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Age-related changes in expression of CTL2/SLC44A2 and its isoforms in the mouse inner ear

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Abstract

The membrane glycoprotein CTL2/SLC44A2 is expressed by supporting cells in the inner ear and has been identified as a target of antibodies that may induce auto-immune hearing loss. To determine if CTL2/SLC44A2 also has roles in inner ear development and to distinguish between isoform-specific roles, we assessed age-related changes in expression of CTL2/SLC44A2 isoforms and protein in the developing murine inner ear. We determined that both isoform p1 and isoform p2 (named for the upstream p1 and proximal p2 promoters that control alternate exons 1a and 1b) were robustly expressed as early as E14 and persisted during embryonic development, but after birth the p1 isoform fell to barely detectable levels while isoform p2 levels were maintained. This trend continued and became even more apparent later in post-natal development and remained in mature ears until at least 6 weeks of age. In aged (18mo old) mice, the level of isoform p1 transcripts rose again to levels similar to the p2 isoform like that seen early in development. At the earliest stage examined, CTL2/SLC44A2 protein was expressed in both immature supporting cells and immature sensory cells, but after birth expression in the sensory cells declined in both the utricle and cochlea and by day P1 expression of CTL2/SLC44A2 was restricted to supporting cells. The changes we observed in isoform distribution are indicative of differential developmental roles and age related changes between the two isoforms of CTL2/SLC44A2 in the inner ear.

Introduction

CTL2/SLC44A2 (CTL2) is a membrane glycoprotein belonging to the solute carrier family of osmolyte transporters. It was discovered in the inner ear as a supporting cell antigen (Zajic, et al., 1991) that is the target of antibody induced inner ear damage (Nair, et al., 1999; Nair, et al., 1997; Nair, et al., 1995). CTL2 has two promoter specific N-terminal isoforms, p1 and p2, that differ from one another only in the first 10–12 amino acids. These isoforms are the result of alternative splicing that encodes either exon 1a (isoform p1) or exon 1b (isoform p2) (Kommareddi, et al., 2010). When we originally cloned CTL2/SLC44A2 from the guinea pig inner ear we found only isoform p1 and identified exon 1a using 5′ RACE (Nair, et al., 2004). This full length isoform which encodes 705 amino acids

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in the guinea pig and 704 in humans did not match the known CTL2 N-terminal sequences in the database (O'Regan, et al., 2000) which at that time showed only the isoform containing exon1b which is driven by the second and more proximal CTL2 promoter. To avoid confusion we named this isoform p2 since it uses the second promoter. (CTL2 isoform p2 maybe identical to what is called CTL2 isoform 1 in the UniProt database.) The full length human isoform p2 encodes 706 amino acids. Subsequent to our identification of isoform p1, two other human CTL2 isoforms called hCTL2a and hCTL2b that differ based on alternate splicing of the C-terminal exons 22 and 23 were reported (Traiffort, Ruat, O'Regan, & Meunier, 2005). hCTL2a is probably identical to CTL2 isoform p2 as both encode 706 amino acids and both use exon 22 (Nair, et al., 2004; O'Regan & Meunier, 2003; Traiffort, et al., 2005). CTL2/SLC44A2 is a prominent and abundant protein in the adult inner ear (Kommareddi, et al., 2007) with expression restricted to supporting cells and not normal hair cells in the adult guinea pig. In vivo binding of antibody to CTL2/SLC44A2 on supporting cells is accompanied by loss of hair cells and hearing loss (Nair, et al., 1999; Nair, et al., 1997; Nair, et al., 1995). Together these observations suggest that CTL2/SLC44A2 has a critical function in maintaining the homeostasis of the inner ear. The purpose of this study was to determine the pattern of CTL2/SLC44A2 isoform expression in the inner ear during development and aging.

