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Expression patterns of FGF receptors in the developing mammalian cochlea

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Abstract

Many studies have shown the importance of the fibroblast growth factor (FGF) family of factors in the development of the mammalian cochlea. There are four fibroblast growth factor receptors (FGFR1–4) and all four are expressed in the cochlea during development. While there are examples in the literature of expression patterns of some of the receptors at specific stages of cochlear development there has been no systematic study. We have assembled a full analysis of the patterns of receptor expression during cochlear development for all four *Fgfrs* using in situ hybridization. We have analyzed the expression patterns from E13.5 through post-natal ages. We find that *Fgfr1*, 2 and 3 are expressed in the epithelium of the cochlear duct and *Fgfr4* is limited in its expression to the mesenchyme surrounding the duct. We compare the receptor expression pattern to markers of the sensory domain (*p27^{kip1}*) and the early hair cells (*math1*).

Keywords

hair cell; support cell; pillar cell; inner ear; sensory epithelium; the organ of Corti

Introduction

The mammalian cochlea contains the primary sensory cells for the auditory system. The mature organ of Corti contains a single row of inner hair cells and three rows of outer hair cells, as well as various types of supporting cells. A great deal has been learned about the normal development of this structure and in the past ten years the molecular mechanisms that regulate its development have begun to be elucidated. The cochlea, in the mouse, forms initially from an out-pocketing of the ventro-medial otocyst at embryonic day (E) 11–12 (Morsli et al., 1998). The precursors of the hair cells and the associated support cells exit the cell cycle between E12–E16, in a apical to basal gradient, and most are generated by E14 (Ruben, 1967). After the cells in the sensory region have completed their last mitotic division, they begin their differentiation as hair cells and support cells in the mid-basal region; the hair cells and support cells continue to differentiate in a temporal gradient that extends both basally and apically, such that the mature complement of hair cells and support cells is achieved in the mouse by E17 to E18 (Ruben, 1967; Sher, 1971; Lim and Anniko, 1985).

Many studies have shown the importance of fibroblast growth factors (FGFs) and their receptors at various stages of cochlear development. 23 different *Fgfs* and 4 FGF receptors (*Fgfr1–4*) have been identified in the mouse and human (Ford-Perriss et al., 2001; Itoh,

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2001; Ornitz and Itoh, 2001; Itoh and Ornitz, 2004; Zhang et al., 2006; Itoh and Ornitz, 2008). In mice, FGF3, FGF10 and FGF8 have been implicated in the early inductive events of the otic vesicle (Mansour et al., 1993; Alvarez et al., 2003) (Wright and Mansour, 2003; Ladher et al., 2005; Martin and Groves, 2006; Ohyama et al., 2007). Mice deficient in both *Fgf3* and *Fgf10* fail to form otic vesicles. Similar defects in the early stages of otocyst development are present in mice with targeted deletion of a specific isoform of *Fgfr2* (FGFR2 IIIB) (Pirvola et al., 2000) and it has been proposed that FGF10 and FGF3 act as the ligands for FGFR2 in otic placode formation and patterning (Pauley et al., 2003; Wright et al., 2003). In the sensory specification phase of the organ of Corti, FGF20 and FGFR1 play a key role (Pirvola et al., 2002; Hayashi et al., 2008). Tissue specific deletion of *Fgfr1* results in severe defects in the development of both hair cells and support cells, and those sensory cells that develop are found in small clusters (Pirvola et al., 2002). Inhibition of FGF20 at this stage causes a reduction in the number of the hair cells and support cells similar to that in the *Fgfr1* deletion (Hayashi et al., 2008). At a later stage of embryonic and neonatal development of the organ of Corti, FGF8, acting through FGFR3, is required for pillar cell differentiation (Colvin et al., 1996; Hayashi et al., 2007; Jacques et al., 2007; Puligilla et al., 2007).

