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Notch signaling regulates the pattern of auditory hair cell differentiation in mammals

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SUMMARY

The development of the mammalian cochlea is an example of patterning in the peripheral nervous system. Sensory hair cells and supporting cells in the cochlea differentiate via regional and cell fate specification. The Notch signaling components shows both distinct and overlapping expression patterns of Notch1 receptor and its ligands Jagged1 (Jag1) and Jagged2 (Jag2) in the developing auditory epithelium of the rat. On embryonic day 16 (E16), many precursor cells within the Kölliker's organ immunostained for the presence of both Notch1 and Jag1, while the area of hair cell precursors did not express either Notch1 and Jag1. During initial events of hair cell differentiation between E18 and birth, Notch1 and Jag1 expression predominated in supporting cells and Jag2 in nascent hair cells. Early after birth, Jag2 expression decreased in hair cells while the pattern of Notch1 expression now included both supporting cells and hair cells. We show that the normal pattern of hair cell differentiation is disrupted by alteration of Notch signaling. A decrease of either Notch1 or Jag1 expression by antisense oligonucleotides in cultures of the developing sensory epithelium resulted in an increase in the number of hair cells. Our data suggest that the Notch1 signaling pathway is involved in a complex interplay between the consequences of different ligand-Notch1 combinations during cochlear morphogenesis and the phases of hair cell differentiation.

Key words: Notch1, Jagged1, Jagged2, Organ of Corti, Inner ear, Rat

INTRODUCTION

The mammalian cochlea is an excellent model system to study signaling mechanisms during development. Development of the organ of Corti involves the differentiation of two types of hair cells: inner hair cells (IHCs) and outer hair cells (OHCs) and four types of supporting cells: Deiters, Hensen, Claudius and pillar. Each hair cell is separated from its neighboring hair cell by apical projections from supporting cells, creating an alternating mosaic that extends along the longitudinal axis of the cochlea. Following the terminal mitotic events during development which end between E14 and E16 (Ruben, 1967), a gradient of differentiation progresses in both directions from the mid-basal region of the cochlea (Lim and Anniko, 1985) turns the newly postmitotic cells into various morphologically and functionally distinct cell types. The primary mechanism specifying the two types of hair cells versus the four types of supporting cells within the organ of Corti remains an important question. The Notch pathway is an evolutionary conserved cell-cell signaling mechanism involved in cell fate decisions during different cellular and developmental processes in *Drosophila*, invertebrates and vertebrates (Artavanis-Tsakonas et al., 1999). In several developmental models, the Notch1 signaling pathway controls the ability of equivalent non-terminally differentiated cells to respond to differentiation signals, by choosing between primary and secondary cell fates or retaining cells as uncommitted precursors, according to the lateral inhibition model (Kopan and Turner, 1996; Lewis, 1996). Notch1 can also signal among non-equivalent cells to promote differentiation (Fleming et al., 1997). The effect of Notch activation has been reported to drive cell fate decisions during development in a wide variety of tissues across many species, ranging from the central nervous of mammals (de la Poma et al., 1997, Wang et al., 1998), chicks (Austin et al., 1995) and frogs (Dorsky et al., 1997) to feather development in chicks (Crowe et al., 1998). In mammals, four distinct types of Notch genes have been cloned (*Notch1-Notch4*) (Ellissen et al., 1991; Weinmaster et al., 1992, Lardelli et al., 1994; Uyttendaele et al., 1996). Notch receptor specific-ligands (Jagged1, Jagged2 and Delta) have also been identified in vertebrates (Lindsell et al., 1995; Shawber et al., 1996; Bettenhausen et al., 1995). Signal mediated cell-cell communications responsible for the normal development of the inner ear has just begun to be defined. Notch signaling has been reported to play a role in the development of vertebrate inner ear in chick and zebrafish (Adam et al., 1998; Haddon et al., 1998). These studies implicate lateral inhibition mediated by Notch1-Delta signaling in the control of the pattern of hair cell differentiation. In mammals, results from a recent study suggest that a lateral inhibition mechanism mediated by

Table 1. Descriptions of the corresponding cDNAs of the antisense, sense-treated and scrambled antisense oligonucleotides used in this study

Oligonucleotide	Region	Position	Sequence
Notch1			
antisense	ANK	5866-5888	5′-CCTCCGCTGCAGGAGGCAATCAT-3′
sense		(23 mers)	5′-ATGATTGCCTCCTGCAGCGCAGG-3′
scrambled antisense			5′-CGTAGTGACTACAGAGCGCTCCC-3′
Jag1			
antisense	Upstream to	381-398	5′-TGGGGACCGCATCGCTGC-3′
sense	DSL	(18 mers)	5′-GCAGCGATGCGGTCCCCA-3′
scrambled antisense			5′-TGCGGTCCCCAACGGTGG-3′

ANK, a region of the intracellular domain of Notch1 that contains 6 ankyrin repeats.
DSL, a region of the extracellular domain of Jag1 that contains a Delta/Serrate/Lag-2 motif.

Notch1 signaling also plays a role in the differentiation of mammalian auditory hair cells (Lanford et al., 1999). The cochlea of Jag2 knockout mice showed an increase in the number of IHCs, while evidence of a role for Notch signaling in OHCs differentiation remained unclear. In addition, a role for the Notch1 receptor, a central element in Notch signaling, in hair cell differentiation has not been directly proven, since targeted disruption of the Notch1 gene results in early embryonic lethality, before E11.5 (Swiatek et al., 1994). The role of the Jag1 ligand in hair cell differentiation has not yet been directly tested in vivo, for the same reason of embryonic lethality of null mutants (Xue et al., 1999). This problem with embryo lethality prompted us to address the question of the functional roles of Notch1 and Jag1 in hair cell differentiation with explants of embryonic and neonatal rat cochleae.

We initially investigated the spatiotemporal expression of Notch1, Jag1 and Jag2 in the embryonic (E16 to E20) and neonatal (P0 to P3) rat organ of Corti. The functional significance of both Notch1 and Jag1 expression was then evaluated selectively and independently by using antisense oligonucleotides to decrease their levels of expression in embryonic and neonatal organ of Corti explants.

MATERIALS AND METHODS

Tissue dissection and culture of the organ of Corti

Organotypic cultures of embryonic and neonatal cochleae were established in vitro by a method described in a previous study (Zine and de Ribaupierre, 1998). Timed-pregnant Sprague-Dawley rats or neonates were killed by cervical dislocation on gestational days 16 (E16), E18 and E20, or on postnatal days 0 (day of birth=P0), P1, P2 and P3. Individual cochleae were isolated, stria vascularis and Reissner's membrane were removed to expose Kölliker's organ or the developing organ of Corti, which were placed individually on a sterile membrane (Millipore) in 800 μl of serum-free medium. The culture medium consisted of Dulbecco's Modified Eagle Medium (DMEM) and Ham's F-12 (Sigma) supplemented with insulin (15 μg/ml), transferrin (20 μg/ml), progesterone (2×10^{-8} M), selenium (3×10^{-8} M) and putrescine (10^{-4} M). All cultures were maintained at 37°C in 5% CO_2 with the culture medium exchanged daily.

Antisense oligonucleotides and treatment of cultures

Antisense, sense and scrambled antisense oligonucleotides were 23 mers in length for Notch1 (AS-Notch1) and 18 mers for Jag1 (AS-Jag1) with phosphorothioate linkages between all bases and were selected from a previously characterized antisense for Notch1 (Austin et al., 1995; Redmond et al., 2000) and for Jag1 (Zimrin et al., 1996). For each antisense sequence, scrambled antisense and sense sequences were used as controls (Table 1). Custom synthesis, HPLC-purification, and gel filtration were performed by MWG-BIOTECH (Ebersberg, Germany). A search of the GenBank Database revealed no homologies between the Notch1 antisense oligonucleotide sequence and other members of Notch receptor family and between Jag1 antisense oligonucleotide and the sequences of other known ligands of the DSL family including Jag2 and Delta1.

Explants were incubated for 5 days either in a normal medium or medium supplemented with either antisense, scrambled antisense or sense oligonucleotides. We used lipofectin (Gibco-BRL) as a cationic lipid to deliver all of the oligonucleotides including control sequences, because this method results in high uptake and stability of phosphorothioate oligonucleotides in the intracellular compartment (Bennet et al., 1992; Capaccioli et al., 1993). Treatment of explants were carried out by the addition of a lipofectin-oligonucleotide complex starting from the first day of culture. All medium was exchanged each day including medium supplemented with the lipofectin-oligonucleotide complexes. At the end of culture period, cochlear explants were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 45 minutes, stained with a TRITC-phalloidin for 1 hour and mounted for confocal microscope observations. Quantitative analysis of supernumerary hair cell production was obtained by measuring the length of sensory epithelium regions with supernumerary hair cells. These supernumerary regions were measured if they contain more than a normally single row of IHCs and three rows of OHCs either in antisense-treated explants or untreated age-matched controls. The numbers of IHCs and OHCs were also determined in the regions of supernumerary hair cells from both antisense-treated explants and the corresponding untreated controls. The mean length of the sensory epithelium with supernumerary hair cells and the number of IHCs and OHCs in these regions were determined for at least 5 explants for each stage studied. Significance was determined using the Student's *t*-test.