We studied mouse embryos and observed that both isoforms are expressed in the auditory and vestibular sensory epithelium during development of the inner ear. In the developing inner ear CTL2 is expressed in the early stages of otic morphogenesis in both sensory and non-sensory cells. CTL2 expression becomes restricted to supporting cells as maturation progresses. We also show that Isoform p1 expression declines as the cochlea matures such that CLT2/SLC44A2 isoform p2 predominates in the sensory epithelium of the inner ear after parturition, but in ears of aging (18 month old) mice the level of isoform p1 rose again to levels seen earlier in development and similar to that of isoform p2.

Methods

Animals

All animal experiments were reviewed and approved by the University of Michigan University Committee on the Use and Care of Animals (UCUCA). CD-1 mice were purchased from Charles River Laboratories International Inc. (Wilmington, MA, USA) and used at embryonic days E14, E17 and E18, newborn-P0, postnatal days P1, P7, P14, P21, and 6 weeks of age. Inner ear tissues from aging mice were harvested from adult CBA/J mice at ages 3 months, 12 months and 18 months(Sha, et al., 2008).

Tissue collection for RNA Isolation—Temporal bones were removed from embryonic, juvenile or adult mice and placed in cold RNAlater for dissection. Inner ear RNA was collected from CBA/J mice used in an aging project (Sha, et al., 2008). Repeat RNA samples from the mice were not available due to the long term nature of the experiment. For the 12 month old mice Dr. Sha had only a small amount of cDNA remaining and we were unable to include the actin control for this time point. The bulla was removed and the organ of Corti was separated from the modiolus and stored in RNAlater at room temperature overnight. The sample was centrifuged, RNAlater removed, and tissue was stored at −80°C. At developmental day P14, in addition to the organ of Corti, the utricles were removed and processed separately for RNA.

Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

To isolate RNA, tissues were dissected in RNAlater (Ambion, Austin, TX), placed in lysis buffer, homogenized by a mortar and pestle and sheared by passing the lysate through a 20-

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gauge needle ten times. Purification of total RNA was done with the RNeasy mini kit (Qiagen, Valencia, CA). An RNA quality check was completed by the University of Michigan Comprehensive Cancer Center Affymetrix and Microarray Core. cDNA was synthesized from total RNA using oligo dT primers with Superscript II reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA). Mouse specific primers were designed for amplifying full length CTL2/SLC44A2 isoform p1 and isoform p2. This insured that we were assessing the complete p1 and p2 isoforms and not a previously unknown variant. Forward and reverse primer sequences are as follows: (MsP1 CTL2/SLC44A2 f — TCGCGCTGGCTTCGGACTCA; MsP2 CTL2/SLC44A2 f — GCGGGTGGCGGCTGTGTC; MsP1 CTL2/SLC44A2 r —GCACAGGGCTGGGCATA CAAG; MsP2 CTL2/SLC44A2r — GGATGGCCAAAGTAGGGGTGAGG). PCR was carried out using 1X GC rich PCR buffer, GC rich resolution buffer, and GC rich DNA polymerase (Roche Diagnostics, Indianapolis, IN). Denaturation occurred at 95°C for 5 minutes after which the reaction was performed for 30 cycles at 95°C for 40 seconds, 60°C for 40 seconds, and 72°C for 3 minute s and 50 seconds. PCR products were represented on a 1% agarose gel using ethidium bromide fluorescence. Amplification of actin was used as a control of the quality and loading of the cDNA as well as a point of comparison for the relative amplification of the p1 and p2 isoforms. Actin primers were purchased from Promega (Cat no. PR-G5740).

