Dual Perspectives

Dual Perspectives Companion Paper: Molecular Identity of the Mechanotransduction Channel in Hair Cells: Not Quite There Yet, by Zizhen Wu and Ulrich Müller

Are TMCs the Mechanotransduction Channels of Vertebrate Hair Cells?

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Sensory transduction in vertebrate hair cells and the molecules that mediate it have long been of great interest. Some components of the mechanotransduction apparatus have been identified, most as deafness gene products. Although prior candidates for the mechanotransduction channel have been proposed, each has faded with new evidence. Now, two strong candidates, TMC1 and TMC2 (transmembrane channel-like), have emerged from discovery of deafness genes in humans and mice. They are expressed at the right time during development: exactly at the onset of mechanosensitivity. They are expressed in the right place: in hair cells but not surrounding cells. Fluorescently tagged TMCs localize to the tips of stereocilia, the site of the transduction channels. TMCs bind other proteins essential for mechanosensation, suggesting a larger transduction complex. Although TMC1 and TMC2 can substitute for each other, genetic deletion of both renders mouse hair cells mechanically insensitive. Finally, the conductance and Ca^{2+} selectivity of the transduction channels depend on the TMC proteins, differing when hair cells express one or the other TMC, and differing if TMC1 harbors a point mutation. Some contrary evidence has emerged: a current activated in hair cells by negative pressure, with some similarity to the transduction current, persists in TMC knock-outs. But it is not clear that this anomalous current is carried by the same proteins. Further evidence is desired, such as production of a mechanically gated conductance by pure TMCs. But the great majority of evidence is consistent with these TMCs as pore-forming subunits of the long-sought hair-cell transduction channel.

Key words: **auditory; hair cell; hearing; mechanotransduction; TMC; vestibular**

Introduction

Sensory hair cells of the inner ear convert the mechanical signals of sound and head movements into electrical signals, which are relayed by the eighth nerve to the brain. The mechanism of this sensory transduction and the molecules that mediate it has been the subject of intense interest for more than four decades. In recent years, several molecular components of the mechanotransduction apparatus have been identified, and there is now a strong pair of candidates for the force-gated ion channels at the heart of the apparatus. Here we discuss progress, setbacks, new evidence, and future directions in the search for some of the most elusive molecules in modern neuroscience.

Mechanotransduction in hair cells has been explained morphologically. A bundle of actin-cored stereocilia extends from the apical surface of each hair cell, and bundle deflection in the excitatory direction causes shearing between stereocilia aligned in the direction of the stimulus. This shearing tensions fine "tip links," which extend between adjacent stereocilia along the excitatory axis. Tip link tension in turn gates mechanically sensitive ion channels located at the tips of stereocilia.

Mechanotransduction has also been described biophysically. Most models include an ion channel that opens in response to force, an elastic gating spring that conveys tension to the channel, and a motor that regulates resting tension and mediates mechanical adaptation. Exquisitely sensitive mechanical measurements have led to estimates for some physical parameters. The channel is thought to undergo a conformational change of 4 nm upon opening; the gating spring compliance is ~1 mN/m; and the motor exerts a resting force of 10–20 pN (Howard and Hudspeth, 1987, 1988).

A general assumption is that these biophysical elements correspond one-to-one to discrete molecular assemblies. Among the molecular components identified thus far are the tip link proteins cadherin 23 (CDH23) and protocadherin 15 (PCDH15) (Kazmierczak et al., 2007). In mature hair cells, CDH23 forms the upper two-thirds of the tip link and PCDH15 forms the lower one-third, although this configuration may vary during development or repair (Indzhykulian et al., 2013). Myosin motors are localized at the upper end of the tip link; in particular, myosins IC and VIIA have been

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implicated in this process (Pan and Holt, 2015; Zhao and Müller, 2015). Additional structural elements at the upper end of the tip link are the proteins USH1G/sans and USH1C/harmonin (Grati and Kachar, 2011); at the lower end, TMIE and LHFPL5/TMHS are essential for mechanotransduction (Xiong et al., 2012; Zhao et al., 2014). Like the tip-link cadherins, most of these were discovered as the products of deafness genes in human or mouse.

