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Cloning and expression analysis of *Fgf5*, *6* and *7* during early chick development

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ABSTRACT

FGFs with similar sequences can play different roles depending on the model organisms examined. Determining these roles requires knowledge of spatio-temporal *Fgf* gene expression patterns. In this study, we report the cloning of chick *Fgf5*, *6* and *7*, and examine their gene expression patterns by whole mount in situ hybridization. We show that *Fgf5*'s spatio-temporally restricted expression pattern indicates a potentially novel role during inner ear development. *Fgf6* and *Fgf7*, although belonging to different subfamilies with diverged sequences, are expressed in similar patterns within the mesoderm. Alignment of protein sequences and phylogenetic analysis demonstrate that FGF5 and FGF6 are highly conserved between chick, human, mouse and zebrafish. FGF7 is similarly conserved except for the zebrafish, which has considerably diverged.

Fibroblast growth factor (FGF) signaling is required for cell migration, proliferation and differentiation during embryonic development (Itoh and Ornitz, 2008). FGFs bind to FGF receptors (FGFR) and heparan sulfate oligosaccharides to activate signal transduction in target cells. FGF signaling is regulated by the amount of FGF ligand available and by the regionally restricted expression of FGFs and FGF receptors (FGFR). The specificity of FGFs is further enhanced by the presence of tissue specific modifications in the heparan chains (Ornitz and Marie, 2002). Alternative splicing of the FGFR mRNA results in generation of specific isoforms. The isoforms are tissue specific, as the b-isoform is generated in epithelial cells and c-isoform in mesenchymal cells (Ornitz and Marie, 2002). FGFs serve as mitogens and mediators of cellular differentiation for many ectoderm and mesoderm derived cells, such as fibroblasts, chondrocytes, smooth muscle cells and vascular endothelial cells (Goldfarb, 1990). All FGFs contain a highly conserved 28 amino acid core region (Ornitz, 2000), and 22 FGFs in various vertebrate species, including human, mouse and chick, have been reported. Due to duplication within the zebrafish genome, multiple copies of *Fgf* genes have arisen, often with divergent roles (Itoh, 2007; Itoh and Ornitz, 2008).

FGF signals across epithelial–mesenchymal boundaries play key roles in induction and patterning of adjacent tissues. Craniofacial development requires FGF signaling, with restricted expression in the nasal and midfacial tissues, suggesting that FGFs play a role in patterning these structures (Bachler and Neubuser, 2001). FGFs are also required for otic placode induction, patterning and differentiation of the otic vesicle, cochlea, vestibular system, and the endolymphatic organ (Schimmang, 2007). The pharyngeal arches and subdivisions of the brain show regionalized expression patterns (Lunn et al., 2007). Somitic mesoderm, pharyngeal endoderm and limb bud mesoderm all required FGF signaling (Alvarez et al., 2003; Couly et al., 2002; Schimmang, 2007; Trokovic et al., 2005).

As a first step in understanding the role of FGFs during development it is essential to determine the gene expression patterns of *Fgf* family members in the developing embryo. Although many FGFs can act redundantly, some that show similarity in their spatio-temporal gene expression are not necessarily functionally redundant. A further complication is that the roles of FGFs with similar sequence can differ in their spatio-temporal expression patterns and mechanism of action between species. For example, *Fgf3* and *Fgf8* are expressed in rhombomere 4 (hindbrain) in the zebrafish embryo and both are required for otic induction (Leger and Brand, 2002; Phillips et al., 2001). In mouse, *Fgf3* is expressed in the neuroectoderm, whereas *Fgf10* is expressed in the head mesenchyme, but both are required for otic induction (Wright and Mansour, 2003). In chick, *Fgf19*, expressed in the cephalic paraxial mesoderm, and *Wnt8c* and *Fgf3*, expressed in the hindbrain, are required for otic induction (Ladher et al., 2005). *Fgf15* is the murine homolog of chick *Fgf19*. Mouse FGF15 and chick FGF19 share very low sequence conservation (32.1%) (Wright et al., 2004), yet they share similar expression domains in some tissues such as the primitive streak, pharyngeal pouches and tail bud. However, *Fgf15* is not expressed in the mesoderm underlying the otic placode and, unlike

Abbreviations: FGF/*Fgf*, Fibroblast Growth Factor; FGFR, FGF receptor; ISH, in situ hybridization.