Immunohistochemistry

The temporal bones were removed from the skull, the cochleae exposed and the organ of Corti region and the utricle were dissected then fixed in 2% paraformaldehyde for 2 hrs. The tissue was washed in PBS and permeabilized in 0.3% triton X-100 before blocking with normal goat serum. Rabbit antiserum (anti CTL2-NT)(Kommareddi, et al., 2009; Nair, et al., 2004) raised to a unique conserved peptide in the N-terminal region of CTL2/SLC44A2 was purified on protein A. The protein A purified CTL2-NT antiserum was used at 1:100. For some experiments CTL2-NT antiserum was further affinity purified on the immunizing peptide and used at 1:10. The monoclonal anti-Myosin VIIa (1:400) antibody developed by D. J. Orten was obtained from the Developmental Studies Hybridoma Bank (The University of Iowa). Primary antibodies were incubated overnight at 4°C, then the tissue was washed with PBS and incubated with secondary goat anti-mouse Alexa-fluor 594, or goat anti-rabbit Alexa-fluor 488 (Invitrogen, Carlsbad, CA) at 1:300. Alexa Fluor 594 labeled phalloidin was used at 1:400. Secondary antibodies and Alexa-fluor labeled phalloidin (Invitrogen, Carlsbad, CA) were incubated for 45 minutes followed by three 5 minute washes in PBS and mounting with Prolong gold (Invitrogen). Images were taken with a Zeiss LSM 510-META Laser Scanning Confocal Microscope mounted on a Zeiss Axiovert 100M inverted microscope.

Results

CTL2/SLC44A2 message was expressed in CD-1 mice as early as E14, and expression remained strong through age of 6 weeks although a shift in isoform prevalence occurred (Figure 1A–1C). The relative expression of CTL2 isoforms 1 and 2 was equivalent on days E14–E18. On day P0 we noted that isoform p2 began to give a stronger amplification signal than isoform p1 (Figure 1A). This shift in relative isoform expression continued to persist during postnatal days 1, 7, 14 and at 6 weeks (Figure 1B). On day P14 we separated the cochlear tissue from the vestibular tissue to determine if the shift in isoform expression was linked to the differentiation program of the vestibular tissue versus the cochlear tissue. However, as shown in Figure 1B, expression of isoform p2 was greater than isoform p1 in both tissues. To determine if the relative difference in isoform expression persisted throughout life, inner ear cDNA from adult and aging CBA/J mice was examined. As in the

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young post partum mice, CTL2/SLC44A2 isoform p2 was more highly expressed than isoform p1 at 3 and 12 months of age, but curiously, in the oldest mice tested (18 months of age), the relative expression of the isoforms changed back to the relative expression levels observed at E14, with increased expression of isoform p1 relative to isoform p2 (Figure 2). We also noted a trend toward greater membrane expression with maturation which is consistent with previous observations in the mature guinea pig inner ear. Table 1 shows the amino acid sequences for the p1 and p2 CTL2 isoforms in human, and mouse and the p1 isoform we identified in guinea pig. To our knowledge the p2 isoform has not yet been identified in guinea pig.

We next tested the expression of CTL2/SLC44A2 protein in the developing inner ear. At E17 and E18, CTL2/SLC44A2 protein is expressed in both developing supporting cells (arrows) and sensory cells (arrowheads) in the utricle (Figure 3) and cochlea (Figure 4). In the utricle, CTL2/SLC44A2 expression is present in both developing hair cells and supporting cells at E17, then gradually declines as the tissue matures (day P1) (Fig 3B), until expression in hair cells is barely detectable (day P7) (Fig. 3C). In the cochlea, some hair cells (especially those outer hair cells in row 3) express CTL2/SLC44A2 on day E17 (Figure 4A). On E18 fewer of the developing hair cells express CTL2/SLC44A2 (data not shown) and by day P1 CTL2/SLC44A2 is nearly exclusively expressed in the supporting cells (Figure 4B with specifically high levels noted in supporting cells that surround the inner hair cells. The typical distribution of CTL2/SLC44A2 observed at P7 approaches the pattern found in mature tissues, with expression restricted to supporting cells and the outer pillar cells which show the strongest expression levels (Figure 4C). The distribution of CTL2/SLC44A2 at P20 (Figure 5) resembles that seen in the mature ear with hair cells being devoid of CTL2/SLC44A2 whereas supporting cells and pillar cells (asterisk) stain positive including distribution to the membrane. The image in Figure 5 is taken at a focal plane lower than the reticular lamina, showing that the antigen is localized not only to the apical surface but is also seen in subapical cytoplasmic regions of supporting cells which is consistent with staining previously observed with the CTL2-NT antibody in the inner ear of the adult guine pig (Nair, et al., 2004).