For the mechanosensory transduction channels themselves, a number of molecules have been considered as candidates over the past 15 years. Each of these candidates faded, however, in the light of additional evidence. TRPN1 (NompC) was proposed as a mechanosensitive channel in zebrafish (Sidi et al., 2003) but was dismissed as the hair cell transduction channel because it is not expressed in higher vertebrates. TRPV4 (VR-OAC) was cloned from vertebrate inner ears (Liedtke et al., 2000), but there is little, if any, expression in hair cells (Scheffer et al., 2015). TRPA1 was an intriguing candidate and satisfied several criteria, including knockdown of transduction current by shRNAs (Corey et al., 2004), but complete TRPA1 knockouts were found to have normal hearing and normal hair cell transduction (Kwan et al., 2006). Likewise, other channels were considered as possible transduction candidates, including HCN1 (Ramakrishnan et al., 2009) and the TRP channels TRPML3 (Grimm et al., 2007; Nagata et al., 2008; van Aken et al., 2008), PKD1 (Steigelman et al., 2011), PKD2 (Fettiplace, 2009), TRPC3/TRPC6 (Quick et al., 2012), and TRPM1 (Gerka-Stuyt et al., 2013), but knock-outs of those genes also lacked definitive hair cell transduction phenotypes (Wu et al., 2016). Although these studies ultimately yielded negative results, they have helped to clarify the criteria that need to be satisfied for definitive identification of the hair cell transduction channel.

Challenges for molecular identification

Why has it been so hard to identify the transduction channel? First, most vertebrate inner ears contain only ~10,000 hair cells (compared with ~10 million photoreceptors in a retina), and large-scale propagating hair cell lines have not been developed. In addition, the number of transduction molecules for each cell is quite small. Physiological studies estimate just 50–200 functional transduction channels per cell; even allowing for multimeric channels and some reserve pool, the total number of subunits is not more than a few thousand per cell. In addition, there are no high-affinity ligands that might be used for purification. The paucity of material has thus far precluded conventional biochemical approaches.

The Tmc gene family in deafness

A strong candidate emerged, however, from identification of a human deafness gene. In three families from Tamil Nadu in India showing recessive deafness, the defective gene was mapped to 9q13-q21, defining a new deafness locus (DFNB7/DFNB11) (Jain et al., 1995). The syntenic region in mouse also harbors a locus responsible for recessive deafness in the *dn* mouse (Deol and Kocher, 1958). Kurima et al. (2002) showed in multiple families that the DFNB7/DFNB11 gene, as well as those for the dominant human deafness DFNA36 and the *dn* mouse, encode a novel protein they called TMC1 (transmembrane cochlear-expressed gene 1, renamed transmembrane channel-like 1). At the same time, another mouse was identified with dominant deafness (*Beethoven*; *Bth*) resulting from a point mutation in *Tmc1* (Vreugde et al., 2002). An integral membrane protein essential for hearing was immediately of great interest.

Yet the path was not clear. *Tmc1* was subsequently dismissed as a transduction channel candidate by most of us when early postnatal mice with *Tmc1* mutations were found to have normal mechanotransduction currents (Marcotti et al., 2006). But *Tmc1* is a member of a gene family comprising eight members in mouse and human (Keresztes et al., 2003), and hair cells express additional members (Kawashima et al., 2011; Scheffer et al., 2015). The possibility of compensation by another *Tmc* gene remained.

Kawashima et al. (2011) then showed that mouse hair cells also express *Tmc2* and that the temporal expression of both *Tmc1* and *Tmc2* matches the onset of mechanotransduction. Deletion of either *Tmc1* or *Tmc2* does not eliminate mechanotransduction in early postnatal mice, consistent with Marcotti et al. (2006), but double knock-outs never have conventional mechanotransduction in hair cells (Kawashima et al., 2011; Kim et al., 2013; Pan et al., 2013). Interestingly, deletion of *Tmc1* and *Tmc2* does not appear to disrupt tip links or alter hair bundle morphology; rather, the deficit seems specific to the mechanotransduction complex itself (Kawashima et al., 2011). Finally, *Tmc2* was found to be downregulated in cochlear (but not vestibular) hair cells near the end of the first postnatal week in mouse, leaving *Tmc1* absolutely required for hearing (but not balance). This changed the game, stimulating additional tests of the TMCs as transduction channel candidates.