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chick FGF19, mouse FGF15 is not required for otic induction (Wright et al., 2004).

In this study, we report the cloning and expression analysis of chick *Fgf5*, *6* and *7* during early development. Targeted fragments of *Fgf5*, *6* and *7* were used to make riboprobes with the least overlap to FGF family members for in situ hybridization (ISH) analysis during early embryogenesis. Non-consensus probes were used to reduce potential cross-reaction between *Fgf* family members. Our results show regionally restricted spatio-temporal expression patterns. *Fgf5* has a novel expression domain in the nascent otic placode, which bears further investigation. *Fgf6* is expressed in the head mesoderm and pharyngeal arches at early stages. *Fgf7* are expressed in the head mesoderm, but excluded from the pharyngeal arches.

1. Results and discussion

1.1. Multiple alignment and phylogenetic analysis

The amino acid sequences of *Fgf5*, *6* and *7* were deduced from NCBI (http://www.ncbi.nlm.nih.gov), and Ensembl (http://www.ensembl.org) database. The four taxa chosen for the phylogenetic study were chick (gg-*Gallus gallus*), mouse (mm-*Mus musculus*), human (hs-*Homo sapiens*) and zebrafish (dr-*Danio rerio*).

Based on multiple alignment analysis, amino acid sequences of FGF5 and FGF6 were highly conserved between all four taxa studied (Fig.1A and B). In zebrafish FGF6a corresponds to the FGF6 sequences in the other taxa. FGF5 and FGF7 are not duplicated in the zebrafish. Although the amino acid sequence of FGF7 indicated high conservation between chick, mouse and human (Fig. 1C), the zebrafish sequence has diverged, suggesting a diverged role for FGF7 in the fish. Further, the amino acid sequences of all three genes showed a higher percent similarity to their murine counterpart, as compared to the human and zebrafish orthologs (Table 1).

A phylogenetic tree of the amino acid sequence of FGF5, 6 and 7 was generated by the Maximum Likelihood (ML) method in the four taxa (bootstrap replicates, $n = 1000$) (data not shown). The same tree topology was deduced by the Neighbor Joining (NJ) method (bootstrap replicates, $n = 1000$). Based on this analysis, FGF5 and FGF6 were more closely related across different vertebrate classes and can be termed as sister groups. FGF7 was an outgroup to both FGF5 and FGF6. For all three proteins, the human and mouse orthologs were more closely related, forming sister clades. The chick orthologs serve as an outgroup for both FGF5 and FGF7. Zebrafish FGF5 and FGF7 were evolutionarily more distant, which corroborated well with our percent similarity and sequence alignment results. Chick and zebrafish FGF6 can be termed sister clades, just as the mouse and human orthologs constitute a sister clade for FGF5 and FGF7.

1.2. Chick FGF5, 6 and 7 bioinformatics analysis

The bioinformatics analyses were performed with Phobius and SignalP 3.0 servers. In addition to Phobius, the Simple Modular Architecture Tool (SMART) from NCBI was used to predict the membrane topology, signal peptides and transmembrane helices (Fig. 1). The Phobius and SMART programs predicted that FGF5 lacks membrane helices and consists of two low complexity segments from amino acid residues 1–14, and the other from amino acid residues 170–183 (Fig. 1A). The SignalP 3.0 results predicted FGF5 to be a non-secretory protein with low probability of having either a signal peptide or signal anchor. On the other hand, the prediction indicated that FGF6 has a signal peptide region (amino acids 1–37), a transmembrane domain (amino acid residues 21–41) and that the remainder of the protein (amino acid residues 38–207) is non-cytoplasmic (Fig. 1B). The signal peptide cleavage site lies between amino acid residues 37 and 38. It was predicted that FGF7 has a cytoplasmic domain (amino acid residues 1–11), short transmembrane domain (amino acid residues 12–31) and a non-cytoplasmic domain (amino acid residues 32–194) (Fig. 1C). The signal peptide region in FGF7 extends from amino acid residues 1–31. The SignalP 3.0 server also predicted a signal anchor for FGF7 and the signal peptide cleavage site between amino acid residues 31and 32.