Discussion

The exact function of CTL2/SLC44A2 in the ear and elsewhere remains unknown. Nevertheless, it is clear that CTL2/SLC44A2 has an important role in the inner ear that is necessary for hair cell survival since antibody to this protein can impair hearing and result in loss of outer hair cells. In this study we linked changes in the expression of this protein in the inner ear cells to the early stages of differentiation of sensory cells and supporting cells. We observed that CTL2/SLC44A2 is expressed in all cells in the developing organ of Corti and the vestibular sensory epithelium at E17 prior to completion of the differentiation into supporting cells and hair cells. By E18 CTL2/SLC44A2 expression is waning in the differentiating hair cells and waxing in the supporting cells. Since this protein is present starting in early development and its expression is sustained into maturity, it is likely to have both developmental and physiological roles.

During development, the timing of this change in molecular profile is coincident with changes in Notch signaling molecules (Artavanis-Tsakonas, Rand, & Lake, 1999) and with acquisition of the more mature morphology of supporting cell. The changes in Notch signaling, which are coincident with lineage maturation and with the change in cell lineage specific expression of CTL2/SLC44A2, suggest that expression of this transporter molecule may be regulated coordinately with or by Notch and its partners, Delta1 and Jagged1(Artavanis-Tsakonas, et al., 1999; Brooker, Hozumi, & Lewis, 2006; Kiernan, et al., 2001). This observation further suggests that CTL2/SLC44A2 is also linked to the changing

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function of the mature supporting cells and sensory cells. However, the genes that maintain CTL2/SLC44A2 expression in mature supporting cells and that suppress its expression in mature hair cells are unlikely to be notch signaling molecules, such as the Notch receptor, or its ligand jagged 2, or HES5 because these are not expressed in the adult auditory epithelium (Batts, Shoemaker, & Raphael, 2009; Hartman, et al., 2009; Kelly & Chen, 2009; Wang, et al., 2010).

A second change in CTL2/SLC44A2 expression happens with development and that change is a switch in the predominant isoform expressed in the murine inner ear. In embryonic ears both CTL2/SLC44A2 isoforms are expressed in the organ of Corti and the relative expression of the isoforms is similar throughout embryonic development of the inner ear as judged by RT-PCR and densitometry. But at the time of parturition isoform p2 becomes relatively more prominent than isoform p1 and this difference is maintained through adulthood until late adulthood (18 months) when the relative expression of the isoforms appear similar. Whether this reversion to embryonic isoform expression is due to age related changes in the inner ear is unknown. CBA/J mice do have variable levels of age related hearing loss (Sha, et al., 2008). The RNA used in this study came from mice that had retained relatively good ABR thresholds up to 18 months of age (Sha, Chen, & Schacht, 2010). We can only speculate that isoform p1 is more important in development in the mouse and isoform p2 is more important in the adult murine inner ear.

In mature ears the normal role of CTL2/SLC44A2 needs to be considered versus its function in response to lesions and reparative/regenerative capability. We previously reported changes in CTL2/SLC44A2 expression at sites of scar formation in ears infused with purified KHRI-3 monoclonal antibody to the CTL2/SLC44A2 protein. We also noted re-expression of this protein in hair cells located within such antibody induced lesions (Nair, et al., 1999). Thus the persistence of CTL2/SLC44A2 in the supporting cells of the mature ear is likely associated with maintenance of the homeostatic mechanisms necessary for hair cell survival, but its expression is also induced after damage at sites of hair cell loss and may have a role in maintaining the integrity of the reticular lamina by facilitating rapid scar formation. The *SLC44A2/CTL2* (MIM 606106) gene maps within a 1.4mb region containing the DFNB15 congenital hearing loss locus on chromosome 19 {Santos, 2006 #721}. This is of interest in light of the findings that antibody to this protein can cause hearing loss. However, Santos et al. found no evidence of functional sequence variants in CTL2 in affected members of their kindreds with this non-syndromic hearing impairment. The scarring mechanism, which is important for maintaining the barrier between endolymph and perilymph, is accomplished by rapid expansion of the supporting cells flanking a degenerating hair cell and involve phagocytosis of hair cell debris occurring at the sites of scar formation by supporting cells (Abrashkin, et al., 2006; Raphael, 2002).