Many lines of evidence have shown the importance of FGF signaling in cochlear development. Although, Wright et al. analyzed expression of FGF receptors at early stages of otic vesicle development (Wright et al., 2003), there is no report that systematically examines the detailed expression patterns of the 4 receptors throughout the later stages of cochlear development. Therefore, we undertook a comprehensive analysis using in situ hybridization of *Fgfr1*, *Fgfr2*, *Fgfr3* and *Fgfr4* at embryonic, neonatal and adult stages of cochlear development. These expression patterns confirm and extend previous reports (Peters et al., 1993; Pirvola et al., 1995; Colvin et al., 1996; Pirvola et al., 2000; Pickles, 2001; Pirvola et al., 2002; Hayashi et al., 2007; Puligilla et al., 2007; Hayashi et al., 2008) and highlight the many critical roles of these receptors in cochlear development.

Results and Discussion

At the earliest stage of cochlear development we analyzed, E13.5, the cochlear duct has not fully extended and so the typical three turns are not yet present. This stage of cochlear development is characterized by the gradual cessation of proliferation in the prosensory region and the specification of hair and support cell progenitors in the presumptive organ of Corti. The cochlear duct expresses both *Fgfr1* and *Fgfr2* at this stage (Figure 1A, B), while *Fgfr3* and *Fgfr4* are not expressed in the epithelium (Figure 1C, D). All probes used in this study will detect both splice forms of the receptors. The expression of *Fgfr1* appears diffuse through the epithelium of the cochlear duct and in the adjacent mesenchyme. Despite the diffuse pattern of staining, this expression is specific as evidenced by the highly specific patterns of expression obtained elsewhere in the embryo (see Supplemental Figure 1A). The expression of *Fgfr2* is confined to the non-sensory regions of the cochlear duct (Figure 1B) and is also expressed in the developing otic capsule. The expression of the receptors is similar at E14.5, though now the three turns of the cochlea are apparent (Figure 1E, F). In addition, at this age, *Fgfr1* expression is highest in the ventral aspect of the duct; this is particularly apparent in the apical part of the cochlear duct (Figure 1E, 3). *Fgfr2* expression at E14.5 resembles that at E13.5, and *Fgfr3* and *Fgfr4* are not expressed in the epithelial cells of the duct (Figure 1C, D, G and H). We confirmed that the all probes showed the specific patterns using E13.5 head and E14.5 eye sections (Supplemental figure1). We use “ventral” to refer to the part of the ductal epithelium that contains the prosensory domain and “lateral” and “medial” to refer to the position relative to the modiolus of the cochlea, which is the central axis of the cochlea.

The expression of *Fgfr1* in the presumptive sensory domain is consistent with previous data demonstrating a critical role for this receptor in prosensory specification. Pirvola's group demonstrated that mice with a conditional deletion in *Fgfr1* in developing cochlea have severe defects in the development of the hair and support cells (Pirvola et al., 2002). We have previously reported similar defects when FGF signaling is inhibited with SU5402, a pan-FGFR inhibitor. Moreover, inhibition of FGF20, a ligand specifically expressed in the prosensory domain at this stage, has similar effects as genetic or small molecule inhibition of FGF signaling (Hayashi et al., 2008). Since none of the other FGF receptors is expressed in the presumptive sensory domain at this stage, it is likely that FGF20 acts through FGFR1 in the process of prosensory specification.

During the next stage of cochlear development, there is a transition from the sensory specification phase to a maturation phase. The hair cells and support cells begin their differentiation in the mid-basal turn at E15.0 and the differentiation proceeds towards the apex and also towards the extreme base. By E15.5, the pattern of expression of the receptors begins to change. *Fgfr1* is still more highly expressed in the ventral aspect of the duct in the more apical turns (Figure 1I), but towards the base of the cochlea, the expression becomes concentrated into a cluster of cells at the lateral aspect of the prosensory domain in the region of the developing outer hair cells (Figure 1I, Figure 2A'). *Fgfr2* expression is similar to the pattern at E14.5 (Figure 1J). *Fgfr4* expression also begins at E15.5; however, this receptor is not expressed in the epithelium, but rather in the mesenchymal cells adjacent to and surrounding the duct (Figure 1L, arrowheads). Figure 2 shows the relationship between the *Fgfr1* and *Fgfr2* expression and the sensory epithelial domain (labeled with p27^{kip1}) and the first developing inner hair cells (labeled with Math1-GFP). The Math1-GFP hair cells are immediately adjacent to the medial aspect of the more highly expressing *Fgfr1*⁺ cells (Figure 2A and A'), and co-extensive with the p27^{kip1}⁺ domain at this age (Figure 2B and B'). By contrast, *Fgfr2* is expressed in cells just lateral to the sensory epithelial domain (Figure 2C and C'). Comparison of the expression patterns for *Fgfr1* and *Fgfr3* with Sox2 shows that *Fgfr3* is expressed at the lateral edge of Sox2 expression (Figure 3A, A' and C, C') whereas *Fgfr3* overlaps more with *Fgfr1* expression (Figure 3B, C and B', C').