The Conserved Domain Search Tool (CDART) of NCBI revealed a FGF domain (cd00058), a receptor interaction site and a heparin-binding site (glycine box) for FGF5, 6, and 7 (Fig. 1, hash tags). The PFAM (FGF) domain was predicted to include amino acid residues 23–152 in FGF5, amino acid residues 83–204 in FGF6 and amino acid residues 65–198 in FGF7.

To determine the possible functions of FGF5, 6 and 7, ExPASy proteomics server, Prosite, was used to determine the various protein domains and functional sites. FGF5 was predicted to contain a serine rich region (amino acid residues 1–14), a proline rich region (amino acid residues 153–181), and an HBGF/FGF family domain (amino acid residues 84–107) (Fig. 1A). Serine rich sequences are fairly common amongst signaling proteins, although the functional role of these regions is not well understood. These regions serve multiple functions, such as flexible linkers between protein domains, mRNA splicing, or act as phosphorylation switches during signal transduction (Sim and Creamer, 2002). In addition to their structural role in forming the correct conformation of the mature protein, the proline rich regions are crucial binding sites for different protein–protein interactions (Williamson, 1994). It was predicted that FGF6 contains a HBGF/FGF family domain from amino acid residues 144–167 (Fig. 1B), whereas in FGF7 this domain was predicted to include amino acid residues 125–148 (Fig. 1C). Further, a prokaryotic membrane lipoprotein attachment site (a post-translational modification) was also predicted for FGF7 at amino acid 32 (not shown).

1.3. Fgf5 gene expression

In mammals FGF5 is a secreted glycoprotein and a member of the FGF4 subfamily (FGF4, 5, and 6) and signals via FGF receptors with specific affinities, shown here in decreasing order of affinity – FGFR1c, 2c > 3c, 4Δ (Zhang et al., 2006). Our bioinformatics analysis predicts that chick FGF5 has a low probability of a signal peptide or signal anchor in its sequence. Therefore, we postulate that chick FGF5 is secreted via the non-classical secretory mechanism similar to FGF2 (Backhaus et al., 2004). *Fgf5* is expressed in numerous mouse tissues over the course of development, including the endoderm, lateral plate mesoderm, skeletal muscle precursors, limb mesenchyme and cranial acoustic ganglion (Haub and Goldfarb, 1991). It is also expressed in the midbrain, dorsal cerebellum and pons, acoustic and dorsal root ganglia, muscle tissue, and the adult brain (Yaylaoglu et al., 2005). Human FGF5 injected into chick embryo limbs using a viral vector increases proliferation of limb fibroblasts, expansion of the perichondrium and connective tissue at the expense of skeletal muscle development (Clase et al., 2000).

Using an extended range of Hamburger and Hamilton (Hamburger and Hamilton, 1951) stages (HH) 4–19 for the ISH analysis, the earliest we detected specific *Fgf5* expression was at HH8 (Fig. 2A). The most notable feature of expression is that *Fgf5* was specifically expressed in the nascent otic placode around the time of induction. The otic placode is a thickening of ectoderm in the hindbrain region that becomes morphologically apparent at around HH9. Induction requires interactions between multiple FGF proteins and WNT8c in the hindbrain, placode and underlying mesoderm (Ladher et al., 2005). The placode invaginates forming