The auditory epithelium differs from the vestibular epithelium in regenerative capability. In the vestibular epithelium supporting cells can undergo transdifferentiation spontaneously and generate new hair cells, whereas in the auditory epithelium supporting cells are unable to perform this task. As such, any difference between supporting cells in the auditory vs the vestibular system are of interest, as they may shed light on the reasons for the differences in the regenerative capability. We noted a subtle difference in the patterns of expression in the auditory versus utricular epithelium suggesting that the switch from CTL2-positive to CTL2-negative hair cells occurs more slowly in the vestibular epithelium. This may have implications related to the difference between these two types of supporting cell vis-à-vis their regenerative capability.

The restricted presence of CTL2/SLC44A2 in supporting cells of the auditory epithelium provides a molecule that could be used for producing targeted Cre mouse lines for inducing

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or repressing gene expression specifically in mature supporting cells or all of the auditory epithelium if activated prior to differentiation. Previous observations associating CTL2/SLC44A2 with auto-immune inner ear disease (Disher, et al., 1997; Kommareddi, et al., 2009; Nair, et al., 1997) may also be related to an immune response to this protein subsequent to a variety lesions in the cochlea.

We also observed a late change in aging animals that suggests a possible role of the dominant embryonic form in the aging inner ear. As the animal becomes aged, hearing may begin to decline possibly creating a greater need for the same types of processes that are carried out during embryonic life.

Curiously the mouse differs from the human and guinea pig in terms of isoform preference in the inner ear. CTL2/SLC44A2 isoform p2 is prominent in the inner ear of newborn and adult mice, whereas in the mature guinea pig and human inner ear the p1 isoform predominates. The reasons for this are not known. There are more sequence differences between the mouse and human isoforms than there are between the human and guinea pig isoforms. Whether these differences account for the use of murine CTL2 isoform p2 and the use of human or guinea pig CTL2 isoform p1 as the isoform used in the mature inner ear remains to be determined.

Further studies are needed to assess how inhibiting expression or overexpressing CTL2/SLC44A2 may affect inner ear development, function, and response to lesions. We postulate that CTL2/SLC44A2 abnormalities will have detrimental effects on development and function of the inner ear. CTL2/SLC44A2 maps in the DFNB68 region suggesting that mutation of CTL2/SLC44A2 could be a cause of congenital hearing loss. Differences in isoform expression and in type and degree of glycosylation of CTL2/SLC44A2 (Kommareddi, et al., 2010) suggest that the isoforms have different functions in different tissues, or in the same tissue at different times in development and maturation.

Conclusion

In conclusion, CTL2/SLC44A2 is expressed as early as E17 in the organ of Corti, in both sensory and non-sensory cells. The non-sensory cells include inner phalangeal cells, pillar cells and Deiters cells. CTL2/SLC44A2 is also expressed in vestibular supporting cells and hair cells, with vestibular hair cells retaining expression longer than their cochlear counterparts.

Acknowledgments

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Research Highlights

- Prior to sensory cell commitment CTL2/SLC44A2 is broadly expressed in the developing inner ear.
- CTL2/SLC44A2 disappears from sensory cells in a gradient consistent with their differentiation.
- Two isoforms of CTL2/SLC44A2 are expressed equivalently in the inner ear until P0.
- At birth isoform p2 becomes more prevalent in the inner ear than the p1 isoform.
- The p2 isoform predominates in young mature animals but p1 increases in ears of aged mice.

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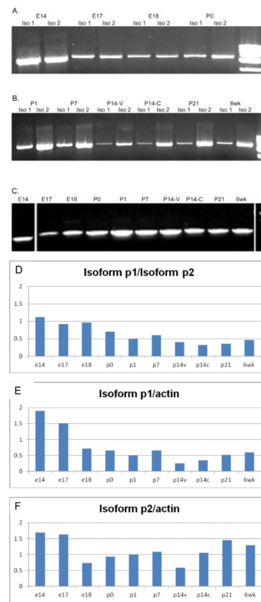

Figure 1.

