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Expression of prestin and Gata-3, -2, -1 mRNA in the rat organ of Corti during the postnatal period and in culture

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ABSTRACT

Based on observations that mutations of GATA-3 are responsible for the HDR-syndrome (hypoparathyroidism, deafness, renal defects) and that GATA-transcription factors have an important role to play in inner ear development, we hypothesized that these transcription factors may be involved in regulatory changes of prestin transcription. To prove this, we examined in parallel the expression of mRNA of prestin and Gata-3, -2 and Gata-1 in the organ of Corti during early postnatal development of rats and in organotypic cultures. Remarkable relations are observed between prestin and Gata-3, -2 expression in organ of Corti preparations *in vivo* and *in vitro*: (i) Gata-3, -2 expression display similar apical–basal gradients as prestin mRNA levels. (ii) The prestin expression increases between postnatal day two and postnatal day eight by a factor of about four in the apical and middle segments and by a factor of two in the basal part. Highly significant Pearson correlation coefficients were observed between Gata-3, -2 mRNA and prestin levels when the data were evaluated by regression analyses. (iii) Parallel changes of prestin mRNA and Gata-3, -2 mRNA levels were observed in response to thyroid hormone and to gemfibrozil application. These observations suggest a regulatory role played by the Gata-3, -2 transcription factors in prestin expression.

1. Introduction

The high sensitivity of the auditory apparatus depends strongly on the functioning of special mechanosensory cells, the outer hair cells (OHCs), which amplify sound-induced vibrations in the organ of Corti. This amplification is mainly the role of prestin, a protein highly expressed in the OHCs but not in the inner hair cells (IHCs; (Zheng et al., 2000). The hearing threshold rises by 40–50 dB, when OHCs are damaged or lost (Ryan and Dallos, 1975). In rats, prestin mRNA expression appears during postnatal days 3–8 along with the development of the hearing function (Belyantseva et al., 2000).

In view of the role prestin has to play in keeping the OHCs functioning it is important to study and understand the factors involved in regulating prestin expression. Because GATA-3 mutations are associated with hearing loss (HDR-syndrome, hypoparathyroidism, deafness, renal defects; Muroya et al., 2001; Ali et al., 2007) and the prestin promoter contains several putative binding sites for GATA-transcription factors (Zheng et al., 2003), we hypothesize that the GATA-transcription factors may be involved in the regulatory changes of prestin transcription. The precise pathogenesis of the developmental anomalies in the inner ear caused by GATA-3 mutations is unknown (Van Esch and Devriendt, 2001). It is assumed that the deafness found in HDR patients is caused by a cochlear disorder reflecting sensorineural and bilateral types (Van der Wees et al., 2004). The hearing loss is more pronounced at the higher frequencies, i.e., at the basal part of the cochlea.

Six vertebrate GATA proteins are known (GATA-1–6). The GATA-1–3 genes are predominantly expressed in hematopoietic cell lines, whereas the GATA-4–6 genes are predominantly expressed in the heart, gut and adrenals. GATA proteins belong to the zinc finger transcription factors and are involved in numerous processes like development of heart, eye, vascular, and hematopoietic systems (Crawford et al., 2002), transcriptional feedback network of sympathoadrenal differentiation (Moriguchi et al.,

Abbreviations: CP, crossing point; DID, differential integrated density; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; HDR, hypoparathyroidism deafness and renal defect syndrome; IHC, inner hair cell; NIHL, noise-induced hearing loss; OC, organ of Corti; OHCs, outer hair cells; PD, postnatal day; PS, promoter set; PPAR, peroxisome proliferator-activated receptor; Tbp, TATA-box binding protein

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2006), pathway selection of olivocochlear neurons (Karis et al., 2001), adipocyte differentiation (Tong et al., 2005), regulation of allergic or inflammatory reactions (Woerly et al., 2003), generation of cytokine environment (Yamane et al., 2005), transcriptional activity of tyrosine hydroxylase (Hong et al., 2006), and inner ear development (Rivolta and Holley, 1998; Lillevali et al., 2007).

Available data as to the localization of Gata-3 and its changes during the postnatal development are subject to controversy. Beyond embryonic days 14–16 immunostained GATA-3 decreases selectively in the hair cells as the latter differentiate progressively from the base to the apex of the developing organ of Corti. In newborn mice, the cochlea remains intensely labeled for GATA-3, but this labeling is restricted to supporting cells such as the inner pillar cells and Deiters cells (Karis et al., 2001). Van der Wees et al. (2004) analyzed GATA-3 expression in the cochlea of newborn mice using Gata-3-driven beta-galactosidase activity (GATA-3-LacZ) and in situ hybridization. GATA-3-LacZ was found in the outer and inner hair cells, neurons of the spiral ganglion, and various supporting cells including Claudius' cells, pillar cells, inner and outer sulcus cells, interdental cells, and cells in the spiral prominence and ligament. These expression profiles in the cochlea remained, throughout adulthood, without any sign of reduced intensity at older ages.

To examine whether GATA-transcription factors are involved in regulation of prestin expression, we compared the expression pattern of mRNA of Gata-3, -2, -1 and prestin in the cochlea during the early postnatal period and in culture. In a previous study, we had shown that two days of culturing resulted in a clear increase of prestin mRNA levels and in the formation of an apical–basal gradient (Gross et al., 2005). These changes are similar to those which were observed *in vivo*. Therefore, in the present study we used the organotypic culture of the organ of Corti to analyze the effects of substances known for their effect on either prestin or Gata-3 expression. One of the best examples for a substance affecting postnatal prestin expression is thyroid hormone (TH; Weber et al., 2002). A substance which affects the Gata-3 expression is gemfibrozil, an agonist of the peroxisome proliferator-activated receptor alpha (PPAR-alpha; Gocke et al., 2009). THs, triiodothyronine (T3) and thyroxine (T4), modulate gene expression by interacting with TH receptors which are members of the nuclear receptor superfamily (Forrest et al., 1996). PPAR-alpha is also a member of the nuclear receptor family of ligand-activated transcription factors. PPARs are involved in regulation of lipid metabolism and transport (Chen et al., 2008). The lipid metabolism of the organ of Corti is important for the prestin function because the fluidity of the OHC lateral plasma membrane is regulated by cholesterol levels (Organ and Raphael, 2009).

2. Materials and methods

2.1. Explant cultures

Apical, middle and basal segments were prepared from the cochlea of 3- to 5-day-old rats on the basis of the work of Sobkowicz et al. (1993) with some modifications (Mazurek et al., 2003). Dissection of the cochlea was performed in buffered saline glucose solution (BSG; 116 mM NaCl, 27.2 mM Na_2HPO_4, 6.1 mM KH_2PO_4, glucose 11.4 mM) at 4 °C under a laminar flow hood using a dissecting microscope (Stemi, SV6, Zeiss, Germany). The fragments were incubated in four-well tissue culture dishes (1.9 cm^2 culture surface per well, Nunc, Wiesbaden, Germany) in 500 µl Dulbecco's Modified Eagle Medium/F12 Nutrient (1:1) Mixtures (DMEM/F12, Gibco, Karlsruhe, Germany) supplemented with 10% fetal bovine serum (FBS, Biochrom AG, Berlin, Germany), 33 mM glucose, insulin-transferrin-Na-selenit-Mix 2 µl/ml (Roche Diagnostics GmbH, Mannheim, Germany), Penicillin 100 U/ml (Biochrom AG, Berlin, Germany) at 37 °C, 24 ng/ml IGF-1 (R&D systems, Minneapolis, USA) and 5% CO_2 in a humidified tissue culture incubator (Lowenheim et al., 1999). L-Thyroxine sodium salt pentahydrate (T4; Sigma) was added at a concentration of 0.5 µM in selected experiments. Gemfibrozil ([5-(2,5-dimethylphenoxy)-2,2-dimethyl-pentanoic acid, IUPAC]; Sigma–Aldrich Chemie, Steinheim, Germany) was added in different concentrations. It is important to place and keep the fragments unfolded on the bottom of the well. Newborn animals (Wistar Han rats) were received from Charles River Laboratories International, Sulzfeld, Germany. All studies were performed in accordance with the German Prevention of Cruelty to Animals Act and permission was obtained from the state Office of Health and Social Affairs Berlin (T0234/00).

2.2. Hair cells

At the end of the culture period (48 or 72 h), the fragments were fixed in 4% paraformaldehyde/0.1 M phosphate buffered saline for 30 min and stained using tetramethyl rhodamine isothiocyanate (TRITC, Sigma, St. Louis, MO, USA) labeled phalloidin. Phalloidin is a specific marker for cellular F-actin and stains stereocilia and the cuticular plate. The Leica DMIL microscope was used to evaluate and count the hair cells (magnification 400×). Each labeled hair cell was counted in the three OHC rows and in one IHC row. Cells were considered missing when there was a gap in the normal geometric array and no stereocilia or cuticular plates were observed. The number of hair cells or gaps was counted per 100 µm OC length over a distance of 500 µm.

