *X. tropicalis igfbp2* was detected in PT1-3, DT1-2, and CT, and was

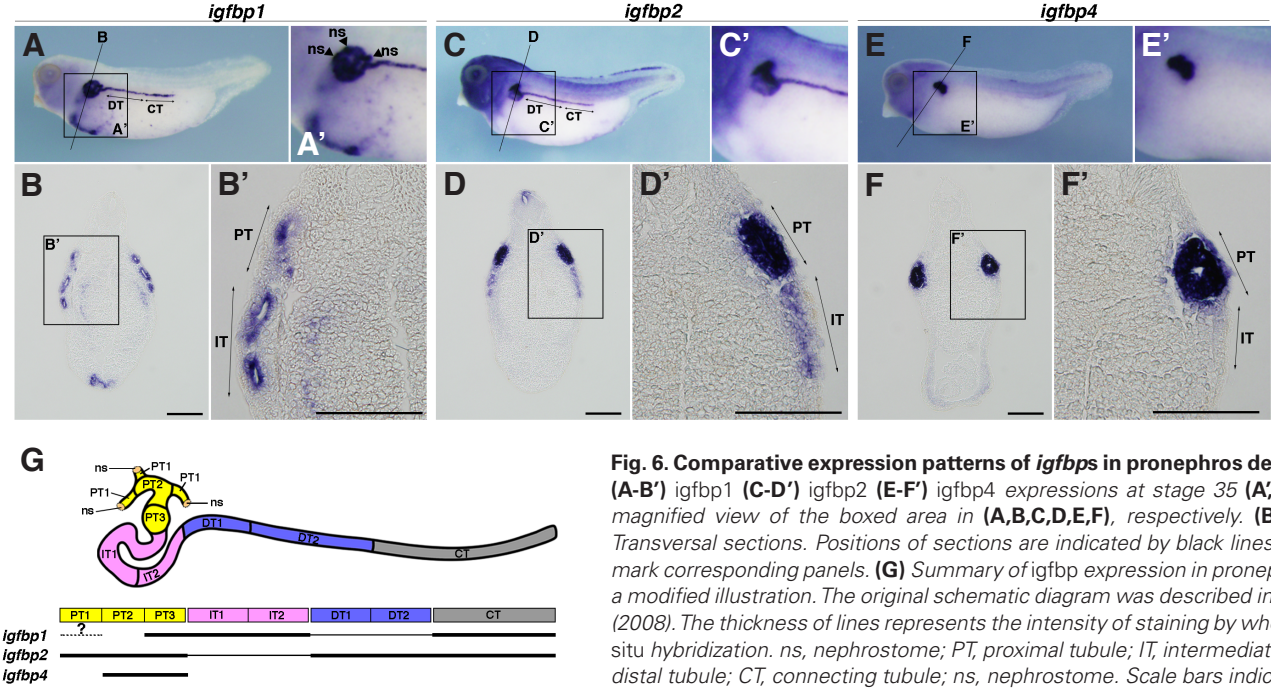


Fig. 6. Comparative expression patterns of *igfbp*s in pronephros development. **(A-B')** igfbp1 **(C-D')** igfbp2 **(E-F')** igfbp4 *expressions at stage 35* **(A',B',C',D',E',F')** *magnified view of the boxed area in* **(A,B,C,D,E,F)**, *respectively.* **(B,B',D,D',F,F')** *Transversal sections. Positions of sections are indicated by black lines, and letters mark corresponding panels.* **(G)** *Summary of* igfbp *expression in pronephros. This is a modified illustration. The original schematic diagram was described in Raciti* et al., *(2008). The thickness of lines represents the intensity of staining by whole-mount* in situ *hybridization. ns, nephrostome; PT, proximal tubule; IT, intermediate tubule; DT, distal tubule; CT, connecting tubule; ns, nephrostome. Scale bars indicate 200 μm.*

TABLE 1

RT-PCR PRIMER SEQUENCES

Gene	Primer sequence	Annealing temperature (℃)	Cycles	Length (bp)	Accession no.	Reference
igfbp1	5'-GCTGCCTGACTTGTGCTCTAAAG-3' 5'-CGAAGAAATGGTGGAATCTGGTC-3'	55	35	265	BC099978	new
igfbp2	5'-ATGAGCAGCAGCGGTCAAAG-3' 5'-TTCACACACCAGCATTCCCC-3'	55	35	244	BC135928	new
igfbp4	5'-CCCTGCCCTTTTGTTGCTTG-3' 5'-GGACTCAATCTCCCCAATCTCAG-3'	55	35	301	XM_002942584	new
igfbp5	5'-TGTGAGCCCTGCGATGATAAAG-3' 5'-TTCTGAGGTGGTCGGTTCTTCG-3'	55	35	288	CR848090	new
odc	5'-GCACATGTCAAGCCAGTTCT-3' 5'-TGCGCTCAGTTCTGGTACTT-3'	60	22	303	NM_001005441	Haramoto et al., 2004
ef1a	5'-TGTAGGAGTCATCAAGGCGGTC-3' 5'-ACAGATTTTGGTCAAGTTGCTTCC-3'	60	22	321	NM_001016692	Fukuda et al., 2010