RT-PCR of full length CTL2/SLC44A2 for isoforms 1 and 2 at different stages of inner ear development. A. PCR was carried out for 30 cycles using cDNA from embryonic days E14 through P0. B. Developmental days P1 through 6 weeks of age. On day P14 we examined vestibular (P14-V) and cochlear (P14-C) tissue separately and observed the same predominance of the p2 isoform over the p1 isoform in both tissues. C. Amplification of actin from the same cDNA samples. Panels A and B, Right lane – 1kb ladder; Panel C, right lane – 100 bp ladder.. The full length CTL2/SLC44A2 cDNA migrates as a 3.4 kb band. Results shown are representative of three different experiments. Panels D-F Relative expression of p1 and p2 isoforms. Fig 1D. shows a comparison of band density of CTL2 p1 and p2 PCR products to each other on days E14 through 6 weeks. The expression of p1 and p2 remains approximately equal through embryonic day 18, with an increase of p2 relative to p1 beginning postnatal day 0. The realtively greater p2 expression continues to increase through P14 but then slightly decreases at P21 through 6 weeks. Fig 1E shows the ratio of band density for isoform p1 relative to actin and Fig 1F shows the ratio of band density for isoform p2 relative to actin. For embryonic days 14, 17, and 18 p1 and p2 have similar ratios when compared to actin. Beginning from postnatal day 0, p2/actin ratio is greater than p1/ actin, with this trend continuing through 6 weeks of age.

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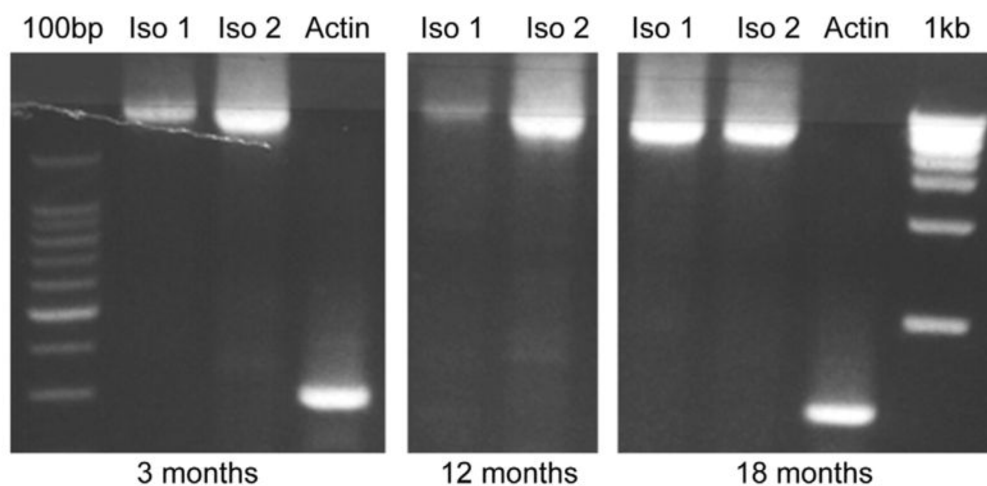

Figure 2.

Relative expression of CTL2/SCL44A2 isoforms 1 and 2 in inner ear of CBA/J mice at ages 3–18 months. The P2 isoform is more abundant at 3 months and 12 months of age in the inner ear; however, at 18 months of age the relative expression is similar for both isoforms which is reminiscent of the embryonic ear. Actin amplification was used as a control for RNA quality. (left lane 100bp; ladder; right lane 1kb ladder)

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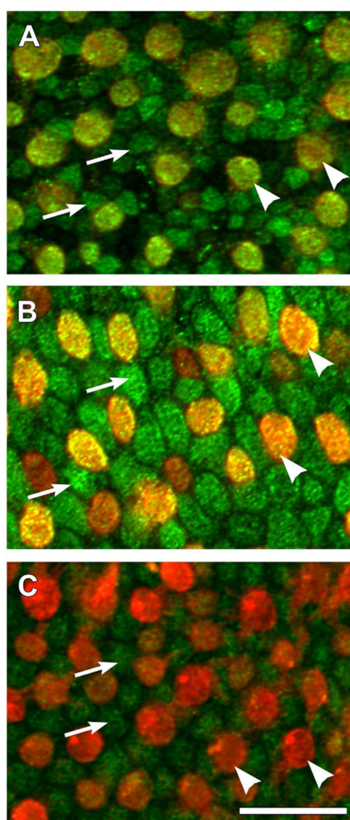

Figure 3.