2.3. Immunohistochemical studies

Samples were fixed with 4% paraformaldehyde (30 min, RT), permeabilized in 0.5% Triton X-100/PBS (10 min; Fluka Chemie AG, Buchs, CH) and then washed in PBS. Nonspecific binding sites were blocked using 4% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) in PBS for 1 h. For prestin staining, samples were incubated (overnight, 4 °C) in the primary antibodies (1:50 dilution of affinity purified polyclonal prestin antibody N-20; Santa Cruz Biotechnology, 200 µg/ml) in a solution containing PBS, 2% NaCl, 1% Normal Donkey Serum, 0.1% Triton X-100 (Weber et al., 2002). After several rinses in PBS, samples were incubated in the fluorescein-(FITC) conjugated AffiniPure Donkey AntiGoat IgG secondary antibody (1:200 dilution, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 60 min (Adler et al., 2003). Samples were mounted using ProLong® Gold antifade reagent (Invitrogen/Molecular Probes, Eugene, OR, USA) and examined with an Axio microscope, Imager A1 (Carl Zeiss AG, Göttingen, Germany). In order to quantify prestin protein expression in the organotypic cultures we used an approach based on the immunocytochemical identification of prestin in the membrane, the visualization of prestin with confocal microscopy and scanning the OHC membranes using the Gelscan software package. Fixed fragments selected for quantitative evaluation were mounted on a glass slide coated with cell and tissue adhesive Cell-Tak (B&D, Bedford, MA) on a small area (about 0.8 cm^2) and circumscribed by an ImmEdge Pen (Vector Laboratories, Burlingame, CA, USA). Thus, all fragments were stained under identical conditions. Confocal optical slices were taken from the middle cell region between the cuticular plate and the nucleus.

Gata-3 staining was performed according to Steenbergen et al. (2002) using GATA-3 (HG3–31) monoclonal mouse anti-GATA-3 #sc-268, Santa Cruz (200 µg/ml, 1:100) as primary antibody. Immunodetection was performed with the respective secondary FITC labeled goat-anti-mouse antibody #115–095-003 (Jackson Dianova, 1:200).

Confocal images of the OHC membranes were acquired with an upright laser microscope (Leica DM 2500) equipped with a 63×

objective (oil-immersion) using the 488-nm line of an argon-ion laser. The Gelscan 5 Professional software (BioSciTec, Marburg, Germany) was used for evaluating the intensity of prestin expression in OHC membranes. The image of the prestin stained OHC membrane was considered to be a lane consisting of a peak (membrane), a valley (cytosol, background) and a peak (opposite site of the membrane). This histogram was used to determine the differential integrated density (DID) of the bands expressed in pixel values (grayscale). The particular advantage of the approach is that it allows the prestin protein expression to be determined in each single segment of the OC. Thirty OHCs per segment were scanned.

2.4. RNA preparation

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The RNA content was determined with the Quant-iT™ RiboGreen RNA Assay Kit (Invitrogen/Molecular Probes, Göttingen, Germany). For RNA preparation, 2–3 segments were pooled.

2.5. RT-PCR

The mRNA expression was measured with the LightCycler (Roche Diagnostics Mannheim, Germany) using real-time polymerase chain reaction as previously described (Gross et al., 2007). The gene expression level was determined by real-time RT-PCR by means of the comparative crossing point (CP) method for differential gene expression using Tata-box binding protein (Tbp) as the housekeeping gene. Tbp is a transcription factor that binds specifically to a DNA sequence called the TATA box. It was used as house keeping gene in several studies (Roon-Mom et al., 2005; Jung et al., 2007; Nygaard et al., 2007).

Primers used for the RT-PCR are (Acc. No., forward, reverse, product length):

Gapdh, AF 106860, 5′-agg tga ccg cat ctt ctt gt-5′; ctt gac tgt gcc gtt gaa ct-5′ (225),

Gata-1, NM 012764, 5′-gaagcgaatgattgtcagca-3′; 5′-ttcctcgtctgg attccatc-3′ (183),

Gata-2, NM 033442, 5′-ccagcaaatccaagaagagc-3′; 5′-cgttggcgtag gtaggatgt-3′ (170),

Gata-3, NM 133293, 5′-gagagcagggacttcctg tg-3′; 5′-catcatgcacc tttttgcac-3′ (209),

Prestin, NM 030840, 5′-cacagagtccgagctacacagtc-3′; 5′-tcagtgcg ctgctgtacaag-3′ (162),

Tbp, NM 001004198, 5′-acccccttgtatccttcacc-3′; 5′-catgatgactgc agcaaacc-3′ (201).

The comparative CP method for analyzing differential gene expression incorporates the amplification efficiencies (E) of the target (Ta) and the reference gene (Ref.: Pfaffl, 2001; Pfaffl et al., 2002). The target gene expression is normalized by the non-regulated reference gene expression according to the following formula:

$$\text{Ratio} = \frac{(E\text{Ta})^{\Delta\text{CPTa[Cali-Sample]}}}{(E\text{Ref})^{\Delta\text{CPRef[Cali-Sample]}}}$$

Symbols: Ratio – expression of target gene normalized to that of reference gene; ΔCPTa [Cali-Sample] – difference of the CP values (normalized to one ng RNA input) of the target genes, of the calibrator (Cali) and experimental samples (Sample); ΔCPRef [Cali-Sample] – difference of the CP values (normalized to one ng RNA input) of the reference gene Tbp, of the calibrator and experimental samples. The following example serves to illustrate the calculation: Ref Tbp: E = 1.99, CPRef Cali = 22.31, CPRef Sample 24.00, ΔCPRef (Cali-Sample) = −1.69, $E\Delta^{CP}$ = 0.3125; Ta Prestin: E = 1.87, CPTa Cali = 27.22, CPTa Sample 26.28, ΔCPTa = 0.94, $E\Delta^{CP}$ = 1.805; Ratio prestin/Tbp = 5.78. An organ of Corti pool from several newborn rats was used as calibrator.

Mean ± standard errors of the mean (SEM) and the Pearson Product-Moment Correlation Coefficient were calculated from the RT-PCR data. The significance of the differences between the different regions and treatment groups was assessed using analysis of variance (ANOVA). A significant ANOVA result was followed by a post hoc Scheffé's or LSD test (StatSoft, Statistika 6.0, Tulsa, USA).

Quality control was carried out by aliquots from an organ of Corti pool from several newborn rats. The variation coefficient in the series was in the range of 5%, the day to day variation coefficient (VC) over a period of about four months was 22% for Tbp, 38% for prestin, 26% for Gata-3, 48% for Gata-2 and 23% for Gata-1 (each n = 22).

Gene2Promoter (www.genomatix.de) was used to locate the promoters for prestin and Gata-3 which were then analyzed by MatInspector (Cartharius et al., 2005).

3. Results

3.1. Prestin and Gata-3, -2 mRNA levels during postnatal development

To exclude hair cell loss as a cause of prestin changes, we first characterized the experimental model in terms of OHC number and quality. Our data showed that the OHCs were well preserved in freshly prepared samples (indicated as *in vivo* in the text below) and in samples cultured for 2–3 days in culture (indicated as *in vitro*; Fig. 1A–F and Table 1). An important aspect of quantifying gene expression is to choose a suitable reference gene. Our data showed that in the present experimental model, glyceraldehyde-3-phosphate dehydrogenase (Gapdh), a gene frequently used as housekeeping gene, is not suited because it increases in culture (Fig. 1G). Instead of Gapdh we used Tbp as reference gene because analyses had shown that the Tbp mRNA expression did not change significantly in either culture (Fig. 1H) or postnatal development (data not shown).

To compare the developmental changes of prestin and Gata-3, -2, we determined their expression in freshly prepared apical, middle and basal segments of the organ of Corti of 2- (PD2), 5- (PD5) and 8- (PD8) day-old newborn rats (Fig. 2A–C). Prestin mRNA levels increased in all segments from PD2 to PD8 by a factor of 4–5 in the apical and middle segments and by a factor of about two in the basal segments. In contrast, the mean mRNA levels of Gata-3 (Fig. 2B) and Gata-2 (Fig. 2C) did not change statistically during development. To analyze the apical–basal expression gradient, we normalized the mRNA levels to the statistical mean of the apical, middle and basal segments of each age group separately. The apical–basal gradient of the prestin is relatively small on PD2 (Fig. 2D, non-significant) and increases during development (PD5, Fig. 2E; PD8, Fig 2F) by a factor of about two. A stable apical–basal Gata-3, -2 gradient exists on all postnatal days tested (Fig. 2D–F; factor 1.5–2.2).

To further characterize the relations between Gata-3, -2 and prestin expression, we correlated the expression levels within each segment group (Fig. 3A–C) and over all segments (Fig. 3D–F). Even though the means of Gata-3, -2 did not change during the postnatal period, a highly significant correlation was observed to occur between Gata-3, -2 and prestin beyond PD2 using an exponential curve fitting approach.