Evidence supporting the TMC channel hypothesis

Multiple lines of evidence are presently consistent with the notion that the hair-cell transduction channel is composed of TMC1 and/or TMC2. The first comes from hereditary deafness: >40 different *TMC1* mutations have been identified, both dominant and recessive, that cause deafness in humans (Kawashima et al., 2015). Similar *Tmc1* mutations cause deafness in mice. Surprisingly, there are no known spontaneous mutations in *TMC2* or *Tmc2* that cause genetic disease in humans or mice.

As mentioned, *Tmc1* and *Tmc2* are expressed at the right time in mouse (Kawashima et al., 2011; Scheffer et al., 2015). *Tmc2* first appears in the vestibular system at about embryonic day 16 (E16), and mechanosensitivity also appears at E16 (Géléoc and Holt, 2003). *Tmc1* expression follows beginning at P3. In the cochlea, *Tmc2* appears at P0-P2, and hair cells become mechanosensitive at P1-P3. *Tmc1* expression follows at P4-P6, as *Tmc2* expression declines.

Tmc1 and *Tmc2* are also expressed in the right place. At the tissue level, both *in situ* localization and RNA-seq revealed that both genes are expressed in auditory and vestibular hair cells, but not elsewhere in the inner ear. For protein localization within hair cells, Kurima et al. (2015) generated mice expressing TMC1 and TMC2 fused to mCherry or GFP. Fluorescent label appeared at the tips of hair cell stereocilia, where the transduction channels are located (Jaramillo and Hudspeth, 1991). Based on Ca^{2+} imaging, transduction channels are notably absent from the tallest stereocilia in mammals (Beurg et al., 2009), and the TMC proteins are also largely absent from the tallest stereocilia. The appearance during development of fluorescently tagged TMC proteins also coincides with the development of mechanosensitivity (Kurima et al., 2015).

TMC1 and TMC2 are part of a complex with other proteins required for mechanotransduction. Biochemical studies have revealed interactions between the tip-link protein PCDH15 and both TMC1 and TMC2 (Maeda et al., 2014; Beurg et al., 2015), and coimmunoprecipitation assays have confirmed the interaction, at least in heterologous cells. The membrane protein LHFPL5 binds both PCDH15 and TMC1 and is essential for the proper targeting of both proteins to the tips of stereocilia (Xiong et al., 2012; Beurg et al., 2015). Similarly, the small membrane protein TMIE binds PCDH15 and LHFPL5 and is essential for transduction (Zhao et al., 2014). All five proteins are required for hearing, and the interaction of TMC1

and TMC2 with so many proteins of the complex suggests they play more than a supporting role.

Importantly, the conductance and selectivity of the transduction channel depend on which TMC is expressed and whether it is mutated (Pan et al., 2013). Hair cells that express *Tmc1* but not *Tmc2* have smaller single-channel conductance and lower calcium permeability, and also show faster adaptation. Those that express *Tmc2* but not *Tmc1* show the opposite, and those that express both have channels of at least four distinct conductances, suggesting heteromultimers (Pan et al., 2013). Perhaps most compelling, a single point mutation in the *Bth* mouse, which substitutes a lysine for a methionine at position 412 of TMC1, also changes the biophysical properties of the transduction channel. Hair cells expressing a single mutant TMC, *Tmc1* with the *Bth* mutation, have lower single-channel conductance (Pan et al., 2013), lower calcium permeability (Pan et al., 2013; Beurg et al., 2015; Corns et al., 2016), and lower sensitivity to block by dihydrostreptomycin (Corns et al., 2016) than cells expressing wild-type *Tmc1*. Although there are methodological differences among these studies, the data from each study are consistent with the hypothesis that TMC1 and TMC2 can function as pore-forming subunits of the transduction channel.

Alternate hypotheses

Nevertheless, there are some contradictory observations, and some confirmatory experiments to be desired. TMCs are clearly required for mechanotransduction, but they might function during development to transport other channel proteins to their correct location (Marcotti et al., 2006; Kawashima et al., 2011). It is difficult, however, to reconcile an exclusive role as a transporter with the observation that the *Bth* mutation causes changes in the channel properties of the transduction channel.