Immunolocalization of Notch1, Jag1 and Jag2 in the developing organ of Corti

Surface preparations

After decapitation of the animal, embryonic and neonatal rat cochleae were rapidly removed and fixed overnight with 4% paraformaldehyde in PBS at 4°C. After fixation, they were rinsed in PBS, and the surface of Kölliker's organ or the organ of Corti was exposed by removing the otic capsule, tectorial and Reissner's membranes and used as surface preparations.

These surface preparations were permeabilized with 1% Triton X-100, washed and blocked with 10% normal donkey serum. After a 2 hour blocking step, the preparations were incubated overnight at 4°C in primary polyclonal antibodies (2-10 μg/ml) of either anti-Notch1 (C-20: goat), or anti-Jag1 (H114: rabbit) or anti-Jag2 (R19: goat)

(Santa Cruz Biotechnology). Some of these polyclonal antibodies have previously been used in many studies (Morrison et al., 1999; Sestan et al., 1999). After rinses in PBS, preparations were incubated with FITC-conjugated donkey anti-goat or TRIC-conjugated donkey anti-rabbit (Jackson Immunoresearch Laboratories) and, in some of them, double-labeling were performed with a secondary antibody mixture.

Paraffin sections

Cochleae were fixed with 4% paraformaldehyde overnight at 4°C, embedded in paraffin using routine procedures and serially sectioned. After an antigen retrieval step carried out by microwave irradiation of deparaffinized sections in 10 mM citric acid, pH 6.0 for 5 minutes, immunolocalization of Notch1, Jag1 and Jag2 were performed as described for the whole-mount specimens. Specificity of antibodies was confirmed by staining sections with a combination of primary antibodies preabsorbed with a 5-fold excess of their peptide antigens. Other specimens were stained with Jag1 antibody that was pretreated with 5-fold excess of Jag2 antigens in order to check for cross-reactivity. As a negative control, the primary antibodies were deleted from the incubation solution.

Confocal laser scanning microscopy

Observations were performed on a Leica confocal laser scanning microscope. Digital images of a compressed Z-series of scans were made using either 40×, 63× or 100× oil objectives. Continuous series of optical sections in the Z plane were saved and recombined to produce a single reconstruction of the entire thickness of the epithelium or of just the apical regions. In all cases, gain and black levels were adjusted to eliminate bleed-through between fluorophore channels. Images used for the figures were processed with either NIH image or Photoshop (Adobe systems Inc., Mountain View, CA) software programs.

Scanning electron microscopy

Explants for surface ultrastructural analysis were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.2) for 2 hours, washed three times in cacodylate buffer, then postfixed for 1 hour with 1% osmium tetroxide. Explants were dehydrated in ascending concentrations of ethanol, critical-point dried from liquid CO_2 and sputter coated with gold. All material was examined in a JEOL 630F scanning electron microscope operating at 5 kV.

RESULTS

Expression of Notch1, Jag1 and Jag2 in the developing cochlea

At E16, a period just before the onset of auditory hair cell differentiation in embryonic cochlea, both anti-Notch1 and anti-Jag1 antibodies stained the population of precursor cells within the presumptive sensory epithelium of Kölliker's organ. (Fig. 1A-C). Notch1 staining was intensely detected in their apical junctional complex between precursor cells. These epithelial precursor cells coexpressed both Notch1 and Jag1 along their lateral membranes. In contrast, the precursor cells located in a region of the sensory epithelium that gives rise to the organ of Corti were not labeled with either Notch1 (Fig. 1A) or Jag1 (Fig. 1B) antibodies. The presence of the spiral vessel just below this region confirms that this is the site of the future organ of Corti. We failed to detect any specific expression of Jag2 in the presumptive sensory epithelium at E16. On both E18 (Fig. 1D-F) and E20 (Fig. 1G-I), the differentiating sensory hair cells become identifiable, temporally coincident with the upregulation of Notch1 and Jag1 expression within the developing organ of Corti. Immunostaining of both Notch1 and Jag1 was restricted to the membranes of the differentiating supporting cells. Notch1 specifically immunolocalized to the supporting cell layer below the differentiating hair cells (Fig. 1D). Jag1 immunostaining showed widespread and linear distribution (Fig. 1E) marking out what appear to be the developing organ of Corti. Its spatial distribution included the supporting cells in addition to their apical expansions that mark the location of the future OHCs. Restricted expression of Jag1 in supporting cells was not obvious in either the E18 or E20 cochlea sections, since these cells are closely apposed to and interdigitated with the differentiating hair cells making it difficult to be sure whether staining was in either the membranes of the hair cells or the apical processes of the supporting cells or both. However, immunostaining of the surface preparations at this stage of maturation (Fig. 2A-C) clearly shows that Jag1 expression is restricted to the apical processes of the supporting cells. At the same developmental time (E18), Jag2 immunostaining was found in the differentiating IHCs (Fig. 2A) and covered both IHCs and OHCs by E20 (Fig. 2D). Double labeling both of surface preparations of the E18 organ of Corti (Fig. 2C) and sections of the E20 cochlea (Fig. 2F), indicated that Jag2 expression was restricted to hair cells while Jag1 labeling was present in the supporting cells. The specific expression patterns of Notch1, Jag1 and Jag2 persisted through the early postnatal period, after the majority of hair cells in the cochlea have completed their differentiation. Double labeling of surface preparations of the newborn organ of Corti with Jag1 and Notch1 antibodies indicated both overlapping and distinct expression patterns (Fig. 1J-L). They were expressed in supporting cells and, predominantly, in dumbbell-shaped apical phalangeal expansions of Deiters' cells and the inner and outer pillar cells (Fig. 3A,B), although a punctuate Notch1 label was also observed around the apical perimeters of the hair cells (Fig. 3A). The rest of the precursor cells located medial to the organ of Corti continue to express both of Notch1 and Jag1 until at least P3. Similarly, within the vestibular sensory epithelium of the P0 rat saccule there was an overlap of Notch1 and Jag1 immunolabeling associated with the supporting cells (Fig. 3D-F). Immunoreactivity was not detected in sections stained with either antibody preabsorbed with its respective antigen and immunoreactivity for Jag1 was not abolished in those specimens stained with Jag1 antibody preabsorbed with Jag2 antigen. Omission of primary antibodies resulted in a complete loss of immunostaining (data not shown).

Effects of Notch1 and Jag1 antisense treatment on hair cell differentiation

The expression patterns of both Notch1 and its ligands Jag1 and Jag2, suggest a possible physiological role of the Notch1 signaling system in hair cell differentiation during cochlea morphogenesis. Since previous analysis has shown that mouse hair cells also express Jag2 and that mice homozygous for a null mutation in this gene have an excess of IHCs, and to lesser degree OHCs (Lanford et al., 1999), we only tested for a physiological role for Jag1 and Notch1.