To compare the correlations between prestin mRNA levels with those of prestin protein we quantified the fluorescence intensity in the OHC membranes stained with FITC labeled antibodies using confocal microscopy and a Gelscan software program (Xia et al., 2008). Confocal images of the whole mount preparations revealed that the three rows of OHCs are clearly labeled, with little or no background staining observed in other regions of the organ of Corti. Prestin was expressed within the lateral plasma membrane of

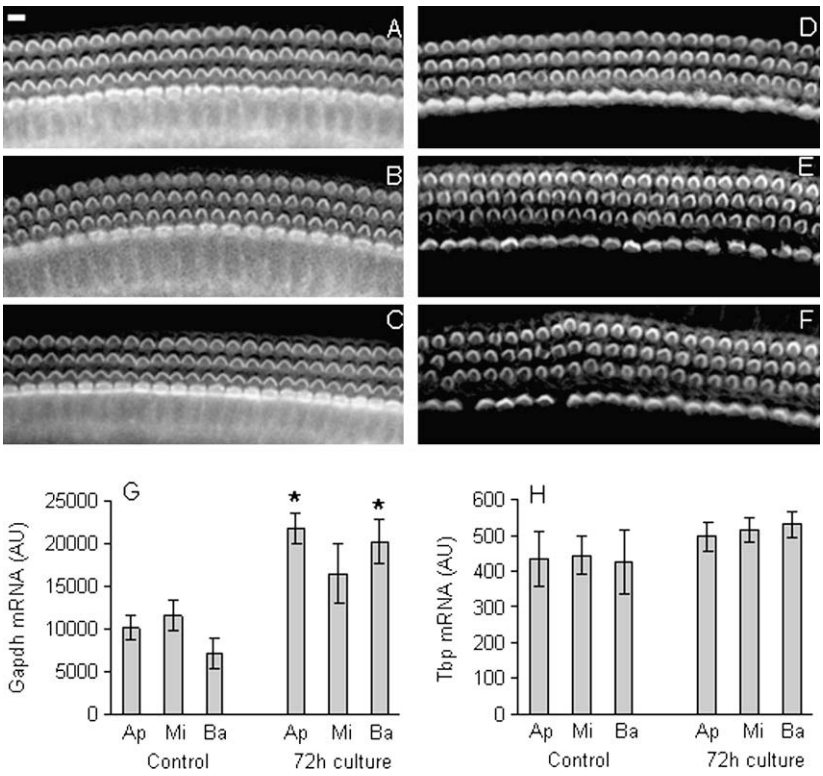

Fig. 1. Images showing the apical, middle and basal parts of rat organ of Corti explants immediately after preparation and after 72 h in culture. (A–C) Freshly prepared tissue; (D–F) tissue after 72 h in culture; (A and D) apical, (B and E) middle and (C and F) basal segments; bar: 10 μm. (G) Expression of Gapdh, (H) expression of Tbp mRNA. The mRNA levels are expressed in arbitrary units per ng total RNA (AU) as described recently (Gross et al., 2007). The data indicate means ± SEM, $n = 6$. Gapdh: apical $^*p < 0.06$, basal $^*p < 0.04$ (Scheffe) versus corresponding control.

Table 1
Number of IHCs and OHCs in freshly prepared OC and after 72 h in culture.

Condition	Apical	Middle	Basal
Control (n = 16)			
IHC	10.4 (8.8–11.2)	10.4 (8.8–11.2)	9.6 (7.2–10.4)
OHC	13.6 (13.1–14.7)	12.9 (12.3–13.6)	12.5 (12.0–13.3)
72-h Culture (n = 9)			
IHC	8.8 (7.2–10.4)	8.8 (7.2–9.6)	8.0 (6.4–9.6)
OHC	12.1 (10.4–13.3)	11.7 (10.7–12.8)	11.2 (9.9–12.5)

This table contains the numbers of IHCs and OHCs (per row and 100 μm OC length; median, 10th and 90th percentile) counted in freshly prepared tissue and after 72 h in culture. In culture, hair cell counting results in a small, non-significant decrease of IHCs and OHCs. Parallel counting of the gaps shows that the apparent hair cell loss results from a certain degree of spreading of the organ of Corti in culture (data not shown).

the OHC cells as previously reported (Fig. 4A; Belyantseva et al., 2000). Both the developmental and the regional patterns of prestin protein expression correspond closely to those of the mRNA expression pattern (Fig. 4B and C).

To analyze the presence and possible changes of GATA-3 protein during postnatal development we immunostained GATA-3 in the apical and basal segments of PD2 and PD8 old rat (Fig. 5). In the apical segments, the three OHC rows and the one IHC row can be easily identified using Hoechst staining (Fig. 5A and C). Gata-3 expression (Fig. 5B and D) is visible in hair cells, but also in adjacent cells; the expression is lower in the PD8 segments (Fig. 5D) than in the PD2 (Fig. 5B) segments. In the basal segments, the region of hair cell nuclei could be better localized using phalloidin staining (Fig. 5I and K). Because of the steric structure of the basal segments, Hoechst staining resulted in overlapping of nuclei. At the level of the stereocilia and below (Fig. 5E–H) no Gata-3 staining was observed. In contrast, a strong staining appeared at the level of nuclei on PD2 (Fig. 5I–J). On PD 8, a faint staining in the nuclei of hair cells and a stronger staining in adjacent cells were observed (Fig. 5K–L). No staining of nuclei could be observed in negative controls (Fig. 8N).

3.2. Prestin and Gata-3, -2 mRNA levels in culture

Fig. 6 shows the mRNA levels of prestin, Gata-3 and Gata-2 in freshly prepared OC tissue of 3- to 5-day-old newborn rats (control) and their changes in 48 h and 72 h cultures. In principle, prestin (Fig. 6A), Gata-3 (Fig. 6B) and Gata-2 (Fig. 6C) mRNA levels increase in all segments. The increase in the apical and middle segments is higher than that in the basal segments (mean fold changes in 72 h culture in the apical segments: 1.7–2.8; mean fold changes in the basal segments: 1.3–1.7).

To make the apical–basal gradient comparable to the *in vivo* data, the mRNA levels were separately normalized to the statistical mean of each segment group (Fig. 6D–F). It was found that an apical–basal gradient develops in culture just like it does in *in vivo* development. In the controls (freshly prepared tissue from 3- to 5-day-old rats, Fig. 6D), a small, non-significant difference of prestin mRNA levels was observed between the apical and basal segments. However, a statistically significant gradient occurs for prestin transcripts after 48 h (Fig. 6E) and 72 h (Fig. 6F) in culture (apical/basal ratio 1.7- to 2.9-fold). A stable apical–basal Gata-3, -2 gradient was found to exist on all days tested (Fig. 6D–F; factor 1.5–2.2).

Similar to the *in vivo* data, the relations between Gata-3, -2 and prestin mRNA were correlated within each segment group (Fig. 7A–C) and over all segments (Fig. 7D and E). In both the controls (PD3–5) and in culture, a significant correlation between Gata-3 and prestin mRNA was observed within the segment groups

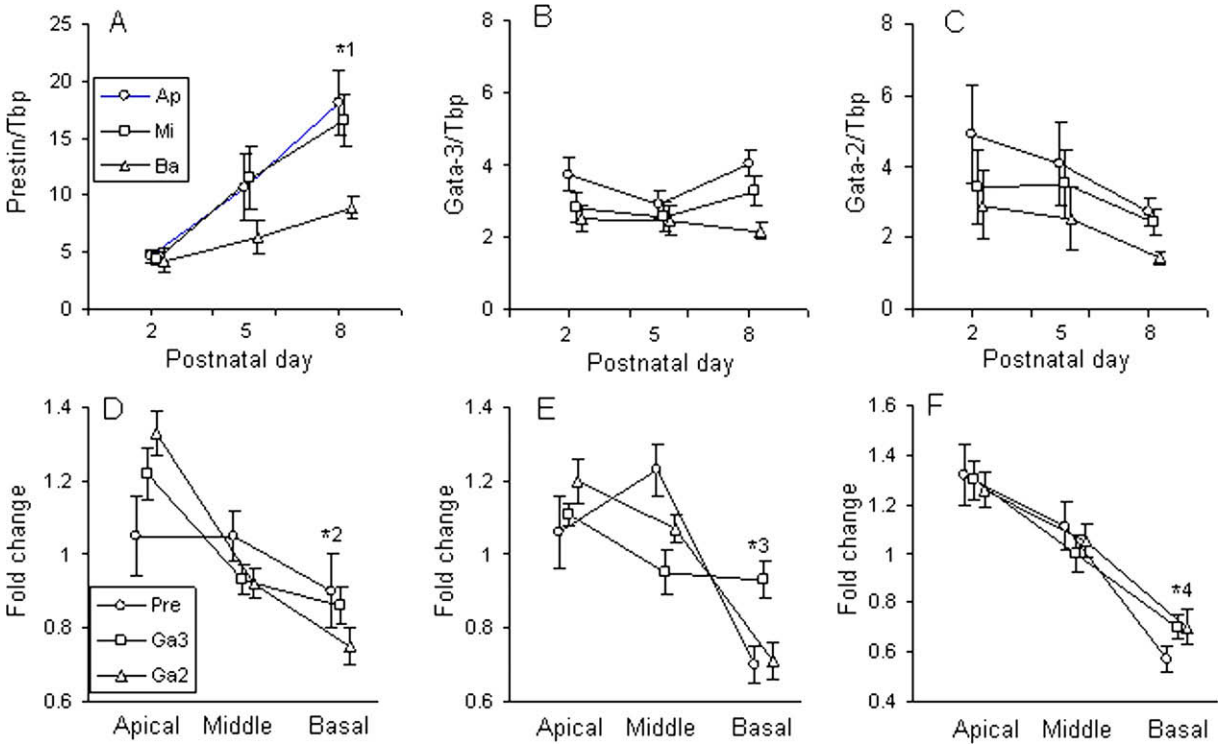

Fig. 2. mRNA levels of prestin and Gata-3, -2 in the apical, middle and basal segments of the organ of Corti during the postnatal development. Data of postnatal development of prestin (A), Gata-3 (B) and Gata-2 (C) are presented as means ± SEM, $n = 6$. Apical–basal gradients of prestin and Gata-3, -2 mRNA levels on PD2 (D), PD5 (E) and PD8 (F) were normalized to the statistical mean of each group. Significances: $^{*1}p < 0.01$ versus PD2; ^{*2}prestin, ns, Gata-3, -2 $p < 0.02$ versus apical; ^{*3}prestin $p < 0.02$ versus middle, Gata-3, -2 $p < 0.02$ versus apical; ^{*4}prestin, Gata-3, -2 $p < 0.02$ versus apical (Scheffe).

and over all segments. Correlations between Gata-2 and prestin mRNA levels reach statistical significance in culture only (data not shown).