Another hypothesis is that TMCs may be structural components of the transduction complex, perhaps as linker proteins that convey force to the channel but are not part of it (Kawashima et al., 2011). Here again, it is difficult to explain how the *Bth* point mutation in a linker protein could produce a change that would alter selectivity, single-channel conductance and sensitivity to dihydrostreptomycin of a different pore-forming protein.

It has also been suggested that TMCs function as accessory or β subunits that modulate the permeation properties of some other, as yet unidentified α subunit. For example, the voltage-gated potassium channel KCNQ1 (Kv7.1) is modulated by KCNE1 (minK), changing both activation kinetics and single-channel conductance, even though KCNE1 does not seem to participate in the pore (Yang and Sigworth, 1998; Van Horn et al., 2011). Yet this may not be a useful precedent: KCNE1 is clearly an accessory protein (KCNQ1 can function without it), whereas TMCs are not; in their absence, there is no conventional mechanotransduction.

Until such an α subunit is identified, it is difficult to support this more elaborate hypothesis in the presence of strong positive data supporting a direct role for TMCs. Furthermore, assigning TMCs a role as accessory or β subunits implies they are dispensable for conventional hair cell transduction, which clearly is not the case.

These and other hypotheses are discussed in more detail in the accompanying *Dual Perspective* article (Wu and Müller, 2016). Although these creative ideas are not without merit, data supporting the alternate hypotheses are lacking and will not be discussed further here.

Anomalous mechanotransduction

The discovery of an unusual, or anomalous, form of mechanosensitivity in hair cells also challenged the hypothesis that TMCs function as pore-forming subunits (Kim et al., 2013). In cells lacking conventional transduction, either from genetic deletion or from acute disruption of transduction proteins, a current can be elicited by negative pressure on or near the apical surface (Beurg et al., 2016). This current has similar ionic selectivity and pharmacology to the normal transduction current, but it persists in *Tmc1* and *Tmc2* double knock-outs, as well as in other mutants that affect conventional transduction (Marcotti et al., 2014). If the anomalous current is carried by the same channels that carry the normal current, just activated in a different way, those channels must not include TMC1 or TMC2. An alternative hypothesis thus suggests that TMC1 is closely associated with unknown pore-forming channel subunits, tightly enough that a TMC1 point mutation changes permeation properties, without TMC1 actually forming the pore itself (Kim et al., 2013). However, this anomalous current does not have exactly the same pharmacology as the normal current (Marcotti et al., 2014), so it may be carried by a different but related channel. Hair cells do express *Tmc3*, *Tmc4*, and *Tmc7* (Scheffer et al., 2015), and the proteins they encode might be expected to have pharmacology similar to TMC1 and TMC2. The emergence of this anomalous current is thus a rather weak argument against a direct role for TMC1 and TMC2 in conventional hair cell transduction.

One hypothesis that could unify these seemingly disparate ideas is that the channel may be a heteromultimeric assembly, with TMC1 and TMC2 as obligate pore-forming subunits together with other as yet unknown pore-forming subunits. If TMC1 and TMC2 are absent, the remaining subunits may form a mechanosensitive channel that is mislocalized.

Definitive tests

What would really nail it? TMCs have already satisfied three (Pan et al., 2013) criteria considered necessary to be force-gated ion channels (Christensen and Corey, 2007). Fulfillment of a fourth criterion, reconstitution in a heterologous system, may be very difficult for hair cell transduction. For instance, channels, such as the bacterial MSCL or the vertebrate PIEZO1, can function in reconstituted lipid bilayers (Kung et al., 2010; Coste et al., 2012), and it would be wonderful to be able to study TMC channels in that way. However, hair-cell transduction requires at least a half-dozen different proteins, the elaborate geometry of the hair bundle, and delivery of force by tip links. It may be many years before hair cell mechanotransduction is reconstituted in a heterologous system. Channel activity of any sort in TMC-transfected heterologous cells would also support the channel hypothesis. Although *Caenorhabditis elegans* TMC-1 has been reported to have channel activity in heterologous cells (Chatzigeorgiou et al., 2013), those observations have not been reproduced. Although heterologous expression has not yielded definitive evidence thus far, the lack of evidence that TMCs form channels should not be taken as evidence that TMCs lack channel activity.