To assess if they have functional roles in the embryonic (E16

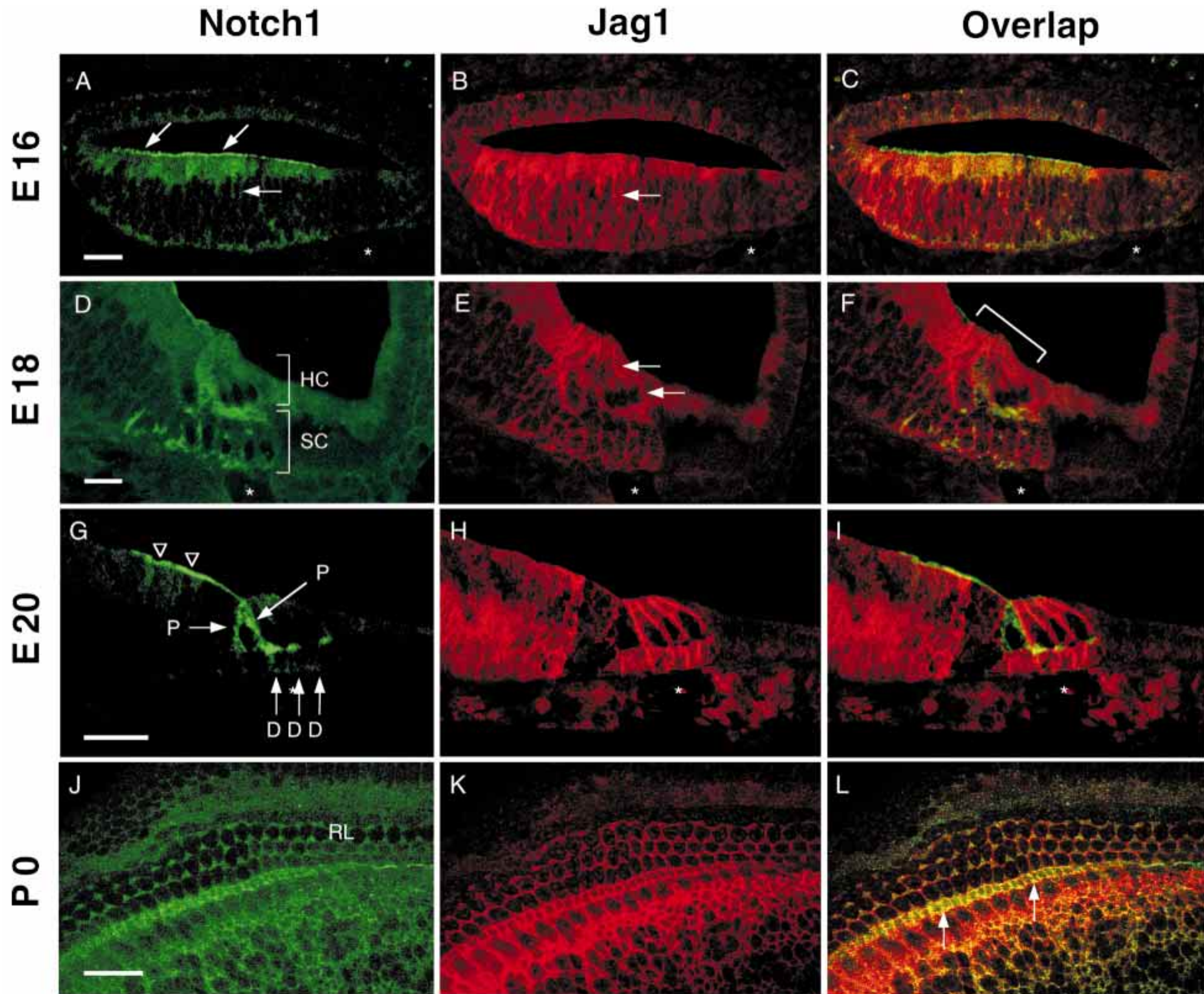

Fig. 1. Confocal images of the expression patterns of Notch1 and Jag1 immunolabeling in the sensory epithelium of the developing cochlea. (A-I) Transverse sections of the embryonic cochlea immunostained with anti-Notch1 antibody (green; A,D,G), anti-Jag1 antibody (red; B,E,H) and a mixture of both antibodies (merged images; C,F,I). Asterisks indicate the position of the spiral vessel, a landmark for the location of the future organ of Corti (A-C) and the differentiating organ of Corti (D-I). (J-L) Immunostained whole-mount surface preparations of newborn organ of Corti processed similarly. (A-C) At E16, Notch1 expression is detected in a most of the progenitor cells that make up Kölliker's organ (KO) which corresponds to the future sensory epithelium (Fig. 1A). The epithelial cells were predominantly immunolabeled for the presence of Notch1 at their apical and basal aspects (arrows). Jag1 expression is observed in the same areas as Notch1 and additionally in the lateral membranes (arrow) of the same population of progenitor cells of the KO (Fig. 1B). Epithelial cells in a region of KO that gives rise to the organ of Corti did not label with either Notch1 or Jag1 antibodies. (D-F) At E18, Notch1 and Jag1 expression patterns mark the prospective developing organ of Corti (brackets in D and F) and were both restricted to the future non-sensory supporting cells (SC). Intense Notch1 expression is detected in the supporting cells just below the developing hair cells (HC). Jag1 has similar pattern but is also expressed in the apical expansions (arrows) of the supporting cells and appear to be in close proximity to the lateral membranes of the nascent hair cells. Expression of neither Notch1 nor Jag1 was observed in the developing hair cells. (G-I) At E20, expression of both Notch1 and Jag1 is restricted to the supporting cells that are in close association with the differentiating hair cells. Punctuate Notch1 expression is present on Deiters' cells (D, arrows) located at the base of OHCs and Pillar cells (P, arrows). Progenitor cells located medial to the IHC region that will form the inner sulcus continue to express Notch1 at their apical regions (arrowheads). There was intense immunostaining for Jag1 on the Deiters' cells and their apical processes that surround the OHCs. (J-L) A whole-mount surface preparation of newborn (P0) organ of Corti showing an overlapping pattern of Notch1 and Jag1expression in apical surface aspects of the supporting cells. Both the receptor and the ligand were immunodetected in the pillar cells (arrows) and phalangeal processes of Deiters' cells at the level of the reticular lamina (RL) with areas of overlap and with some areas with only Notch1 immunostaining. Progenitor cells located medial to the IHC region continue to express both Notch1 and Jag1. All surface preparations of this figure are from the middle turn of the cochlear duct. Bar, 20 µm for each age group.

to E20) and neonatal (P0 to P3) development of hair cells, specific antisense oligonucleotides were used to decrease the levels of Notch1 and Jag1 in organotypic cultures of Kölliker's organ and the organ of Corti. A series of oligonucleotide and lipofectin concentrations were tested and it was determined that the addition of antisense oligonucleotides-lipofectin complexes (5 µM and 3 µg/ml, respectively) provided optimal inhibition with no evident toxicity to the organotypic explants. All treatments were carried

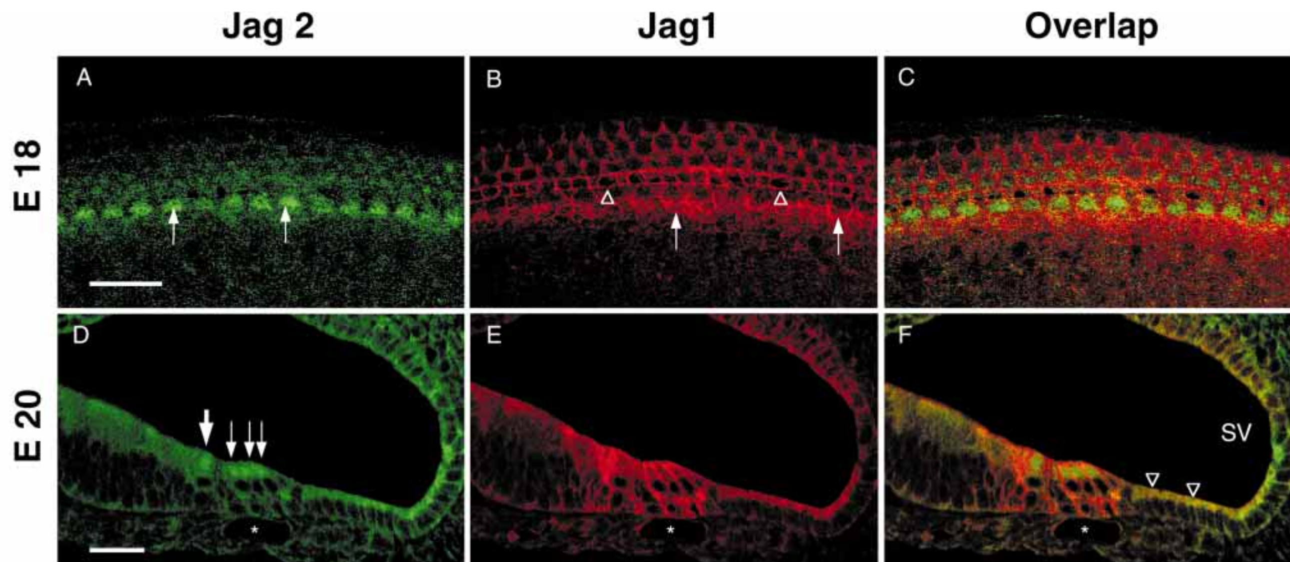

Fig. 2. Confocal images of the expression patterns of Jag1 and Jag2 on surface preparations of E18 organ of Corti (A-C) and on transverse sections of E20 cochlea (D-F). (A-C) Initial expression of Jag2 in the developing organ of Corti is detected at this stage (E18) and was mainly restricted to a single row of differentiating IHCs (arrows) with some light staining in the region of the future OHCs. In contrast, Jag1 staining exhibits a mosaic like-pattern corresponding to the phalangeal processes of Deiters' cells; the supporting cells that surround the IHCs (arrows) and the pillar cells (arrowheads). (D-F) At E20, Jag2 immunostaining was observed in both IHCs (large arrow) and OHCs (small arrows), while Jag1 staining was distributed throughout the supporting cell population. Jag1 and Jag2 were coexpressed in the supporting cells (arrowheads) located lateral to the OHC region and stria vascularis (SV). Bar, 20 μm for each age group.