To compare the GATA-3 protein expression with that of *in vivo* samples, we immunostained GATA-3 in the apical and basal segments of PD5 rats and in samples after two days in culture

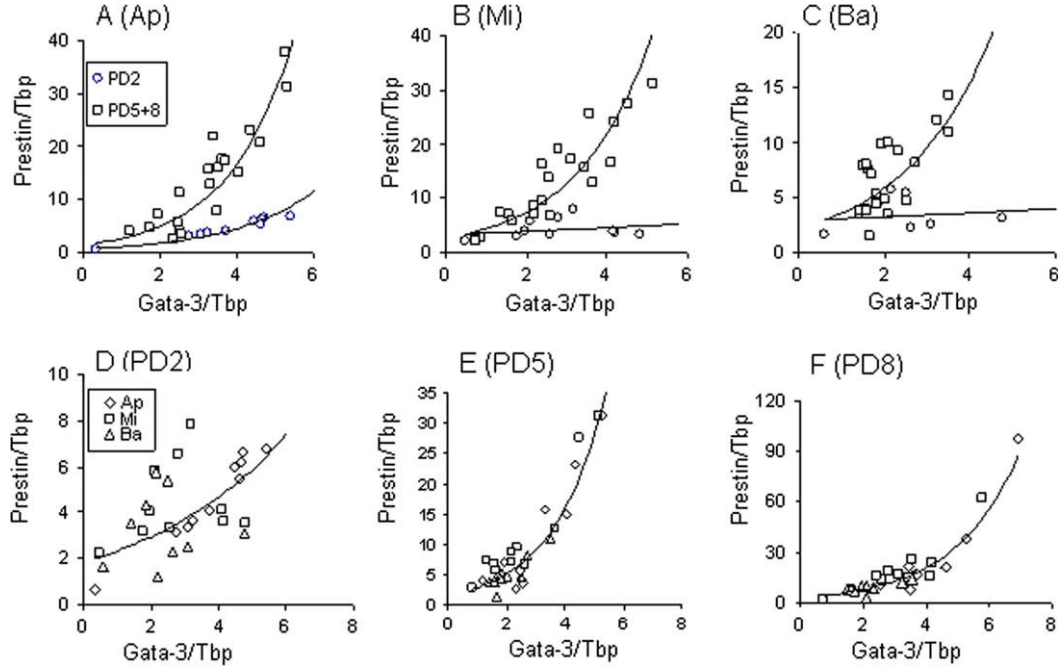

Fig. 3. Pearson correlation analysis of RT-PCR data of Gata-3 and prestin within each segment group (A–C) and over all segments (D–F) during postnatal period. (A) apical, (B) middle, (C) basal segments; (D) PD2, (E) PD5 and (F) PD8. The following exponential equations fitted best to the experimental data. (A–C) PD2: Ap, $y = 0.69e^{0.49x}$ ($p < 0.01$); Mi, $y = 3.30e^{0.08x}$ (ns); Ba, $y = 2.90e^{0.05x}$ (ns); PD5 and PD8 data were combined because no significant differences were observed; Ap, $y = 1.51e^{0.60x}$ ($p < 0.01$); Mi, $y = 2.53e^{0.54x}$ ($p < 0.01$); Ba, $y = 2.20e^{0.49x}$ ($p < 0.01$). (D–F) ($n = 28$–30), PD2, $y = 1.86e^{0.23x}$ ($p < 0.01$), PD5, $y = 1.73e^{0.55x}$ ($p < 0.01$), PD8, $y = 2.94e^{0.49x}$ ($p < 0.01$).

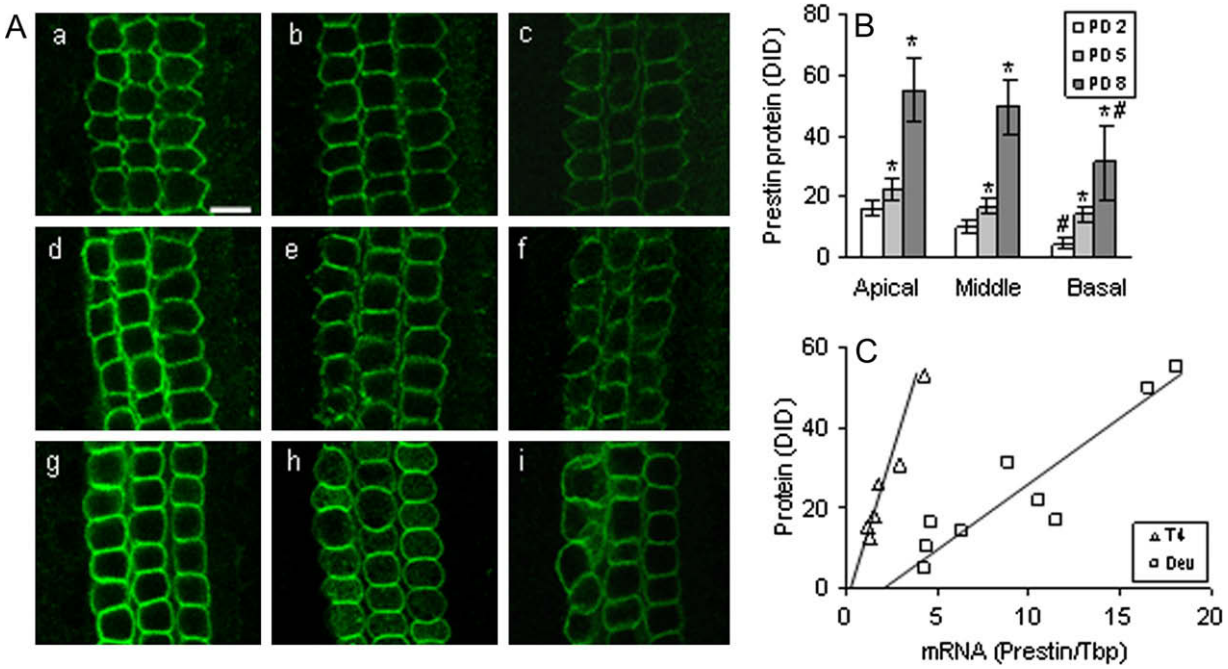

Fig. 4. Postnatal changes of prestin expression of the apical, middle and basal turns of the organ of Corti. (A) prestin immunoreactivity in the OHCs; (a, d and g) apical; (b, e and h) middle; (c, f and i) basal segments of PD2 (a–c), PD5 (d–f) and PD8 (g–i). Scale bars: 10 μm. (B) prestin protein levels expressed as differential integrated density (DID) in the organ of Corti outer hair cells of PD2, PD5 and PD8 old rats. $^{*}p < 0.02$ versus PD2; $^{\#}p < 0.05$ versus apical segment of the corresponding postnatal day (Scheffe). (C) Correlation between mRNA and protein levels of prestin. mRNA levels are expressed as prestin/Tbp ratio, protein levels are expressed as DID. Square – data from Fig. 2 (Dev – development, means from the apical, middle and basal segments); regression follows the equation $y = 3.1x - 4.9$; $r = 0.92$, $p < 0.01$, $n = 9$; triangles – data from Fig. 6 (controls and T4 treated samples, means from the apical, middle and basal segments); regression follows the equation $y = 12.2x - 0.9$, $r = 0.98$, $p < 0.01$, $n = 6$.

(Fig. 8). In the apical segments, an intensive staining of GATA-3 was observed in hair cells, but also in adjacent cells in controls as well as after two days in culture (Fig. 8A and B). In culture, the fluorescence intensity appears even more strongly compared to the controls (Fig. 8C and D). In the basal segments, no GATA-staining was observed at the level of stereocilia and below (Fig. 8E and F). In contrast, a strong staining appeared at the level of nuclei after two days in culture (Fig. 8K and L).

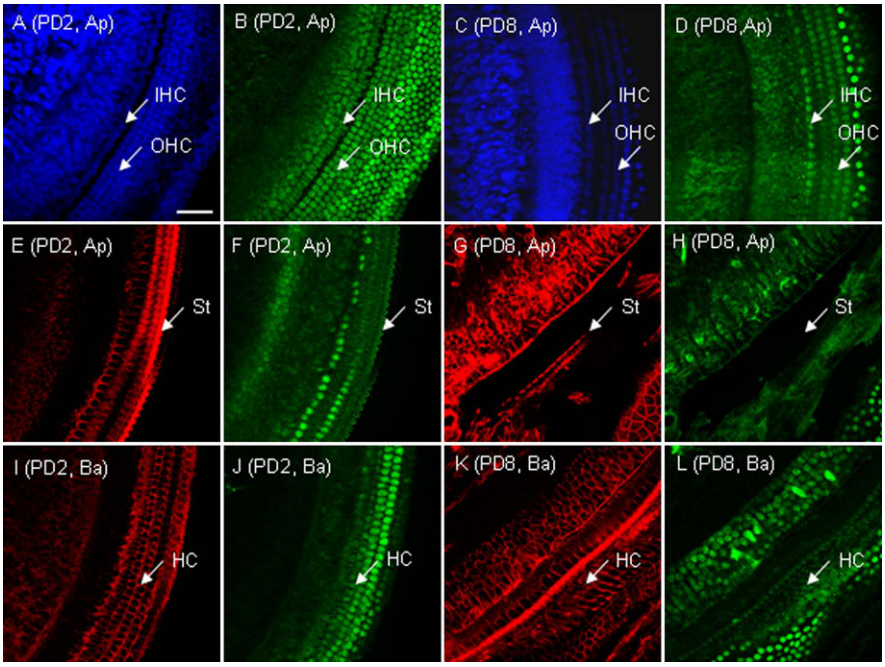

Fig. 5. GATA-3 immunoreactivity of cell nuclei in the apical and basal segments of the organ of Corti of two and eight day old rats. (A) Hoechst stained hair cell nuclei of the apical segment of PD2 old rats; (B) Gata-3 staining of the same section; (C) Hoechst stained hair cell nuclei of the apical segment of PD8 old rats; (D) Gata-3 staining of the same section; (E) PD2, phalloidin stained basal segment of the organ of Corti focused on the stereocilia level; (F) Gata-3 staining of the same section; (G) PD8, phalloidin stained basal segment of the organ of Corti focused on the stereocilia level; (H) Gata-3 staining of the same section; (I) PD2, phalloidin stained basal segment of the organ of Corti focused on the hair cell nuclei; (J) Gata-3 staining of the same section; (K) PD8, phalloidin stained basal segment of the organ of Corti focused on the hair cell nuclei; (L) Gata-3 staining of the same section; Scale bars: 10 μm.