Epi-fluorescence of whole-mounts of the utricle stained for CTL2/SLC44A2 (green) and Myosin VIIa (red). A. On embryonic day 17, CTL2 is present in the supporting cells (arrows) and hair cells (arrowheads). B. On day P1, CTL2 is present in the supporting cells and a sub-population of hair cells. C. at P7, CTL2 in the utricle is present in the supporting cells and remains visible in a sub-population of hair cells. Bar = 20 μm.

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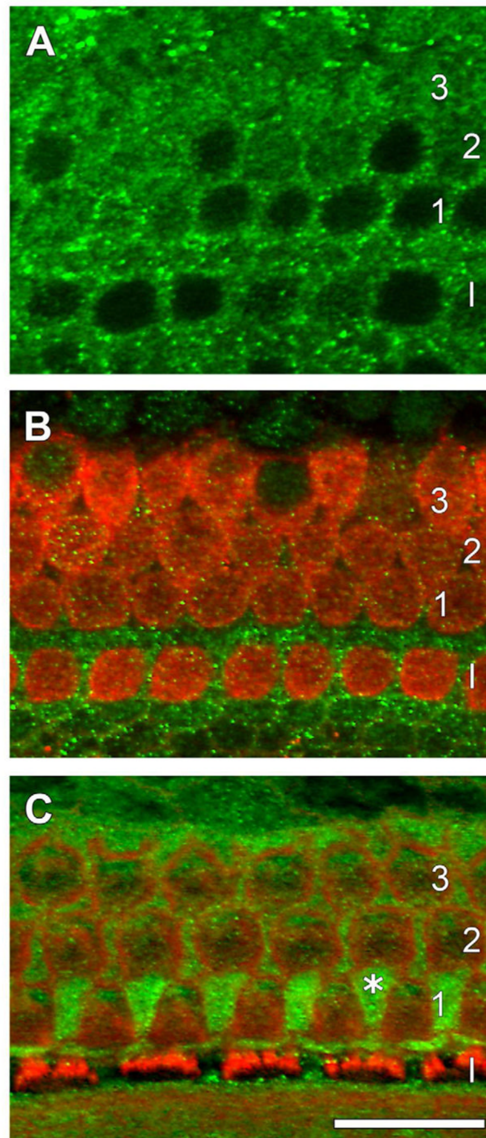

Figure 4.

CTL2/SLC44A2 expression (green) in whole-mount of early developing murine cochlea counterstained with Myosin VIIa (red in B) or phalloidin (red in C). A. Peptide purified CTL2-NT antibody staining of E17 mouse inner ear showing CTL2/SLC44A2 signal in the pillar cells, the inner phalangeal cells, and first row Deiters cells. Many Hair cells are also CTL2/SLC44A2 positive. B. In P1 organ of Corti, maturing hair cells exhibit strong Myosin VIIa. A few hair cells (row 3) are still CTL2/SLC44A2 positive. The second and third row of outer hair cells (OHCs) contained more CTL2/SLC44A2 positive cells than the first row. All supporting cells express CTL2/SLC44A2, including the pillars and inner phalangeal cells. C. At P7, expression of CTL2/SLC44A2 is robust in all supporting cells, with the inner and outer pillars (asterisk) staining most intensely, whereas hair cells are negative. Bar = 20 μm (I=inner hair cells; 1=first row OHC; 2= second row of OHC; 3=third row OHC.)