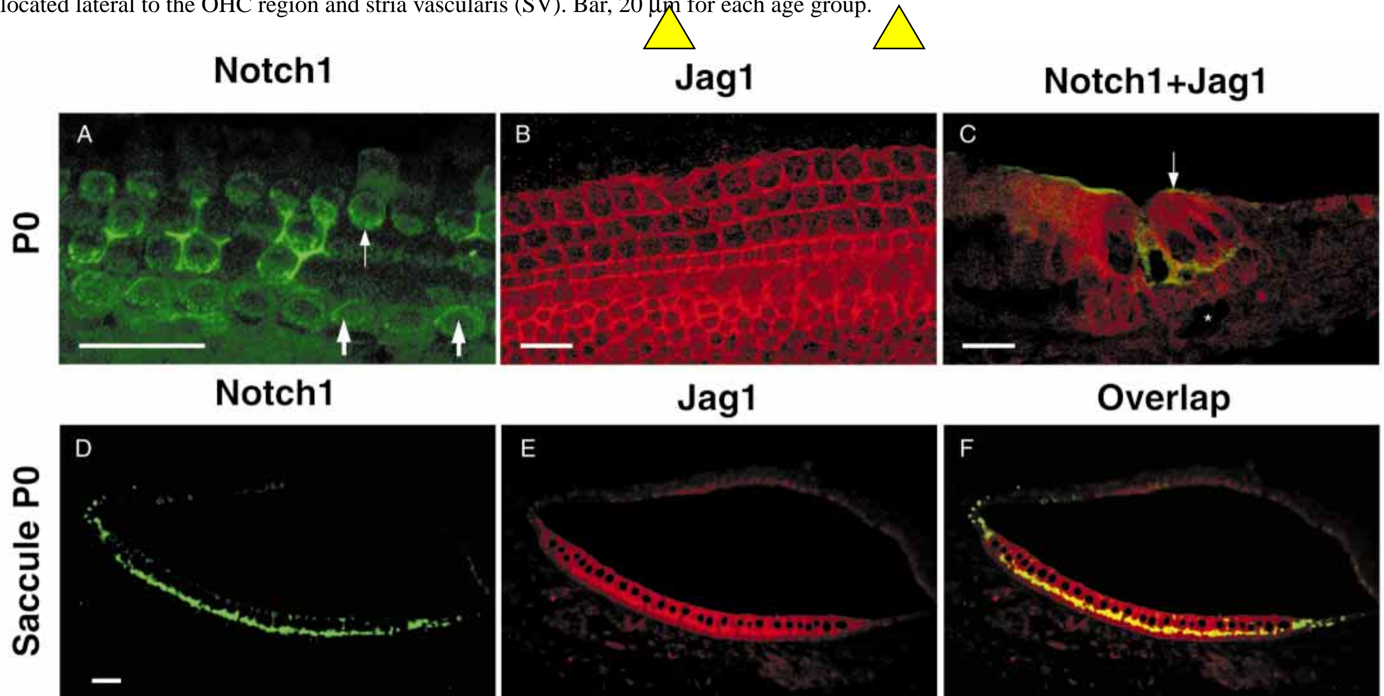

Fig. 3. Confocal images of expression patterns of Notch1 and Jag1 in the auditory (A-C) and vestibular sensory (D-F) epithelium in newborn (P0) inner ears. (A) Surface preparation showing intense Notch1 staining in the apical processes of the supporting cells and a punctuate staining in some IHCs (large arrows) and OHCs (small arrow). (B) Jag1 expression was restricted to supporting cells. (C) Double labeling of a cochlear cross-section, showing Notch1 staining of the supporting cells and the apical region of the hair cells (arrow). (D-F) Saccular epithelium cross-sections with Notch1 labeling (D) restricted mostly to the basal portion of the supporting cells and Jag1 labeling (E) throughout the entire basal to apical extensions of the supporting cells. (F) Overlapping label appears yellow and is restricted to the basal portion of the supporting cell layer and to cells that immediately border the supporting cells. Bar, 20 μm in all panels.

out by the addition of the oligonucleotide-lipofectin complexes containing either antisense, sense or antisense scrambled sequences continuously present from the first until the last day of culture. At the end of the culture period, specimens were labeled as whole-mount preparations with fluorescent-labeled phalloidin to visualize the pattern of hair cell differentiation.

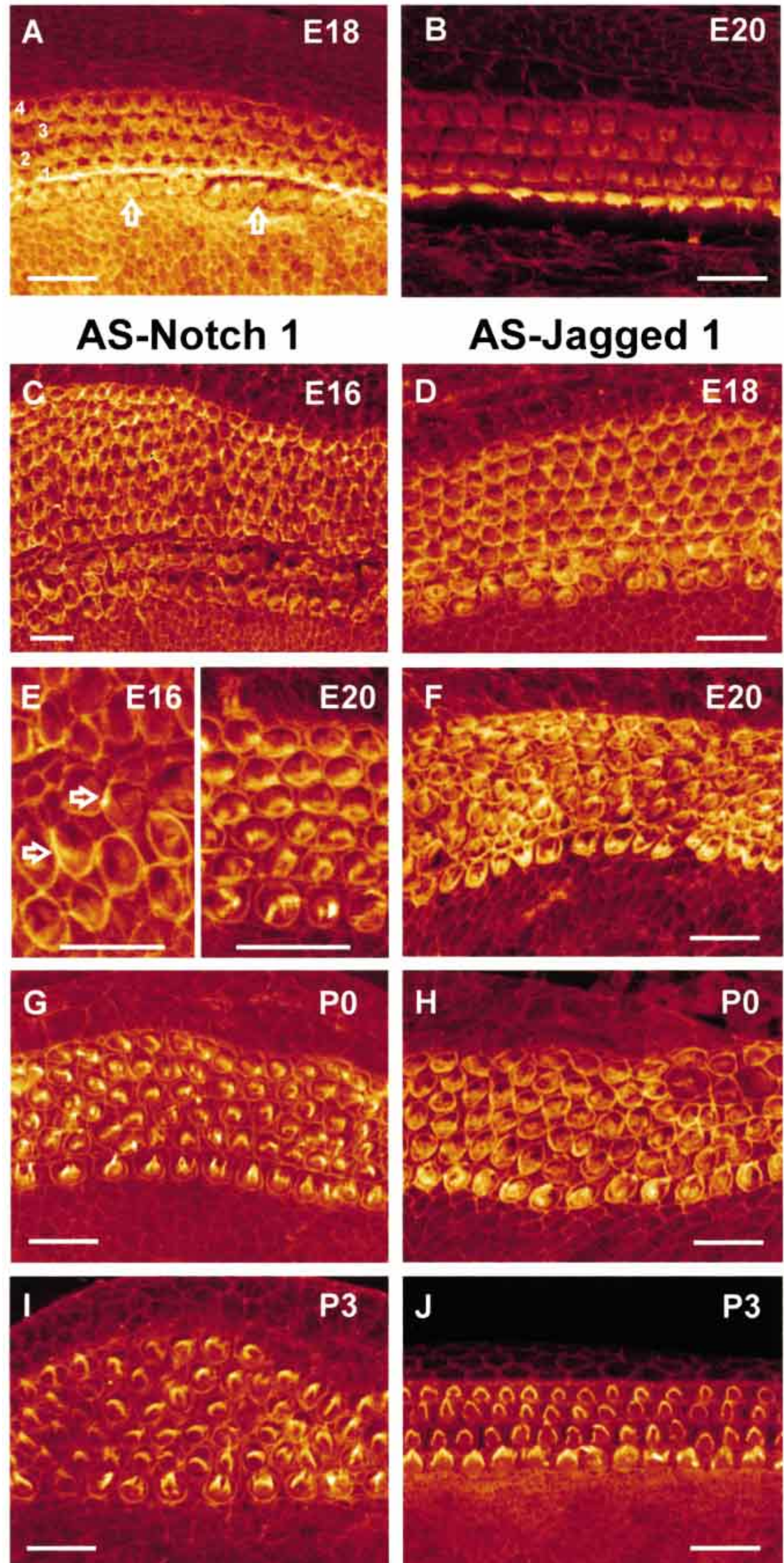

Fig. 4. Confocal images of phalloidin-stained cochlear explants maintained 5 days in vitro in either normal medium (A,B), treated with AS-Notch1 (C,E,G,I) or treated with AS-Jag1 (D,F,H,J). (A) An E18 untreated explant after 5 days in normal medium. There is a characteristic single row of IHCs (arrows) and three (right side) to four rows of OHCs (left side; numbered). (B) An E20 untreated explant after 5 days in normal medium. A single row of IHCs and three rows of OHCs are present. (C) An E16 explant after 5 days in medium containing AS-Notch1. Hair cells are densely packed together and are arranged in two to three rows of IHCs and six to eight rows of OHCs. (D) An E18 explant after 5 days in medium containing AS-Jag1. There are two rows of IHCs and five to six rows of OHCs. (E) Left-side image. A higher magnification of C at the level of the IHC region showing contacts between supernumerary hair cells (arrows), suggesting an absence of supporting cells between these sensory cells. (E) Right-side image. An E20 cochlear explant treated with AS-Notch1. At this stage of maturation, the area of extra hair cells is restricted to the region of the OHCs. (F) E20 explant treated with AS-Jag1. Extra hair cells are observed only in the OHC region of the explant. (G) A newborn (P0) explant treated with AS-Notch1. Four to five rows of OHCs are seen with their stereociliary bundles. Hair cells appear to be less densely packed together than in the AS-treated embryonic cochlear explants. (H) A newborn explant treated with AS-Jag1 showing 4 to 5 rows of OHCs. (I) The apical turn of a P3 explant treated with AS-Notch1. The only cochlear region with supernumerary hair cells was is located in the apical region of the explant as shown here; the rest of the cochlear coil is unaffected by this AS-treatment by this stage of maturation. (J) The basal turn of P3 cochlear explant treated with AS-Jag1. In the basal turn, this treatment does not result in the production of any supernumerary hair cells. The normal configuration of the organ of Corti is preserved with one row of IHCs and three rows of OHCs, and with normal polarity of stereociliary bundles. Explants are from basal (J) middle (A-H) and apical (I) turns. Bars, equal 20 μm.