Also at E15.5, *Fgfr3* begins to be expressed in the developing sensory region, specifically in the basal two turns of the cochlea (Figure 1K, arrows). The expression of *Fgfr1* and *Fgfr3* overlaps at this stage. This can be best observed by examination of the expression of these receptors in relation to the developing inner hair cells, labeled with Math1 (Figure 4). The panels in Figure 4A, A' show expression of *Fgfr1*, Figure 4B, B', the expression of *Math1* on adjacent sections, and Figure 4C, C' the expression of *Fgfr3*, also on adjacent sections. The in situ expression patterns were pseudo-colored and overlapping images were generated and are shown in Figure 4D, E, and F. From these images, the overlap between the highest region of expression of *Fgfr1*, and that of *Fgfr3* can be seen, and it appears that the *Fgfr3* expressing cells are a nested subset of the high *Fgfr1* expressing cluster. The position of these cells immediately adjacent to the first Math1 expressing cells (inner hair cells) suggests that the presumptive pillar cells, outer hair cells and Deiters' cells express the two receptors at this stage of development.

As early as E15, there exists the possibility that *Fgfr3* may be able to compensate for loss of *Fgfr1*, since there is substantial overlap in their expression. As noted above, loss of function of *Fgfr1* leads to defects in both hair cell and support cell development, and therefore *Fgfr3* cannot replace the early functions of *Fgfr1*. In fact, rather than a decline in the number of hair cells and support cells, targeted deletion of *Fgfr3* leads to mice with an over-production of hair cells and support cells in the apical two thirds of the cochlea (Hayashi et al., 2007; Puligilla et al., 2007), suggesting that FGFR3 may actually interact to reduce signaling through FGFR1. One way this could occur is if activation of FGFR3 led to increased

Sprouty2 expression, which could then reduce the effectiveness of signaling through both the FGFR1 and the FGFR3; however, we found no changes in the expression of *Sprouty2* in our analysis of *Fgfr3* mutants (Hayashi et al., 2007). An alternative possibility is that FGFR1 and FGFR3 compete for the FGF ligands. While our expression data show that these two receptors are expressed in the same cells at this stage of development, and the genetic evidence indicates the presence of an interaction, the nature of that interaction remains elusive.

At E16.5, *Fgfr1* expression continues to be highest in the cells in the lateral part of the prosensory domain, the region that will give rise to outer hair cells, pillar cells and Deiters' cells. However, this receptor is still expressed in most of the rest of the ductal epithelium as well (Figure 5A and A'). *Fgfr2*, by contrast, is expressed in a very similar region of the epithelium as that at earlier embryonic stages, lateral to the prosensory region (Figure 5B and B'). By E16.5, *Fgfr3* expression is no longer confined to the base (Figure 5C and C'), but is now extended through most of the cochlea (though still absent from the most apical turn). At each point along the base-to-apex axis, *Fgfr3* is expressed in a subset of cells in the sensory domain, the differentiating outer hair cells, pillar cells and Deiters' cells. At E18.5, most hair cells and support cells have differentiated (and can be labeled with antibodies to Prox1 and Myo6+, respectively), except at the apical tip. Figure 5E–H show the expression of the receptors at this stage, in relation to the Myo6 labeled hair cells (Figure 5E'–H'). By E18.5, *Fgfr1* is expressed only weakly in the sensory region, although expression is maintained and even up-regulated in other regions of the epithelium, most notably the greater epithelial ridge (GER) and Hensen's cells (Figure 5A, A', E). The expression of *Fgfr3* is still robust in the sensory epithelium (Figure 4C, C', G), and is now more clearly confined to the pillar cells and Deiters' cells. *Fgfr2* shows the same expression pattern as at earlier stages (Figure 5B, B', F), and *Fgfr4* is not expressed in the epithelium, but is maintained in the surrounding mesenchyme (Figure 5D, D', H).