detectable in DT1-2 (Fig. 6 C–D' and G). High expression of *X. tropicalis igfbp4* was detected only in PT2-3 (Fig. 6 E–F' and G). Our results showed that at least two *igfbps* were co-expressed in all pronephric domains, suggesting that redundant function of *igfbp* genes is required in early pronephric kidney development.

In conclusion, we identified *X. tropicalis igfbp* genes and examined their spatial and temporal expression patterns during early embryonic development. Such distinct expression patterns of *igfbp* genes suggest divergent roles in embryonic development. Redundancy and tandem repeats in the genome make it difficult to understand their *in vivo* function. The genome of *X. tropicalis* has fewer *igfbp* genes than mammalian genomes. This small number of *igfbp* genes indicates that *X. tropicalis* is suitable for loss of function analysis of the Igfbp family. Our study will facilitate functional analysis of the Igfbp family during embryonic development.

Materials and Methods

Genome Analysis

Using Metazome v3.0 (http://www.metazome.net/) and the Xenbase genome browser (*X. tropicalis*-ver. 7.1) (http://gbrowse.xenbase.org/fgb2/gbrowse/xt7_1/?), the upstream and downstream flanking genes of *igfbp* orthologs were compared between *H. sapiens*, *M. musculus*, and *X. tropicalis*. The neighbor joining phylogenetic tree was calculated using MacVector 11.1.0 software.

Cloning of X. tropicalis igfbp *genes*

X. tropicalis igfbp sequences were RT-PCR amplified from total cDNA using gene-specific primers. Gene-specific primers were as follows: *igfbp1* (NM_001033946) (F, 5′-CACACTCGAGATGGCTAGGGAGAACATCTC-3′; R, 5′-CACATCTAGACTATTCTTGAACATTAAGGTAC-3′), *igfbp2* (NM_001100237) (F, 5′-CACAGAATTCATGGGGCTCAGCCGGTACCTG-3′; R, 5′-CACATCTAGACTACGGGGCCCGCTGAGTATG-3′), igfbp4 (XM_002942584) (F: 5′-CACAGAATTCATGTCTGGAAACTGCCACCC-3′; R, 5′-CACACTCGAGTCATTCCTTTCCCCTCTCAG-3′), and igfbp5 (NM_001016042) (F, 5′-ATGGAAATGTTGGTGCCAGC-3′; *igfbp5* R, 5′-TCATTCTGTGTTGCTGCTATC-3′). The PCR products were digested with *Xho*I/*Xba*I for *igfbp1*, *Eco*RI/*Xba*I for *igfbp2*, *Eco*RI/*Xho*I for *igfbp4* and cloned into the corresponding site of the pCS2p vector. The PCR product for *igfbp5* was cloned into the *Stu*I site of the pCS2p vector.

RT-PCR

Total RNA was extracted from *X. tropicalis* embryos and adult tissues (Nigerian line) using ISOGEN (Nippon Gene, Toyama, Japan) by homogenization (Physcotron, Microtec Co., Ltd., Chiba, Japan). First-strand cDNA was synthesized from 1 μg of total RNA using Superscript III reverse transcriptase (Life Technologies, Carlsbad, CA, USA). *Ornithine decarboxylase* (*odc*) and *elongation factor 1α* (*ef1α*) were used as internal controls. Reverse transcriptase negative (RT-) reactions indicated the absence of genomic DNA contamination. Primer sequences, sizes of PCR products, and cycling numbers are described in Table 1.

Embryos and whole-mount in situ *hybridization*

X. tropicalis embryos were obtained by artificial fertilization, and cultured in 0.1× Steinberg's solution (Haramoto *et al*., 2004). The embryos were staged according to Nieuwkoop and Faber (1956). Whole-mount *in situ* hybridization was carried out as previously described (Sive *et al*., 2000). DIG-labeled antisense RNA probes were synthesized with T7 polymerase (Promega, Madison, WI, USA) using the following plasmids: pCS2p-igfbp1, pCS2p-igfbp2, pCS2p-igfbp4, and pCS2p-igfbp5. After whole-mount *in situ* hybridization, embryos were embedded in paraffin, sectioned into thin slices (10 μm), and observed under an optical microscope (BX51; Olympus, Tokyo, Japan).

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Author contributions

Conceived and designed the experiments: YH, YI. Performed the experiments: YH, TO, and ST. Wrote the paper: YH.

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