Effects of Notch1 antisense treatment

Confocal microscopic observations revealed that the addition of AS-Notch1 to the culture medium for a period of 5 days, resulted in regions of the organ of Corti explants with many extra rows of IHCs and OHCs (Fig. 4C,E,G,I). Control explants (i.e. untreated, sense-treated and scrambled antisense-treated) always showed only one row of IHCs and three or rarely four rows of OHCs (Fig. 4A). The supernumerary regions were characterized by five to eight rows of OHCs and two to three rows of IHCs in AS-Notch1-treated organ of Corti cultures explanted during embryonic days E16 and E20 (Fig. 4C,E). The effect of AS-Notch1 treatment on regions with extra rows of hair cells was directly related to the age of the explants (Fig. 5A). There was a progressive decline in the length of the organ of Corti occupied by supernumerary hair cells when the age of the treated explants was varied between E16 and P3. Almost the entire sensory epithelium (i.e. base to apex) was affected by AS-Notch1 when treatment started on E16. AS-Notch1 treatment of E16 explants, resulted in an average, for each cochlea, of a region of approximately 1900 μm in length with extra hair cells (paired untreated control: 220 μm). In embryonic (E16 to E18) AS-Notch1-treated explants, regions with supernumerary hair cells extend to areas in both the basal and apical coils and include extra rows of both IHCs and OHCs. In embryonic untreated control explants, there was variability in the rate of spontaneous occurrence of supernumerary hair cell regions, which often contained four rows of OHCs and the length of these regions was small in comparison to AS-Notch1-treated explants. In contrast, AS-Notch1 treatment of organ of Corti explants established in the early postnatal period (P0 to P3), resulted in regions with supernumerary hair cells restricted to patches (average length: P0, 870 μm; P1, 730 μm) within the region of the mid-apical turn. In this responsive region, only one to two extra rows of OHCs appeared in the AS-Notch1-treated cultures (Fig. 4G) with no extra rows of IHCs. AS-Notch1 treatment of P3 explants resulted in only limited patches with supernumerary OHCs formed (average length: 250 μm) which were restricted to the area of the apical turn (Fig. 4I). The rest of the cochlear coil in these P3 explants was unaffected by treatment with AS-Notch1. No changes were seen in control cultures established between P0 and P3 treated with either sense or scrambled antisense sequences for Notch1 when compared to untreated control cultures. The normal pattern of a single row of IHCs and three rows of OHCs were observed in all of the control cultures. As a result of the supernumerary hair cell regions, there was a significant increase in the spatial packing density of hair cells in AS-Notch1-treated cultures compared to the three types of control explants (i.e. untreated, sense-treated and scrambled antisense-treated). Hair cells were often tightly packed together without any visible evidence of separation by supporting cells (Fig. 4E, open arrows). A comparison of the mean number of hair cells in the supernumerary regions of AS-Notch1-treated embryonic explants showed that the number of IHCs and OHCs were significantly higher (Fig. 5C). For example, there was almost double the number of both IHCs and OHCs in the supernumerary regions of AS-Notch1-treated explants from E16 and E18 cochleae compared to untreated controls. In E20-treated explants, there was only a significant increase in the number of OHCs as compared to untreated controls. For a correct comparison between experimental and control embryonic explants (E16 to E20), hair cell counts were made on age-matched untreated control explants that also presented patches with supernumerary hair cells. The effect of AS-Notch1 treatment on the mean number of supernumerary hair cells was related to the age of the treated explants. There was a progressive decrease of supernumerary hair cells when the beginning of AS-Notch1 treatment varied between E16 and E20 (Fig. 5C). Treatment with AS-Notch1 resulted in average of 150 OHCs and 52 IHCs in 200 μm length of the supernumerary regions of E16 explants (paired untreated controls: 78 OHCs and 24 IHCs), while the same antisense treatment of E18 explants resulted in 140 OHCs and 45 IHCs (paired untreated controls: 82 OHCs and 26 IHCs). Treatment with AS-Notch1 on E20 explants resulted only in the formation of supernumerary OHCs, while the number of IHCs was unaffected. Similar treatment on postnatal explants (i.e. P0 to P3) has an effect that is less robust to the results from the E20 AS-Notch1-treated explants.

Effects of Jag1 antisense treatment

Treatment of organ of Corti explants with AS-Jag1 resulted qualitatively in the same effects as observed after treatment with AS-Notch1. Microscopic observations revealed that the addition of AS-Jag1 (5 μM) to the culture medium for a period of 5 days, resulted in regions of the organ of Corti explants with many extra rows of hair cells (Fig. 4D,F,H). However, the overall length of supernumerary hair cell regions with extra hair cells (Fig. 5B) and the density of hair cells in these regions (Fig. 5D) that resulted from AS-Jag1 treatment was lower when compared to age-matched AS-Notch1 explants (Fig. 5A,C). Addition of AS-Jag1 to E18 cultured cochleae resulted in an average, for each cochlea, of a region approximately 1300 μm in length with extra hair cells (paired untreated control: 200 μm), while treatment with AS-Notch1 resulted in an average of 1900 μm (paired untreated control: 220 μm) of the explant with extra hair cells. There was an average of 126 OHC and 35 IHCs in the supernumerary regions of AS-Jag1 E18 treated explants. This average increased to 140 OHCs and 45 IHCs in the supernumerary regions of AS-Notch1-treated E18 explants.

Scanning electron microscopy analysis

Other specimens (Fig. 6) from the same antisense treatment experiments as those studied by confocal microscopy (Fig. 4), were fixed and analyzed by SEM. After 5 days in culture, untreated E18 control explants developed a normal sensory epithelium with one row of IHCs plus three rows of OHCs (Fig. 6A). Each hair cell was separated from its neighbors by intercalated supporting cells creating a regular pattern (Fig. 6B, arrows). This highly ordered sensory cell pattern was altered in E18 organ of Corti cultures treated with AS-Jag1 for 5 days, due to the production of supernumerary hair cells organized in rows within this sensory receptor epithelium (Fig. 6C, arrowheads). High magnification SEM micrographs revealed that hair cell polarity was not homogenous as indicated by the position of their kinocilia in the regions of Corti's organ containing the supernumerary hair cells (Fig. 6D). Additionally, these regions contain pairs of OHCs that appeared to be in direct contact with one another at their apical surfaces without any interposed supporting cells. In newborn AS-Notch1-treated cultures, the regular configuration of the organ of Corti is less altered than in embryonic explants treated