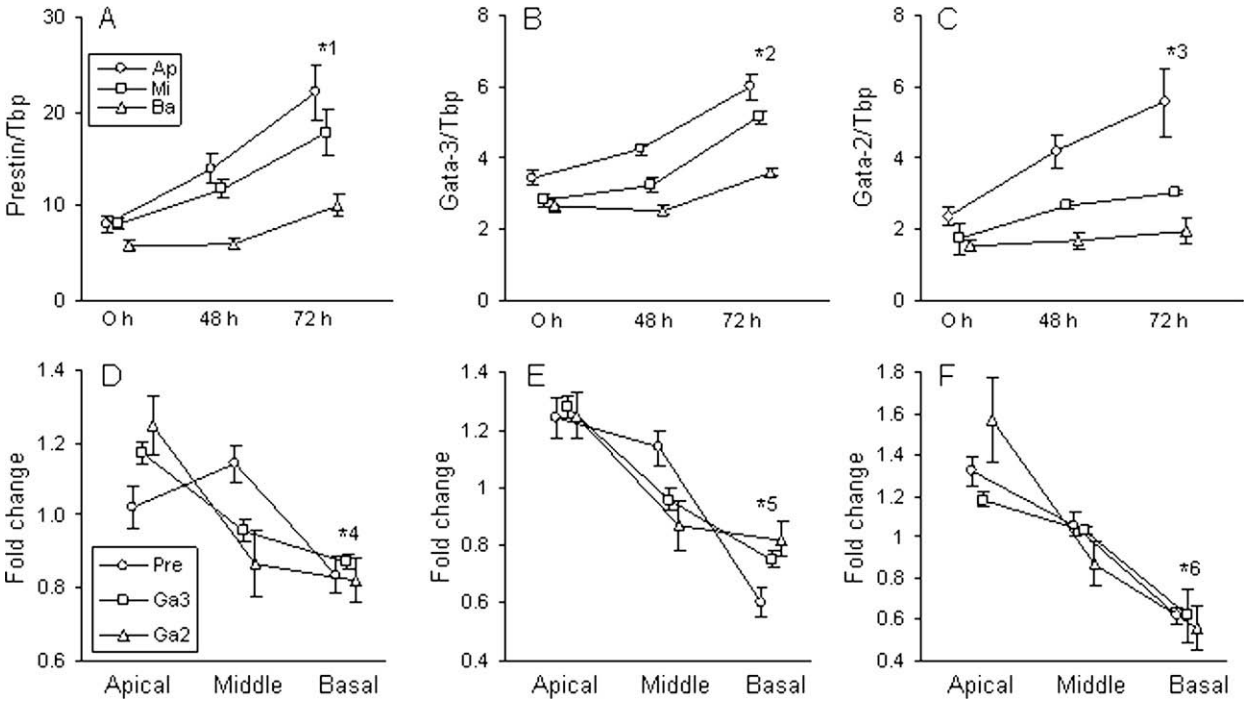

Fig. 6. Changes of mRNA levels of prestin and Gata-3, -2 in the apical, middle and basal segments of the organ of Corti in culture. (A) Prestin, (B) Gata-3, (C) Gata-2. (D–F) The apical–basal gradients of prestin and Gata-3, -2 mRNA levels on PD3–5 (D, freshly prepared tissue as control), after 48 h (E) and 72 h (F) in culture. Data are presented as means ± SEM, $n = 6$. [*1]prestin $p < 0.001$ versus 0 h; [*2,3]Gata-3,-2 $p < 0.02$ versus 0 h; [*4]prestin ns, Gata-3,-2 $p < 0.02$ versus apical (Scheffe). [*5,6]$p < 0.02$ versus apical for prestin and Gata-3,-2.

3.3. Thyroid hormone induces prestin and Gata-3 expression in parallel

T4 was previously found to be an important enhancer of prestin expression *in vivo* during the early postnatal period (Weber et al., 2002). To analyze whether T4 affects the expression of Gata-transcription factors in culture, the co-expression pattern of prestin and Gata-3, -2 was analyzed (Fig. 9). In 48-h-old cultures, applying T4 induced, the increase of prestin and Gata-3 mRNA levels in the

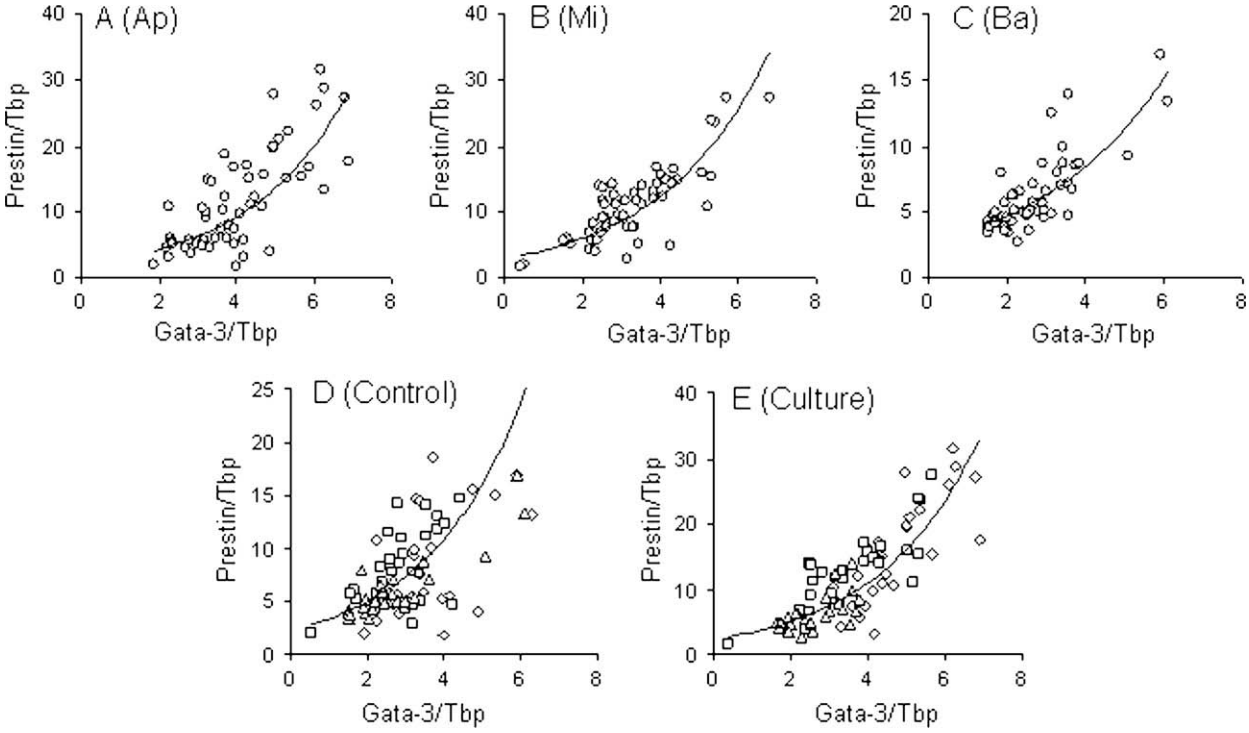

Fig. 7. Pearson correlation analysis of RT-PCR data of Gata-3 and prestin within each segment group (A–C) and over all segments (D and E) of freshly prepared tissue and cultures. (A) apical, (B) middle, (C) basal segments; (D) freshly prepared tissue, (E) cultures (rhombus – apical, square – middle, triangle – basal). The following exponential equations fitted best to the experimental data: Ap, $y = 2.67e^{0.27x}$; Mi, $y = 3.03e^{0.32x}$; Ba, $y = 2.62e^{0.28x}$ ($p < 0.01$ each). (D) Control, (E) OC after two days in culture. The following equations fitted best to the experimental data: control, $y = 2.94e^{0.27x}$ ($p < 0.01$), culture, $y = 2.41e^{0.38x}$ ($p < 0.01$).