After birth, the cells of the organ of Corti continue to mature. The tunnel of the organ of Corti flanked by the pillar cells, opens first in the base at approximately postnatal day 7 (P7) and the animals begin to hear after P10. At P0 and P3 (Figures 6 and 7, respectively) the expression of *Fgfr1* is now nearly absent in the sensory domain. However, the signal is up-regulated in the GER (presumptive inner sulcus and border cells) and at the lateral edge of the sensory epithelium in Hensen's cells and possibly Claudius' cells (Figures 6 and 7, A and A'). The expression of *Fgfr2* is similar to that at earlier stages, occupying a domain lateral to the developing sensory epithelium with high levels of expression in the developing Claudius' cells, outer sulcus and spiral prominence (Figures 6 and 7, B and B'). This receptor appears to be expressed at lower levels in the developing stria vascularis and Reissner's membrane (Figures 6 and 7, B and B'). At both P0 and P3, *Fgfr3* shows robust expression in the pillar cells and Deiters' cells, though at P3 the expression appears to be stronger in the pillar cells than the Deiters' cells (Figures 6 and 7, C, C'). After P7, obtaining reliable signal from in situ hybridization is difficult due to the calcification of the cochlear capsule. We could only obtain consistent results with *Fgfr3* (see Supplemental Figure 2). We found *Fgfr3* to be robustly expressed in the pillar cells and Deiters' cells into adulthood. The role of *Fgfr3* in the differentiation of the pillar cells is now well established. FGF8 expressed by the inner hair cells activates FGFR3 in the adjacent pillar cells and this is necessary and sufficient for their differentiation. Over-activation of this signaling, either by ectopic expression of *Fgf8* or addition of FGF17 to explant cultures of cochlea (Jacques et al., 2007), or by targeted deletion of the inhibitor of FGF signaling *Sprouty2*, causes an increase in the number of pillar cells (Shim et al., 2005). In addition mutations in *Fgfr3* which cause over-activation also result in increased numbers of pillar cells (Mansour et al., 2009). Our results are consistent with this interpretation, since at the stages of pillar cell differentiation, *Fgfr3* is the only FGF receptor expressed in these cells.

One of the more striking findings of our analysis is the robust and consistent expression of *Fgfr2* in the presumptive stria vascularis and spiral prominence from E13.5 to P3. A similar conclusion was reached by Pickles using microdissection and RT-PCR in newborn mice (Pickles, 2001), and Pirvola's group showed a similar pattern at E16 (Pirvola et al., 2000). Although a role for *Fgfr2* in early cochlear development and otic induction has been well established, it is not known whether it has a more specific function in the development of the stria or spiral prominence. Conditional deletions of *Fgfr2* in the myelinating glial cells of the spiral ganglia leads to progressive hearing loss (Wang et al., 2009). FGF16 is expressed very specifically in the area of the developing spiral prominence from E14.5 (Hatch et al., 2009) so this may be an important ligand for FGFR2. Conditional deletions of this receptor specifically in the non-sensory regions of the cochlear duct may shed light on potential additional roles for this receptor in inner ear development. In the region of the developing spiral prominence the *Fgfr2* receptor could be deleted using the *Fgf16-Cre* developed in the Mansour lab (Hatch et al., 2009).