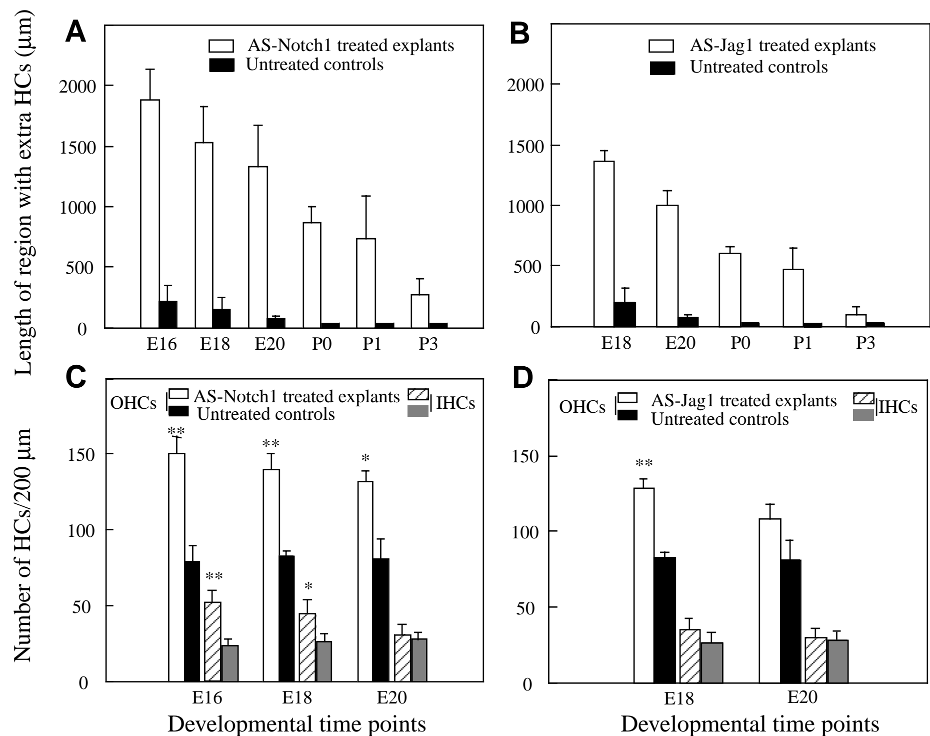

Fig. 5. Comparisons of the length of sensory epithelial regions with supernumerary hair cells and the number of IHCs and OHCs in these regions between cochlear explants established between E16/E18 and postnatal day (P3) and maintained in normal medium (untreated controls) or with AS-Notch1 or AS-Jag1 for 5 days. (A) The average length of the sensory epithelium that was occupied by supernumerary hair cells in cochlear explants established between E16 and P3 and treated with AS-Notch1. (B) The average length of the sensory epithelium with supernumerary hair cells in cochlear explants established between E18 and P3 and treated with AS-Jag1. (C) The average number of IHCs and OHCs in the supernumerary regions from cochlear explants established between E16 and E20 and treated with AS-Notch1. (D) The average number of IHCs and OHCs in the supernumerary regions from cochlear explants established between E18 and E20 and treated with AS-Jag1. Each data point represents the mean number of supernumerary hair cells from a minimum of 5 cochlear explants. Statistical confidence was determined using Student's *t*-test (*, $P<0.05$; **, $P<0.01$). Error bars present s.e.m. There was a significant increase in the size of supernumerary hair cell regions after treatment with either AS-Notch1 or with AS-Jag1 as compared to data obtained from paired untreated control explants. At any given developmental stage, the length of the explants region with supernumerary hair cells was larger with AS-Notch1 treatment than with AS-Jag1 treatment. The significant increase in hair cell number in the supernumerary hair cell regions was mostly due to an increase in OHCs number. In early embryonic explants (i.e. E16 and E18), a significant increase in IHCs number was also observed in AS-Notch1-treated explants. In both the E18 and E20 explants, the overall number of hair cells was higher in AS-Notch1-treated explants compared to AS-Jag1-treated explants.

with antisense Jag1, although a few stereociliary bundles did exhibit aberrant orientations (Fig. 6E). Similar to confocal microscopy observations, only one or two rows of extra OHCs resulted from AS-Notch1 or AS-Jag1 treatments of early postnatal organ of Corti explants. The hair cells in these Notch1 antisense-treated postnatal explants are separated from each other by intercalated supporting cells (Fig. 6F, open arrow).

DISCUSSION

The mammalian organ of Corti has one of the most highly organized patterns of cells of any sensory epithelium, with hair cells and supporting cells arranged in a precise periodic pattern. There is compelling evidence from both mammals and non-mammals that hair cells and supporting cells share a common progenitor (Kelley et al., 1995; Fekete, 1996). Studies of how their production is controlled during development are crucial towards understanding the causes of deafness or to attempt to induce hair cell regeneration. Recent studies in chick and zebrafish suggest that lateral inhibition mediated by Delta-Notch signaling controls the pattern of hair cells differentiation in the ear (Adam et al., 1998; Haddon et al., 1998). In mammals, evidence of the implication of Notch signaling in hair cell differentiation comes from the analysis of cochleae from Jag2 null mutants (Lanford et al., 1999). Jag2 null mutants develop an excess of IHCs (i.e. two rows instead of one) and, to a lesser extent, some supernumerary OHCs. These investigators reported the expression of the Notch ligand Jag2 in nascent hair cells which is thought to inhibit their immediate neighbors in the developing sensory epithelium, which express the Notch1 receptor, from also differentiating in hair cells. However, a role for the Notch signaling pathway in the differentiation of OHCs within the mammalian cochlea was not convincingly demonstrated, and the cochlea of these Jag2 mutants suggests that other ligands of the Notch1 may also participate in the regulation of auditory hair cell differentiation. The analysis of the Jag2 null mutants did not provide any direct evidence of a role for the Notch1 receptor in patterning of hair cells within the mammalian cochlea.

In the developing rat cochlea, evidence based on phallodin staining and SEM observations of the developmental time course of hair cell differentiation in vivo, suggest that it occurs from E18 to P0 in a base-to-apex gradient (Romand et al., 1993; Zine and Romand, 1996). By E18, shortly after the terminal mitosis of the hair cell progenitors (Ruben, 1967), IHCs differentiate first in the basal turn from the pool of progenitor cells. The first OHCs were observed to differentiate on E20 in the basal turn and on P0 in the apical turn of the cochlea. During these sequential differentiation phases, Notch1, Jag1 and Jag2 show both complementary and overlapping expression patterns at several sites within the developing rat cochlea. Both Notch1 and Jag1 are expressed homogeneously throughout precursor cells of Kölliker's organ