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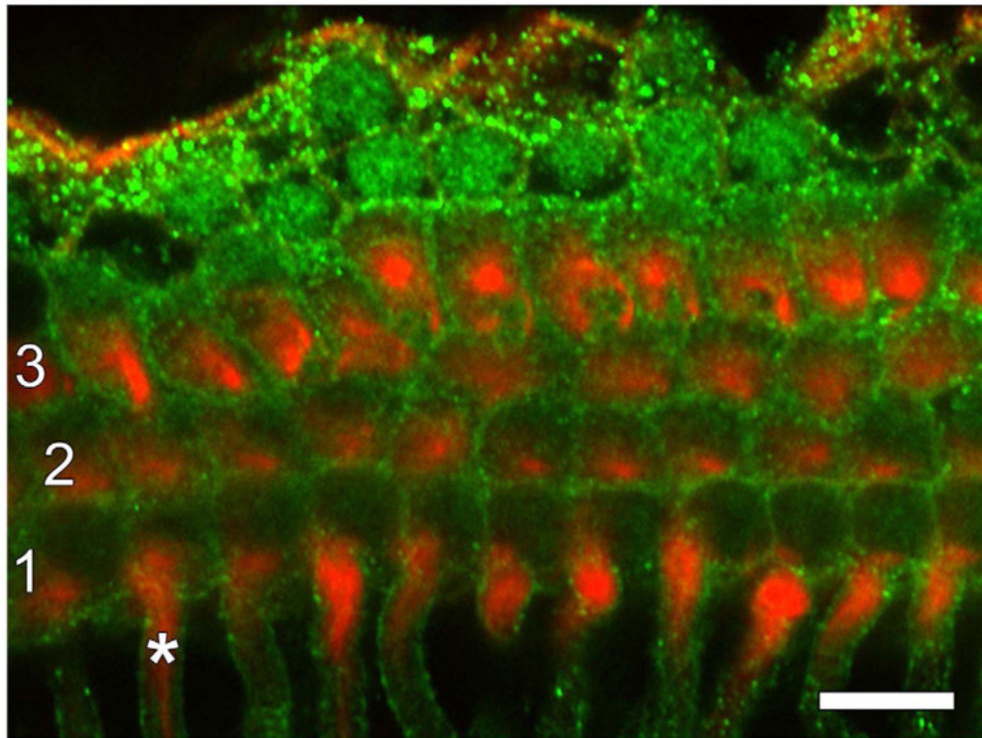

Figure 5.
CTL2/SLC44A2 (green) expression in supporting cells of P20 organ of Corti, counterstained with phalloidin (red) shown at a focal plane below the hair cell level. Actin cores in Deiters and pillar cells are prominent. CTL2/SLC44A2 is present along the membrane of the Deiters cells and pillar cells areas in this focal plane. Asterisk marks the pillar cells; rows of outer hair cells are indicated with numbers 1–3. Bar = 20 μm.

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Table 1

Comparison of CTL2 promoter 1 (p1) and promoter 2 (p2) isoforms from different species

P1 isoform	Exon 1a (10aa) Amino acid sequence
Human P1 CTL2	MEDERK----NGAYGTPQKYDPT*
Guinea pig P1 CTL2	MEDQRK----YGAYGTPQKYDPT
Mouse P1 CTL2	MEDDRK----DAVYGTPQKYDPT
P2 Isoform	Exon 1b (p2 Isoform) Amino acid sequence
Human P2 CTL2**	MGDERPHYYGKHGTPQKYDPT-(12aa)
Guinea pig P2 CTL2	MGGDRPH—YGLHGTPQKYDPT-(11aa)-
Mouse P2 CTL2	MGKDSQNYYGKHGTPQKYDPT-(12aa)

*Exon 1a and 1b encoded amino acids are in black, except for differences which are in red. Exon 2 encoded amino acids are in blue and are highly conserved across these species.

The P2 isoform has two C-terminal isoforms.

The human and guinea P2 isoform we studied in the inner ear and is the same as the isoform1 in the UniProt database and it differs from the isoform 2 in UniProt by an alternative C-terminal splice variant.