A summary of the expression of the FGF receptors during key stages of cochlear development is shown in Figure 8. It is clear that the two important receptors as far as sensory cell development are *Fgfr1* and *Fgfr3*. Based on its expression pattern it is likely that *Fgfr2* plays a role in the development of outer sulcus, spiral prominence and stria vascularis. Expression of *Fgfr4* was confined to the mesenchyme at all stages of development and the morphological development of the cochlea is normal in *Fgfr4*^{-/-} animals (Birmingham-McDonogh, unpublished data). Our results, together with those of a number of investigators, have identified several key roles for FGF signaling during cochlear development. The results of this study further consolidate our understanding of these signaling molecules and suggest that there are additional, undiscovered roles for FGFs in auditory development.

Experimental Procedures

Mice

All mice were housed by the Department of Comparative Medicine; all procedures were carried out in accordance with the guidelines of the animal care and use committee at the University of Washington. Timed pregnant and non pregnant female mice (Swiss-Webster) were purchased from Harlan (Indianapolis, IN). To obtain *Math1*-GFP expressing embryos, male mice carrying the *Math1*-GFP transgene were mated with Swiss-Webster females. Pregnant mothers were euthanized and we used the Theiler staging system (Theiler, 1989) to accurately stage the embryos at the time of harvest (<http://genex.hgu.mrc.ac.uk/Atlas/intro.html>). For the postnatal animals, P0 is defined as the day of birth.

In situ hybridization

Whole heads of embryos were fixed in a modified Carnoy's solution for 6 hours at room temperature. The samples were washed and dehydrated in 100% ethanol overnight at 4°C, and then were embedded in paraffin and 6 µm sections were collected. At least 3 animals were examined at each time point. The hybridization was carried out according to Hayashi et al. (Hayashi et al., 2007) and Digoxigenin (DIG)-labeled probes were prepared according to the manufacturer's manual for DIG-11-UTP (Roche, Indianapolis, IN). The in situ product was visualized using anti-DIG alkaline phosphatase conjugated secondary antibody (Roche) and NBT (nitroblue tetrazolium)/BCIP (5-bromo-4-chloro-3-indolyl phosphate). After color development, the slides were fixed with 4% PFA for 1 hour. cDNA coding for mouse *Fgfr1* and *Fgfr2* were gift from Dr. P. Soriano (Mt. Sinai school of medicine of New York university, NY). NT 1-2526 of *Fgfr1* clone (Gene Bank ID; NM_010206) was sub-

cloned into pCRII vector (Invitrogen). cDNA of *Fgfr2* (NM_010207, nts 891–2634) was sub-cloned into pCRII. *Fgfr3* clone was purchased from OpenBiosystems Inc. Huntsville, AL (clone ID5708838), probe was prepared using *Xho*I restriction endonuclease which generates a probe of 2206 NTs. The probes used for *Fgfr1-3* will not distinguish between the two splice variants. cDNA for mouse *Fgfr4* was gifted from Dr. A. McMahon (Harvard University, Boston, MA), and sub-cloned into pBlueScriptII (NM_010207, nts 891–2634). *Math1* clone was purchased from OpenBiosystems Inc (2,144 bp, clone ID 6530849). cDNA encoding Sox2 (530 bp) was a gift from Dr. Kondoh (Osaka University, Japan).

Immunofluorescence

Paraffin embedded and sectioned tissue was used after de-waxing or after in situ hybridization. Tissue sections were incubated with 10% fetal bovine serum and 1% nonfat dry milk in PBS/0.1% Triton X-100 (PBST) for 30 minutes. Sections were incubated overnight at 4°C with the following primary antibodies and dilutions: rabbit anti-Myo6, 1:1000 (Proteus Biosciences, Ramona, CA); mouse anti-p27^{kip1}, 1:100 (BD Transduction Laboratories, San Diego, CA); or chicken anti-GFP, 1:200 (Abcam, Cambridge, MA). The sections were rinsed with PBST (PBS with 0.1% Triton X100), incubated for 4 hours with a fluorescent-conjugated secondary antibody, rinsed with PBST, and mounted in Fluoromount G (Southern Biotechnology, Birmingham, AL).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Expression patterns of *Fgf* receptors in the E13.5, E14.5 and E15.5 cochlea
 A–D show micrographs of adjacent sections of the middle region of the E13.5 cochlear duct showing in situ localization of *Fgfr* 1–4, respectively. E–H show adjacent sections of the E14.5 cochlea. Levels (half-turns) of the cochlear duct are numbered from the base (turn 1) to the apex (turn 3). I–L show adjacent sections of the E15.5 cochlea with the same numbering as at E14.5. Scale bars= 100 μ m.