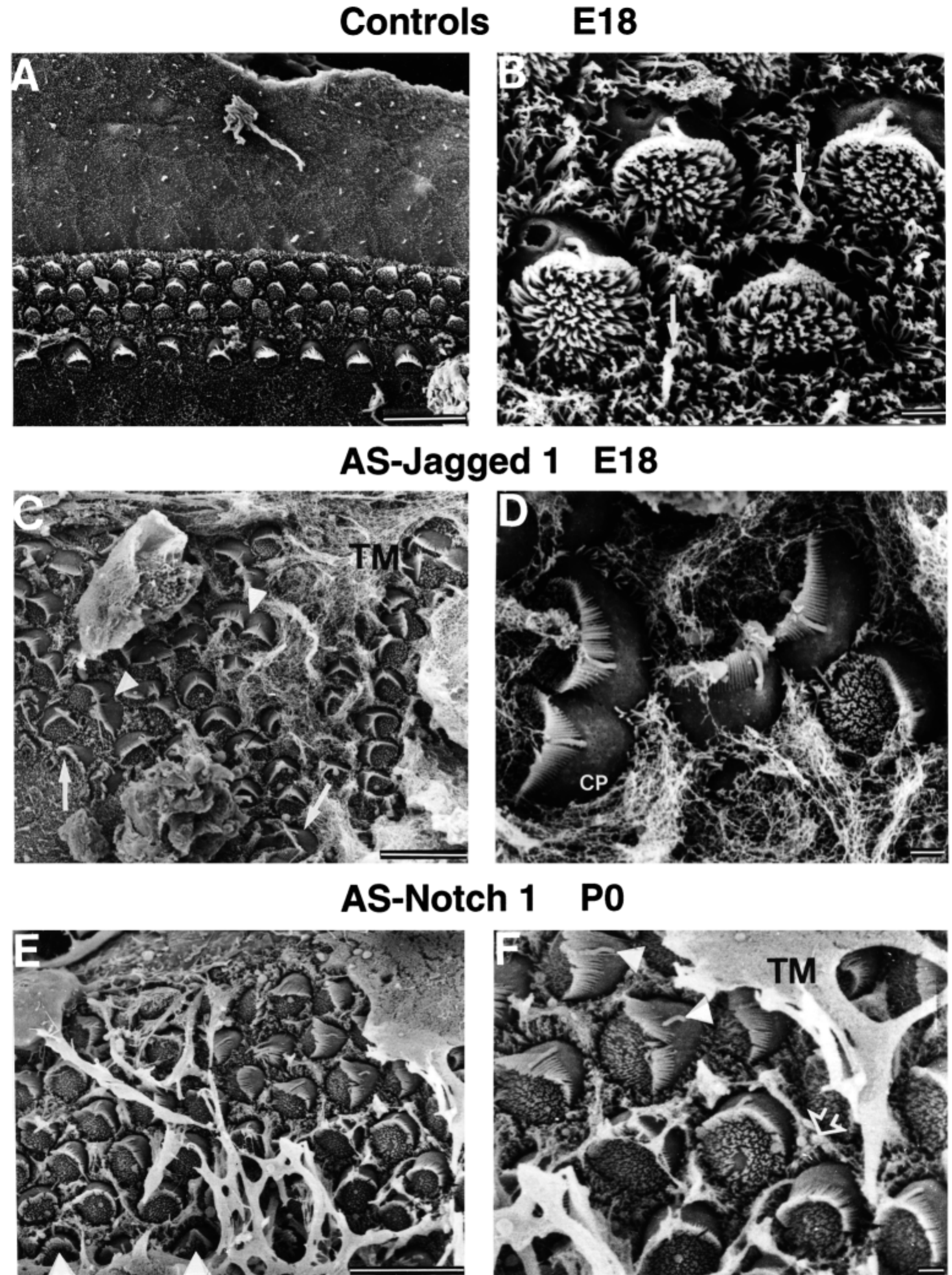

Fig. 6. Scanning electron micrographs of cochlear explants maintained 5 days in vitro in either normal medium (A,B), or treated with AS-Jag1 (C,D), or with AS-Notch1 (E,F). (A) The mid-cochlear turn of an E18 untreated organ of Corti explant. Hair cells are arranged in three orderly rows for OHCs and a single row for IHCs, showing uniform orientation of their stereociliary bundles. (B) Higher magnification at the level of the second and third rows of OHCs from the control explant shown in A. Each OHC is separated from its neighbor by the apical processes of supporting cells, with each of these cells topped by numerous microvilli and a single kinocilium (arrows). (C) The mid-cochlear turn region from an E18 explant treated with AS-Jag1. Its regular organization has been lost due to the addition of many extra OHCs. The IHCs are barely visible (arrows) due to regrowth of a tectorial membrane (TM). The regular polarity of hair cell stereociliary bundles one to another has been disrupted (arrowheads). (D) Higher magnification of OHC region from C has been rotated by 90° to show two pairs of OHCs that appear to be in direct contact at the level of their cuticular plates (CP), without evidence of any intervening supporting cell processes. (E) The mid-cochlear turn region of a newborn (P0) AS-Notch1-treated explant. At this stage of explantation, only 1 to 2 additional rows of OHCs develop on the lateral edge of the organ of Corti, with a normal spatial packing density. The IHC row (arrowheads) shows no sign of extra hair cells. The surface of the organ of Corti is partly covered by fragments of the regrown tectorial membrane. (F) Higher magnification of the OHC region depicted in E shows that supernumerary OHCs are separated by intervening supporting cell processes (open arrow), in contrast to the embryonic organ of Corti cultures treated with AS-Notch1. The polarity of hair cells as indicated by the positioning of their kinocilia (arrowheads), showed only slight variations in sensory hair cell polarity. Bar, 10 μm (A,C,E), 1 μm (B,D,F).

at E16 (period just before hair cell differentiation and after terminal mitoses) with the exception of the region of Kölliker's organ that correspond to the future organ of Corti where Notch1 and Jag1 expression was either very weak or absent. Between E18 and E20, a developmental period that corresponds respectively to IHCs and OHCs differentiation, expression of both Notch1 and Jag1 was upregulated within the developing organ of Corti. Their expression now demarcates the developing organ of Corti showing both an overlap and a distinct pattern of distribution associated with the differentiating supporting cells. However, Jag2 expression was restricted to the nascent hair cells. This expression pattern was in accordance with a study (Lanford et al., 1999) that reported an upregulation of Jag2 gene expression in the nascent hair cells in the cochlea of embryonic mice. Jag2 expression continues in hair cells, and Notch1 and Jag1 expression in supporting cells of the developing organ of Corti through P3, although early in postnatal development, Notch1 also begin to be detectable in the hair cells. This unconventional expression of Notch1 in terminally differentiated hair cells had also been

reported in a recent study (Lewis et al., 1998). However, we did not observe any detectable expression of Notch1 in the hair cells during embryonic development from E18 to E20. This divergence could be related to either the use of different methods of detection and/or to a low expression of Notch1 in differentiating hair cells that was below the limit of sensitivity of our immunocytochemical technique. Together, the results from our expression study show an overlap of the expression of Notch1 and Jag1 in Kölliker's organ at E16, followed by an upregulation of the expression of either Notch1, Jag1 and Jag2 coincident with the initiation of hair cell differentiation. Jag2 was first expressed between E18 and E20 in the developing hair cells while the expression of Notch1 and Jag1 increased within the developing organ of Corti and was associated with the non-sensory supporting cells. In double-labeling experiments, Jag2 expression in the nascent hair cells appeared precisely defined by Notch1 and Jag1 expression domains suggesting that the three genes are functionally coupled in some way.

To assess if Notch1 and Jag1 have a functional role in hair cell development in the mammalian cochlea, as suggested by the analysis of inner ears from Jag2 mutants (Lanford et al., 1999), we used antisense oligonucleotides to decrease their levels of expression in embryonic and neonatal organ of Corti explants. The results from these experiments indicated that perturbation of the Notch1signaling pathway by decreasing either Notch1 or Jag1 expression resulted in an excess of hair cells in the developing organ of Corti. However, the overall density of supernumerary hair cells in addition to the length of the sensory epithelium containing extra hair cells were significantly decreased in AS-Jag1-treated explants as compared to AS-Notch1-treated age-matched explants. In embryonic AS-Notch1-treated explants, the number of hair cells rows was much greater (5 to 8 rows, OHCs; 2 rows, IHCs) than controls (3 to 4 rows, OHCs; 1 row, IHCs). Many regions of these explants contain hair cells that appear to directly contact each other at their apical borders without any separation by supporting cells. This observation suggests that supernumerary hair cells have been produced at the expense of supporting cells (e.g. Deiters' cells), as a result of a tissue-specific decrease of Notch1 activity. In early postnatal explants, although one to two extra rows of OHCs developed in restricted patches after AS-Notch1 treatment, hair cells were observed to be separated by supporting cells. In these explants, supernumerary OHCs may arise through the transformation of nearby differentiating supporting cells (e.g. Hensen's cells), since almost all precursor cells in this period have been committed to a final phenotype. In addition, this type of supporting cell was also observed to express Notch1 and is juxtaposed to OHCs. This observation raises the possibility that, besides its role in the determination of cell fate in embryonic cochlear development, Notch1 may also be involved in the maintenance of the differentiated state and acts to prevent supporting cells from generating new hair cells during postnatal development. The role of Notch in terminally differentiated tissue is not known but it has been speculated that Notch may confer some degree of developmental plasticity (Ahmad et al., 1995). The comparison of the effects of AS-Notch1 and AS-Jag1 on the density of supernumerary hair cells and on the length of the sensory epithelium occupied by these cells supported the presence of other ligands operating within the developing sensory epithelium that may interact with Notch1 to regulate hair cell differentiation. Recent works have revealed the expressions of Jag2 (Lanford et al., 1999) and Delta1 (Morrison et al., 1999) in the developing auditory hair cells are coincidental with their initial phases of determination. However, a functional role for Delta1 in the control of mammalian hair cell differentiation has not been reported. In our culture experiments, we could not eliminate the possibility that a decrease of Jag1 expression may result indirectly from a downregulation of the expression of Notch1, e.g. in feed-back regulation loops within the developing cochlear sensory epithelium. According to this mechanism, the level of Notch activation in a cell would depend on the level of ligands expressed by its neighbors, and vice-versa, this would give rise to feedback loops that would correlate the fate of adjacent cells to control the pattern of differentiation (Heitzler and Simpson, 1991; Wilkinson et al., 1994).

Our results provide an in vitro assay of Notch signaling function during the early stages of mammalian organ of Corti development and fit with the in vivo analysis of the cochleae from embryonic mice homozygous for a null mutation of Jag2 gene (Landford et al., 1999) suggesting a role for lateral inhibition mediated by Notch1-Jag2 signaling in the control of hair cell differentiation. In addition, the uncommon expression pattern of Notch1 in terminally differentiated hair cells, its coexpression with Jag1 in supporting cells, in combination to the presence of Delta1, Jag2 (in hair cells) suggest a complexity of sensory hair cell differentiation events that are a consequence of the heterogeneity in the expression of multiple ligands in the developing cochlea. Moreover, variations in the polarity of the stereociliary bundles between supernumerary hair cells observed by SEM suggest that Notch signaling may also play a role in controlling hair cell polarity. The Wnt signaling pathway has been reported to be required for the developmental control of the correct cell polarity in *Drosophila*, invertebrates and vertebrates (Moon et al., 1997). Moreover, Dishevelled protein, known to be a key downstream component of Wnt signaling has been reported to bind and interact with the intracellular domain of Notch receptor in *Drosophila* (Axelrod et al., 1996; Copper and Bray, 1999). This suggests that there might be some interplay between the mechanisms that control cell fate determination and hair cell stereocilia polarity in the embryonic mammalian cochlea. These hypotheses remain to be tested in future studies.

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REFERENCES

Adam, J., Myat, A., Le Roux, I., Eddison, M., Henrique D., Ish-Horowicz, D. and Lewis, J. (1998). Cell fate choices and the expression of Notch, Delta and Serrate homologues in the chick inner ear: parallels with *Drosophila* sense-organ development. *Development* **125,** 4645-4654.