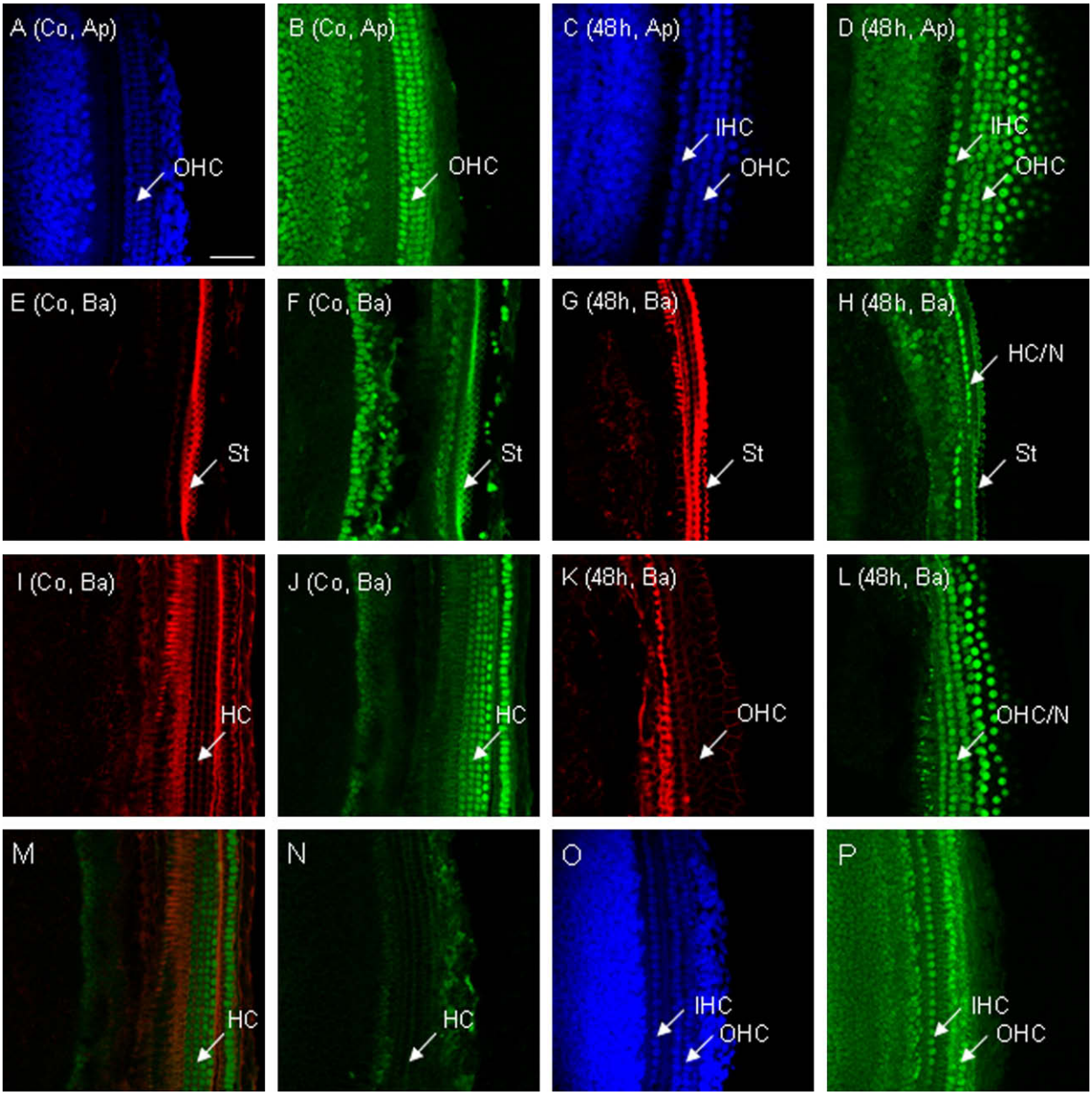

Fig. 8. GATA-3 immunoreactivity of cell nuclei in the apical and basal segments of freshly prepared organ of Corti from 3- to 5-day-old newborn rats and of samples cultured for 48 h. (A) Hoechst stained hair cell nuclei of the apical segment from freshly prepared tissue; (B) Gata-3 staining of the same section; (C) Hoechst stained hair cell nuclei of the apical segment after 48 culture; (D) Gata-3 staining of the same section. (E) Phalloidin stained basal segment of the freshly prepared organ of Corti on the level of stereocilia; (F) Gata-3 staining of the same section; (G) Phalloidin stained basal segment of the organ of Corti on the level of stereocilia after 48 h culture; (H) Gata-3 staining of the same section; (I) Phalloidin stained basal segment of freshly prepared organ of Corti on the level of hair cell nuclei; (J) Gata-3 staining of the same section; (K) Phalloidin stained basal segment of the organ of Corti on the level of nuclei after 48 h culture; (L) Gata-3 staining of the same section; (M) Merge of I and J; (N) negative control (staining without the Gata-3 specific antibody); (O) Fig. A focused on the nuclei of the IHCs; (P) Gata-3 staining of the same section. HC/N, nuclei of hair cells. Scale bar 10 μm.

apical and middle segments of the organ of Corti to be nearly two times as high as in the control cultures (Fig. 9A–C). In contrast, the level of Gata-2 did not change significantly. When Gata-3, -2 levels were correlated to the prestin levels, a close correlation was observed for Gata-3 (Fig 9E), and a less strong, but statistically significant one was found for Gata-2 (Fig. 9F).

To analyze whether the T4-induced increase of prestin mRNA is associated with the increase of the protein level, we immuno-labeled prestin protein in the organotypic cultures and quantified the fluorescence intensity of the OHC walls (Fig. 9D). Analyses of the differential integrated density (DID) of membrane staining in the various segments confirmed that the fluorescence intensity significantly increases in the apical and middle segments following T4 application and decreases from the apical to the basal segments. This is in good agreement with the mRNA levels and previous data (Belyantseva et al., 2000). A high correlation coefficient between prestin mRNA and protein levels was calculated (Fig. 4C).

3.4. Gemfibrozil decreases prestin and Gata-3 mRNA expression

Gemfibrozil, an activator of PPAR-alpha and a lipid-lowering drug is known to be a substance that stimulated the expression and DNA-binding activity of GATA-3 (Gocke et al., 2009). For this reason, we hypothesized that gemfibrozil affects the expression of Gata-3 and prestin in our model. As expected, under control conditions the expression level of both increased in all segments

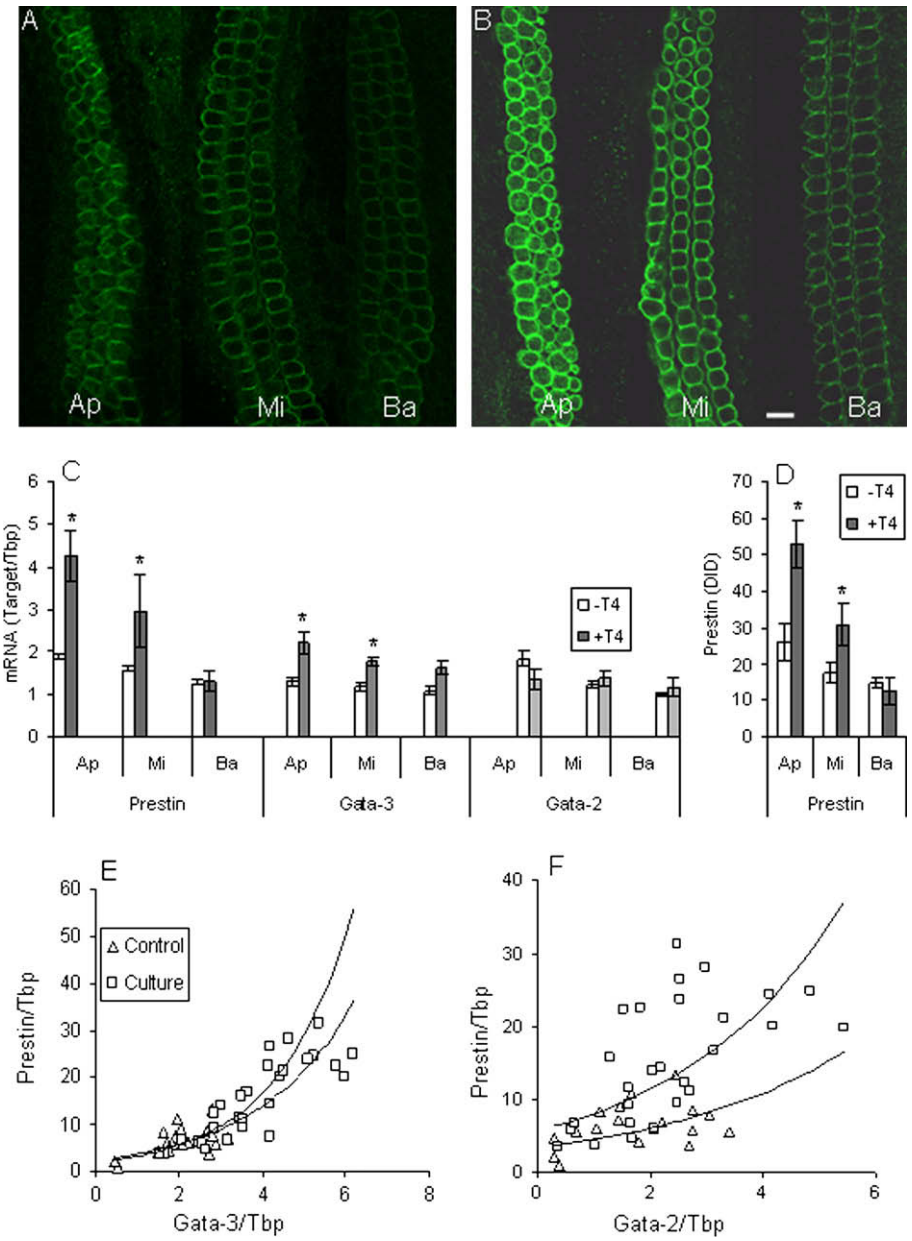

Fig. 9. Effects of the thyroid hormone on the expression of prestin and Gata-3, -2. A and B are confocal images of prestin protein in OHCs of control cultures (A) and cultures grown under the influence of 0.5 μM T4 (B). Scale bars, 10 μm. (C) Data indicate the mRNA levels in controls (−T4) and T4 treated (+T4) segments in 48 h old cultures expressed as fold changes as compared to freshly prepared tissue. The data indicate mean ± SEM, $n = 25$ (controls) and $n = 6$ (experimental). (D) data indicate the prestin protein levels expressed as differential integrated density (DID) of the pixel intensity (relative units). Significances: prestin mRNA, Ap, $p < 0.000$, Mi, $p < 0.03$ (Scheffe); Gata-3 mRNA, Ap, $p < 0.006$ (Scheffe), Mi and Ba $p < 0.008$ (LSD); Prestin protein, Ap and Mi $p > 0.000$ (Scheffe). (E and F) Pearson correlation analysis of RT-PCR data of Gata-3 and Gata-2. Control includes all samples (apical, middle, basal) not treated with T4 and culture includes all samples treated with T4. The following equations fitted best to the experimental data: Gata-3, control, $y = 1.81e^{0.55x}$ ($p < 0.01$); culture $y = 2.35e^{0.44x}$ ($p < 0.01$); Gata-2, control $y = 3.37e^{0.29x}$ (ns); culture $y = 5.74e^{0.34x}$ ($p < 0.01$).

during 48 h culture (Fig. 10). Low gemfibrozil concentrations (50 and 250 μM) had no effect on prestin (Fig. 10A) or Gata-3 (Fig. 10B) expression. However, at one mM gemfibrozil, Gata-3 and prestin expression were strongly inhibited by about 50% (Fig. 10).