Figure 2. Temporal and spatial relationship between *Fgfr1* and *Fgfr2* expression and the sensory epithelium

Shown are micrographs of adjacent sections of the E15.0 cochlea from a Math1-GFP transgenic mouse taken at low magnification (A, B, C) and high magnification of the 2nd turn (A'B'C'). The cochlear turns are numbered from the base (1) to apex (4). Following in situ hybridization for *Fgfr1* or *Fgfr2*, the sections were processed for immunofluorescence with antibodies against p27^{kip1} (red) to identify the sensory domain and GFP (green) to label the developing hair cells. The arrow points to the GFP+ cells in A', B' and C', while the p27^{kip1} domain is shown as a black bar. Scale bars= 100µm

Figure 3. Comparison of *Fgfr1* and 3 with *Sox2* at E15.5

A–C show expression of *Sox2*, *Fgfr1* and *Fgfr3* respectively. A'–C' are higher magnification images of the basal turn shown boxed in A–C. The dark staining areas marked with asterisks are nonspecific background.

Figure 4. Temporal and spatial relationship between *Fgfr1* and *Fgfr3* expression and the developing hair cells

Shown are micrographs of adjacent sections of E15.0 cochlea after in situ for *Fgfr1* (A, A'), *Math1* (B, B') or *Fgfr3* (C, C'). Panels D–F are pseudo-colored combinations to show the relationship among the expression patterns. The expression domain of *Fgfr3* appears to be nested within the expression domain of *Fgfr1* (F), while *Math1* expression in the developing inner hair cell medial to both *Fgfr1* (D) and *Fgfr3* (E) suggests that both these receptors are expressed in the developing outer hair cells and supporting cells. In panels A, B, and C, the cochlear turns are numbered from the base (1) to apex (4). The mid- basal turns (turn 2) are shown in panels A', B' and C'. Scale bars= 100μm

Figure 5. Expression patterns of *Fgf* receptors in the E16.5 and E18.5 cochlea

A–D show sister sections of the E16.5 cochlea at low magnification; the levels (half-turns) of cochlear duct are numbered from the base (1) to apex (4). The basal turns (turn 1) are shown in panels A', B', C' and D'. E–H show the basal turns (turn 1) from sections of the E18.5 cochlea. The inner hair cells (arrows in E'–H') and the outer hair cells (arrowheads in E'–H') were labeled with anti-Myo6 antibody after *in situ* hybridization. Scale bars= 100µm

Figure 6. Expression patterns of *Fgf* receptors in the P0 cochlea

Panels A–D show sister sections of the P0 cochlea. The levels (half-turns) of cochlear duct are numbered from the base (1) to apex (4). The basal turns (turn 1) are shown at higher magnification in panels A', B', C' and D'. Scale bars= 100μm

Figure 7. Expression patterns of *Fgf* receptors in the P3 cochlea

Panels A–D show sister sections of the P3 cochlea. The levels (half-turns) of the cochlear duct are numbered from the base (1) to apex (5). The basal turns (turn 1) are shown in panels A', B', C' and D'. The inner hair cells (arrows in A'–D') and the outer hair cells (arrowheads in A'–D') were labeled with anti-Myo6 antibody after *in situ* hybridization (A''–D''). Scale bars= 100µm

Figure 8. Schematic of *Fgfr* expression through development

The expression of the four receptors is color-coded and shown at four developmental stages. *Fgfr1* expression is depicted in blue with the darker blue representing more robust expression. *Fgfr2* is represented in orange with the dots of color depicting less expression. *Fgfr3* is shown in purple and *Fgfr4* is depicted with grey lines, this expression is throughout the mesenchyme immediately surrounding the duct. The black dotted line denotes the developing sensory region within the epithelium.