Ahmad, I., Zagouras, P. and Artavanis-Tsakonas, S. (1995). Involvement of Notch-1 in mammalian retinal neurogenesis: association of Notch-1 activity with both immature and terminally differentiated cells. *Mech. Dev.* **53,** 73-85.

Artavanis-Tsakonas, S., Rand, M. D. and Lake, R. J. (1999). Notch

signaling: Cell fate control and signal integration in development. *Science* **284**, 770-776.

Austin, C. P., Feldman, D. E., Ida, J. A. Jr. and Cepko, C. L. (1995). Vertebrate retinal ganglion cells are selected from competent progenitors by the action of Notch. *Development* **121,** 3637-3650.

Axelrod, J. D., Matsuno, K., Artavanis-Tsakonas, S. and Perrimon, N. (1996). Interaction between Wingless and Notch signaling pathways mediated by Dishevelled. *Science* **271,** 1826-1832.

Bennet, C. F., Chiang, M. Y., Chan, H., Shoemaker, J. E. E. and Mirabelli, C. K. (1992). Cationic lipids enhance cellular uptake and activity of phosphorothioate antisense oligonucleotides. *Mol. Pharmacol.* **41,** 1023-1033.

Bettenhausen, B., Hrabé de Angelis, M., Simon, D., Guenet, J. L. and Gossler., A. (1995). Transient and restricted expression during mouse embryogenesis of Dll1, a murine gene closely related to *D*rosophila Delta. *Development* **121,** 2407-2418.

Capaccioli, S., Di Pasquale, G., Mini, E., Mazzei, T. and Quattrone, A. (1993). Cationic lipids improve antisense oligonucleotide uptake and prevent degradation in cultured cells and in human serum. *Biochem. Biophys. Res. Commun.* **197,** 818-825.

Copper, M. T. and Bray, S. J. (1999). Frizzled regulation of Notch signalling polarizes cell fate in the Drosophila eye. *Nature* **397,** 526-530.

Crowe, R., Henrique, D., Ish-Horowicz, D. and Niswander, L. (1998). A new role for Notch and Delta in cell fate decisions: patterning the feather array. *Development* **125,** 767-775.

de la Poma, J. L., Wakeham, A., Correia, K. M., Samper, E., Brown, S., Aguillera, R. J. Nakano, T., Honjo, T., Mak, T. W., Rossant, J. and Conlon, R. A. (1997). Conservation of the Notch signaling pathway in mammalian neurogenesis. *Development* **124,** 1139-1148.

Dorsky, R. I., Chang, W. S., Rapaport, D. H. and Harris, W. A. (1997). Regulation of neuronal diversity in the Xenopus retina by Delta signaling. *Nature* **385,** 67-70.

Ellissen, L., Bird, J., West, D. C., Soreng., A. L., Reynolds, T. C., Smith, S. D. and Sklar, J. (1991). TAN-1, the human homolog of the *D*rosophila Notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. *Cell* **66,** 649-661.

Fekete, D. M. (1996). Cell fate specification in the inner ear. *Curr. Opin. Neurobiol*. **6,** 533-541.

Fleming, R. J., Purcell, K. and Artavanis-Tsakonas, S. (1997). The NOTCH receptor and its ligands. *Trends Cell. Biol*. **7,** 437-441.

Haddon, C., Jiang, Y. L., Smithers, L. and Lewis, J. (1998). Delta-Notch signalling and the patterning of sensory cell differentiation in the zebrafish ear: evidence from the mind bomb mutant. *Development* **125,** 4637-4644.

Heitzler, P. and Simpson, P. (1991). The choice of cell fate in the epidermis of Drosophila. *Cell* **64,** 1083-1092.

Kelley, M. W., Talreja, D. R. and Corwin, J. T. (1995). Replacement of hair cells after laser microbeam irradiation in cultured organs of Corti from embryonic and neonatal mice. *J. Neurosci.* **15,** 3013-3026.

Kopan, R. and Turner, D. L. (1996). The Notch pathway: democracy and aristocracy in the selection of cell fate. *Curr. Opin. Neurobiol*. **6,** 594-601.

Lanford, P. J., Lan, Y., Jiang, R., Lindsell, C., Weinmaster, G., Gridley, T. and Kelley, M. W. (1999). Notch signaling pathway mediates hair cell development in mammalian cochlea. *Nature Genetics* **21,** 289-292.

Lardelli, M., Dahlstrand, J. and Lendahl, U. (1994). The novel Notch homologue mouse Notch3 lacks specific epidermal growth factor-repeats and is expressed in proliferating neuroepithelium. *Mech. Dev*. **46,** 123-136.

Lewis, A. K., Frantz, G., Carpenter, D., de Sauvage, F. J. and Gao, W. Q. (1998). Distinct expression patterns of Notch family receptors and ligands during development of the mammalian inner ear. *Mech. Dev*. **78**, 159-163.

Lewis, J. (1996). Neurogenic genes and vertebrates neurogenesis. *Curr. Opin. Neurobiol*. **6**, 3-10.

Lim, D. J. and Anniko, M. (1985). Developmental morphology of the mouse inner ear. A scanning electron microscopic observation. *Acta Otolaryngol. Suppl*. **422**, 1-69.

Lindsell, C. E., Shawber, C. J., Boulter, J. and Weinmaster, G. (1995). Jagged: A mammalian ligand that activates Notch1. *Cell* **80,** 909-917.

Moon, R. T., Brown, J. D. and Torres, M. (1997). WNTs modulates cell fates and behavior during vertebrate development. *Trends Genet*. **13,** 157-162.

Morrison, A, Hodgetts, C., Gossler, A., Hrabé de Angelis, M. and Lewis, J. (1999). Expression of Delta1 and Serrate 1(Jagged1) in the mouse inner ear. *Mech. Dev*. **84,** 169-172.

Redmond, L., Oh, S. R., Hicks, C., Weinmaster, G. and Ghosh, A. (2000). Nuclear Notch1 signaling and the regulation of dendritic development. *Nature Neurosci*. **3,** 30-40.

Romand, R., Zine, A. and Hafidi, A. (1993). Ontogenesis of F-actin in hair cells. *Cell Motil. Cytoskeleton*. **25,** 213-222.

Ruben, R. J. (1967). Development of the inner ear of the mouse: a radioautographic study of terminal mitosis. *Acta Otolaryngol. Suppl*. **220,** 1-44.

Sestan, N., Artavanis-Tsakonas, S. and Rakic, P. (1999). Contact-dependant inhibition of cortical neurite growth mediated by Notch signaling. *Science* **286,** 741-746.

Shawber, C., Boulter, J., Lindsell, C. E. and Weinmaster, G. (1996). Jagged2: a serrate-like gene expressed during rat embryogenesis. *Dev. Biol*. **180,** 370-376.

Swiatek., P. J., Lindsell, C. E., Franko del Amo, F., Weinmaster, G. and Gridley, T. (1994). *Notch*1 is essential for postimplantation development in mice. *Genes Dev*. **8,** 707-719.

Uyttendaele, H., Marazzi, G., Wu, G., Yan, Q., Sassoon, D and Kitajewski. J. (1996). Notch-4/int-3, a mammary protooncogene, is an endothelial cell-specific mammalian notch gene. *Development* **122,** 2251-2259.

Wang, S., Sdrulla, A. D., diSibio, G., Bush, G., Nofziger, D., Hicks, C., Weinmaster, G. and Barres, B.A. (1998). Notch receptor activation inhibits oligodendrocyte differentiation. *Neuron* **21,** 63-75.

Weinmaster, G., Roberts, V. J. and Lemke, G. (1992). Notch2: A second mammalian Notch gene. *Development* **116,** 931-941.

Wilkinson, H. A., Fitzgerald, K. and Greenwald, I. (1994). Reciprocal changes in expression of the receptor *lin*-12 and its ligand *lag*-2 prior to commitment in a C. elegans cell fate decision. *Cell* **79,** 1187-1198.

Xue, Y., Gao., X., Lindsell, C. E. Norton, C. R., Chang, B., Hicks, C., Gendron-Maguire, M., Rand, E. B., Weinmaster., G. and Gridely, T. (1999). Embryonic lethality and vascular defects in mice lacking the Notch ligand Jagged1. *Human Mol. Genetics* **8,** 723-730.

Zimrin, A. B., Pepper, M. S., McMahon, G. A., Nguyen, F., Montesano, R. and Maciag, T. (1996). An antisense oligonucleotide to the Notch ligand Jagged enhances fibroblast growth factor-induced angiogenesis *in vitro*. *J. Biol. Chem*. **271,** 32499-32502.

Zine, A. and Romand, R. (1996). Development of the auditory receptors of the rat: a SEM study. *Brain Res*. **721,** 49-58.

Zine, A. and de Ribaupierre, F. (1998). Replacement of mammalian auditory hair cells. *NeuroReport* **9,** 263-268.