3.5. No correlations exists between prestin and Gata-1

Under all conditions tested, the response of Gata-1 is completely different from that of Gata-3 and Gata-2 (Table 2). First, in both *in vivo* and *in vitro*, Gata-1 expression levels are much lower than those of Gata-3, -2, amounting to about 10% of the latter. The level of expression did not change during development *in vivo* and decreased in culture. No changes in the regional expression (apical–basal gradient) were found *in vivo* or *in vitro*.

4. Discussion

The molecular mechanisms that regulate the expression of the prestin gene are poorly understood. Prestin expression and incorporation in the plasma membrane begin from postnatal day zero and increase progressively in a time course coinciding with that of electromotility (Belyantseva et al., 2000). Here, we have shown for the first time that there is a close correlation between the mRNA levels of Gata-3, -2 on one side and prestin on the other side in both *in vivo* and *in vitro*, which points to a role of both two transcription

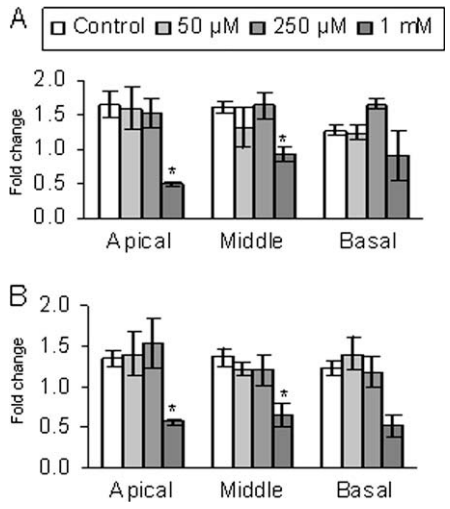

Fig. 10. Effects of gemfibrozil on the expression of prestin (A) and Gata-3 (B) mRNA levels. Data indicate the fold changes in culture compared to freshly prepared tissue. The data indicate means ± SEM, $n = 3–6$. Prestin, apical-$^*p < 0.001$, middle-$p < 0.01$ versus control (LSD). Gata-3, apical-$^*p < 0.001$, middle $^*p < 0.01$ (LSD).

Table 2
Gata-1 expression in the apical, middle and basal segments of the organ of Corti.

Condition	Apical	Middle	Basal
In vivo			
PD2	0.50 ± 0.08 (10)	0.95 ± 0.24 (10)	0.63 ± 0.12 (10)
PD5	0.41 ± 0.06 (10)	0.48 ± 0.13 (10)	0.57 ± 0.19 (10)
PD8	0.40 ± 0.06 (10)	0.63 ± 0.09 (10)	0.55 ± 0.07 (10)
In vitro			
Control	0.45 ± 0.03 (15)	0.44 ± 0.06 (15)	0.56 ± 0.08 (15)
48 h	0.09 ± 0.02 (12)*	0.12 ± 0.03 (11)*	0.07 ± 0.01 (12)*
72 h	0.04 ± 0.01 (4)*	0.04 ± 0.03 (4)*	0.08 ± 0.06 (4)*

Data indicate the Gata-1/Tbp ratio. Data indicate means ± SEM (number of independent experiments).
* $p < 0.01$ versus corresponding controls.

factors may have to play in regulating prestin expression. Remarkable relations are observed between prestin and Gata-3, -2 expression in OC preparations *in vivo* and *in vitro*: (i) Gata-3, -2 expression display similar apical–basal gradients as prestin mRNA levels. (ii) The prestin expression increases between PD2 and PD8 by a factor of about four in the apical and middle segments and by a factor of two in the basal part. Whereas the mean Gata-3, -2 levels remain unchanged during postnatal development, their expression increases in culture. (iii) Highly significant Pearson correlation coefficients were observed between Gata-3, -2 mRNA and prestin levels when the data were evaluated by regression analyses.

The finding of a parallel apical–basal gradient of prestin and Gata-3, -2 *in vivo* and *in vitro* may be indicative of a regulatory mechanism, but may also simply be an expression of morphometric features of the hair cells. It is known that at any developmental stage the apical hair cells are longer than the basal ones (Sato et al., 1999). Assuming that hair cells resemble a cylinder, an apical–basal gradient of hair cell volume in the range of about two can be calculated. This could explain the apical–basal gradient of prestin mRNA levels on PD5 and PD8 because prestin is expressed in the OHCs only. However, it can hardly explain the apical–basal gradient of Gata-3 and Gata-2 mRNA levels because the cellular expression patterns of GATA-3, -2 are less specific. A selective decrease of GATA-3 staining in hair cells in newborn mouse cochlea rats was observed by Rivolta and Holley (1998) and Karis et al. (2001). In contrast, van der Wees et al. (2004) have shown that the GATA-3 expression is maintained in hair cells and that it is expressed in several adjacent cells. Our RT-PCR and immunostaining data confirm GATA-3 to be present in hair cell nuclei during the postnatal period. The expression levels appear lower in the hair cells of the basal segment as compared to those of the apical segment, and they appear to be lower on PD8 than on PD2.

The developmental kinetics and the significant Pearson correlation coefficients between Gata-3, -2 mRNA and prestin levels cannot be explained by morphometric changes of the hair cells and suggest that Gata-3, -2 play a functional role in prestin expression during the postnatal period. During development, the OHCs in the middle and apical regions show an increase in length and a decrease in width, so that no changes in cell volume and in prestin level can be expected during development (Sato et al., 1999; Abe et al., 2007). Our results about the developmental time course of prestin expression are in agreement with the observations in the literature. Using a freeze-fraction technique, Souter et al. (1995) found that intramembrane protein particles (IMP), the putative OHC motor elements, were present at low density at PD2 and increased in density from 2200 IMP/µm^2 at PD2 to 4131 IMP/µm^2 at PD8. Oliver and Fakler (1999) studied the expression of prestin during postnatal development by measuring the voltage-dependent charge transfer (non-linear capacitance, $C_{non\text{-}lin}$) reflecting functional expression of prestin. Basal and apical turns of Wistar rats were analyzed. On the PD0, $C_{non\text{-}lin}$ was not detected in OHCs of basal turn of the cochlea, while it was present in apical OHCs. $C_{non\text{-}lin}$ increased gradually during postnatal development. Belyantseva et al. (2000) used antibodies to compare the postnatal expression pattern of prestin with the development of electromotility in OHCs. Prestin incorporation in the plasma membrane begins fromPD0 and increases progressively. Judice et al. (2002) found a prominent longitudinal gradient of prestin in both, mice and rats which increased in staining intensity from base-to-apex using in situ hybridization technique. Reisinger et al. (2005) quantified the amount of mRNA coding for prestin between PD0 and PD9 and used a fragment of 18S rRNA as reference. A clear increase of prestin mRNA levels was observed. Abe et al. (2007) quantified the expression of prestin and Gapdh using a two-step RT-QPCR. Both the absolute and the normalized levels of prestin mRNA increased between PD5 and PD10, the time period studied. The results were consistent with the non-linear capacitance, and electrophysiological indicator of prestin protein. In the present study, we used Tbp as a house keeping gene. Regardless of which technique or housekeeping gene is used to normalize the data, the relative trends are the same. The increase of the mRNA levels during postnatal development reflects an increase of transcript numbers per cell. This increase correlates closely with the density of prestin protein in the plasma membrane.

In contrast to prestin, remain the mean Gata-3, -2 mRNA levels unchanged during postnatal development. In culture, the expression levels increases, most probably as response to the culture stress. Nevertheless, high Pearson product-moment correlation coefficients were found between Gata-3, -2 and prestin mRNA over all segments and within each segment group. The close correlation appears to exist beyond PD2, i.e., at the time the prestin synthesis starts *in vivo* and *in vitro* (Fig. 3) Remarkably, this correlation can be observed also under the influence of thyroid hormones (Fig. 9E and F) and gemfibrozil (data not shown).

The concordance between mRNA and protein expression findings indicates that prestin protein expression is regulated significantly by transcription (Fig. 4C). A similar relationship seems to exist for Gata-3. The question arises of how GATA-3 could be able to regulate the prestin expression during postnatal development while its expression in the hair cells remains unchanged or decreases. Based on the understanding of the mechanism of transcription and the structure of the prestin promoter, it seems likely that prestin expression needs multiple transcription factors,

GATA-3, -2 being two of these (Tamura et al., 1996; Zheng et al., 2003). The absolute level of the transcription factors may not be the critical factor as indicated by the existence of transcription modules (Cartharius et al., 2005). Genes do not have to be highly expressed to play regulatory roles (Hildebrand et al., 2007). The exponential regression between Gata-3, -2 and prestin indicate that different prestin levels require a different threshold of Gata-3/-2. The regulation of the prestin expression may involve distinct and also overlapping effects of Gata-3 and Gata-2 as observed during inner ear development.

It may be surprising, that the transcription factors Gata-3, -2 which are important regulators of inner ear morphogenesis including cell proliferation (Lillevali et al., 2007) regulate the prestin expression in the outer hair cells during postnatal development. However, changes in the function of genes used primarily to control proliferation in the nervous system are not unusual. For example, differentiated neurons after having withdrawn from the cell cycle, use cell cycle proteins alternatively to control synaptic plasticity (Arendt, 2005). Moreover, Gata-3, -2 seem to be involved not only in the regulation of prestin expression, but also in the regulation of prestin-associated genes or genes expressed in adjacent cells and which are important for OHC function. This conclusion arises from the multiple functions of GATA-3, -2 transcription factors, and the presence in various cells of the cochlea, the apical–basal gradients, and the close cooperation of the outer hair cells with the adjacent cells. For example, GATA-3 may be involved in the cholesterol homeostasis. Gemfibrozil affects the prestin and Gata-3 expression via PPAR, a member of the nuclear receptor family. PPAR plays an important role in the regulation of genes involved in lipid metabolism and transport (Chen et al., 2008). This is important for the prestin function because the fluidity of the OHC lateral plasma membrane is regulated by cholesterol levels. Interestingly, there are quantitative differences in lipid lateral mobility among the apical, lateral and basal regions of the OHC (Organ and Raphael, 2009). It is possible, that GATA-3 mediates some effects of T4 recently identified in thyroid hormone deficient mice. In a mouse model of secondary hypothyroidism, several novel defects of hormone deficiency related to outer hair cell function, potassium recycling and deafness were observed (Mustapha et al., 2009).

A general precondition for involvement of transcription factors in gene expression is the existence of response elements in the promoters of the corresponding target genes. The presence of several GATA response elements in the prestin promoters was identified (Table 3). GATA family transcription factors bind to GATA-containing sequence motifs (Murphy and Reiner, 2002). Of the six Gata factor family members, only Gata-2 and Gata-3 are expressed in the nervous system (Moriguchi et al., 2006). Among the six GATA factors, the sequence homology is the highest for GATA-3 and GATA-2 (Ferreira et al., 2007). In the inner ear, both are expressed in an overlapping manner (Lillevali et al., 2007). Sequences containing an AGATCTTA motif are bound by GATA-2 and -3 with high affinity, while the GATA-1 binding is very weak (Ko and Engel, 1993). The co-expression changes of Gata-3 and prestin in response to T4 could have their basis in the presence of a thyroid hormone response element (Table 4). Gemfibrozil interacts with the PPAR-alpha. PPAR-alpha and PPAR-gamma have recently been shown to negatively regulate GATA-3 in various cell types within the immune system (Woerly et al., 2003).

Table 3
Presence of GATA-binding sites in the promoters of prestin gene.

Gene/PS	Start	End	Sequence
PS3 (GATA-1)	608[b]	620	tgaaGATAggcct
PS1 (GATA-1)	331[a]	343	tgcaGATAatttt
PS1 (GATA-1)	320[b]	332	caagGATAtgggt
PS1 (GATA-3)	179[a]	191	taaTGATcatatt

Three alternative promoter regions have been mapped for prestin (Cartharius et al., 2005). Promoter set 3 (PS3) is localized at (−550 to 101), PS2 at (−21,513 to −20,913) and PS1 at (−26,042 to −25,437) from the ATG site. No GATA-binding site was found in promoter set PS2.
[a] (+) Strand.
[b] (−) Strand.

Table 4
Presence of thyroid hormone binding sites in the prestin and Gata-3 genes.

Gene/PS	Start	End	Sequence
Prestin/PS3[a]	131	155	cgtgAGGTCAgttTAAGGACAatct
Gata-3/PS3[b]	768	792	agaaaaggtgagcaAAGGagagggt
Gata-3/PS3[a]	967	985	cgctGGGTGAgccaccaTCACCCggcg

[a] (+) Strand.
[b] (−) Strand. PS, promoter set. In Gata-1 and Gata-2, no thyroid hormone binding sites were found.

In a previous study, we investigated the prestin mRNA expression in adult guinea pigs and rats (Mazurek et al., 2007). Similar to what was observed for the immature cochlea, a clear base-to-apex gradient in the prestin mRNA expression was found to exist. An unilateral impulse noise exposure (167 dB peak sound pressure level for 2.5–5 min) produced an increase in the number of prestin RNA transcripts at a mean noise-induced hearing loss of about 15–25 dB indicating an upregulation in the intact outer hair cells. Replicate analyses of samples of these experiments showed a significant correlation between Gata-3 and prestin expression in the apical and middle segments (Fig. 11). Thus, GATA-3 may play a role for prestin expression also in adult animals. Although we have evidence that mRNA and protein are correlated during postnatal development, it remains uncertain whether prestin protein is differentially distributed in the apical-to-basal gradient in adult cochleae. Liberman et al. (2002) showed that targeted deletion of prestin in mice resulted in loss of outer hair cell electromotility. RT-PCR analysis of the deleted region showed that the amount of prestin mRNA in heterozygous (+/−) cochleae was roughly half that in normal (+/+) cochleae. In contrast, Cheatham et al. (2005) showed that cochlear function in mice with only one copy of the

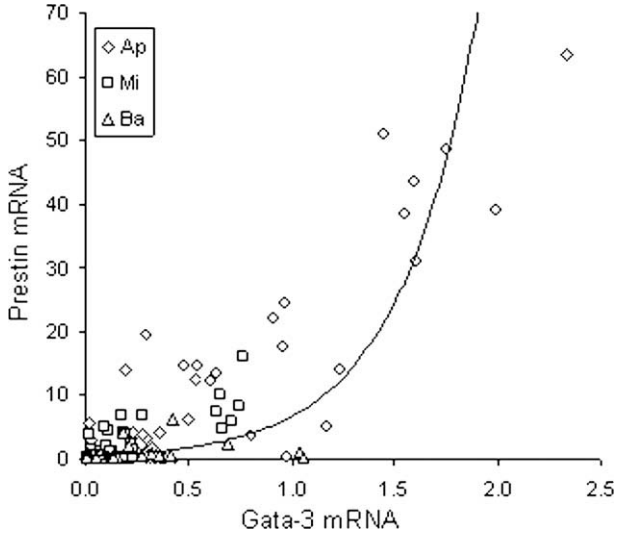

Fig. 11. Pearson correlation analysis of RT-PCR data of Gata-3 and prestin of the apical, middle and basal segments of the organ of Corti of adult rats. The following equation fits to the experimental data: $y = 0.53e^{2.60x}$, $r = 0.67$, $n = 40$, $p < 0.01$. The samples originate from our previous study (Mazurek et al., 2007). They had been kept at -80 °C. Prestin and Gata-3 mRNA levels were determined again in parallel and expressed in arbitrary units (Gross et al., 2007). A close correlation of the previous and the present prestin mRNA levels indicate the integrity of the samples (data not shown).

prestin gene was indistinguishable in the two genotypes as was electromotility. Although prestin mRNA was less than in wildtype controls at all ages tested, near normal protein levels were observed using immunocytochemistry and western blot analysis.

The question of the biological significance of the apical–basal prestin gradient arises together with that of why changes of expression are more pronounced in the apical part than in the basal one of the organ of Corti (Ashmore, 2008). This is an open question, because there are not enough data available of conditionally induced changes of mRNA, protein levels and electromotility in wildtype animals. In addition, stability and function of prestin protein is affected by posttranscriptional changes like phosphorylation (Cheatham et al., 2005). Additional experiments are necessary to understand the functional consequences of changes in prestin mRNA expression. Nevertheless it is interesting to note that sound-evoked basilar membrane vibrations in the high-frequency region of prestin (−/−) mice cochleae are, surprisingly, as sensitive as those of their prestin (+/+) siblings (Mellado Lagarde et al., 2008). The fact that prestin is differentially expressed at different frequency sites of the organ of Corti appears to reflect an evolutionary adaptation of the basal membrane properties and of OHC features in the regions concerned. Perhaps, this differential expression of prestin is involved in the frequency-dependent coupling of the basilar membrane motion and the inner hair cell stereociliar shear as discussed recently (Santos-Sacchi, 2008).

The present results show Gata-1 to respond differently from Gata-3, -2. The role of GATA-1 in inner ear development or in prestin expression is not clear. In general, Gata-1 shows a very low expression level and a behavior opposite to that of Gata-3, -2. For example, in culture, Gata-3, -2 expression was up-regulated, whereas Gata-1 was down-regulated. The question of whether these features play a role in regulating prestin expression remains open. In erythroid differentiation, a down-regulation of the GATA-1 gene is needed for erythroid terminal maturation (Zheng et al., 2006).

Altogether, the expression data of prestin and Gata-3 over multiple conditions strongly suggest a regulatory role played by the GATA-transcription factors in prestin expression. This is an important observation for understanding the regulation of prestin expression under different pathological conditions and may be important for explaining symptoms like hearing loss, hyperacusis or tinnitus. More generally, stress and injuries of the inner ear may induce Gata-transcription factors which could then modulate the expression of prestin in the outer hair cells. The hearing loss due to GATA-3 haploinsufficiency has been shown to be peripheral in origin, and a strong outer hair cell malfunctioning was identified in the hearing loss HDR-syndrome (van Looij et al., 2005). It is possible that GATA mutation is associated with changes in prestin expression.

Acknowledgments

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