Alternate lines of evidence, such as an atomic structure of a TMC protein, would be extremely valuable. Even if a structure does not immediately reveal a permeation pathway (the channel is likely to be closed in the absence of tension), it would guide further mutagenesis that might reveal pore residues. In the meantime, structure–function studies in native cells, based on transmembrane topology (Labay et al., 2010), cysteine mutagenesis, and cysteine-modifying reagents, might reveal functional domains for permeation and gating. If additional

TMC1 mutations are identified that affect permeation properties, it would be more difficult to argue they indirectly affect the conduction pathway in a different protein through a conformational effect.

In conclusion, there is a growing body of evidence supporting the hypothesis that TMC1 and TMC2 are pore-forming subunits of the hair-cell transduction channel and very few published data that are inconsistent with the hypothesis. Nonetheless, many questions remain that will keep the field busy for years to come. What is the channel stoichiometry, and do other TMCs or other pore-forming subunits participate? What are the roles of the essential proteins TMIE and LHFPL5, and how do they associate with TMC1 and TMC2? Are there additional accessory subunits required to form the transduction complex? How does PCDH15 attach to the channel to convey force? Is the channel simply anchored in the lipid, or is there a distinct tether protein that attaches it to the actin core of a stereocilium? Which residues line the channel pore, and how is cation selectivity determined? And perhaps at the core of hearing, what is the force-dependent conformational change that gates the channel? Answers to these questions will surely provide a wealth of new insight into one of nature's most exquisite mechanosensors.

Response from Dual Perspective Companion Authors–Zizhen Wu and Ulrich Müller

Are TMC proteins pore-forming subunits of transduction channels?

The mechanotransduction channel of hair cells has been a hot topic for decades. Holt and Corey argue that TMC1 and TMC2 are pore-forming subunits of the mechanotransduction channel. They describe alternative hypotheses as "creative ideas," but these hypotheses are supported by strong scientific evidence. Although we do not rule out TMC1/2 as pore-forming subunits of transduction channels, we state that conclusive proof is lacking. This is important; many candidates have been proposed to be the transduction channel by circumstantial evidence (e.g., TRPA1) (Corey et al., 2006) but have been ruled out subsequently (Bautista et al., 2006; Kwan et al., 2006). Other proteins, including LHFPL5/TMHS and TMIE, need to be considered; not to mention other channel components that are not yet identified. We believe that irrefutable evidence is lacking that TMC1/2 are ion channels, and we emphasize the importance not to jump to premature conclusions.

We briefly address arguments put forward by Holt and Corey in support of TMC1/2 as pore-forming subunits of the transduction channel as follows:

1. "Mutations in TMC1 cause deafness."

Mutations in >100 genes cause deafness without affecting components of transduction channels.

2. "The expression pattern of TMC1 is consistent with a role in the transduction channel."

Although this is correct, similar localization also implicates other proteins, such as LHFPL5/TMHS, TMIE, and whirlin.

3. "TMC forms a protein complex with LHFPL5/TMHS and PCDH15."

This statement is incorrect. Biochemical experiments have so far failed to identify interactions between TMC with LHFPL5/TMHS (Beurg et al., 2015) and also with TMIE (Zhao et al., 2014). Regarding PCDH15, immunoprecipitation experiments show that TMC1 binds to PCDH15 when expressed in heterologous cells (Maeda et al., 2014; Beurg et al., 2015); but when protein distribution is examined by immunocytochemistry, TMC1 is in an intracellular compartment and PCDH15 at the cell surface (Beurg et al., 2015). Are the biochemical interactions physiologically relevant or an artifact (e.g., complexes that form after cell lysis)?

4. "Mutations in TMC1 change the conductance and ion selectivity of the channel."

Holt observed changes in conductance (Pan et al., 2013), but Fettiplace could not confirm these findings (Beurg et al., 2015). Changes in ion selectivity are small; similar changes can be caused by mutations in proteins that do not contribute to the channel pore (see Wu and Muller, 2016, *Dual Perspective* companion article). Moreover, mutations in LHFPL5/TMHS affect the conductance of the transduction channel (Xiong et al., 2012; Beurg et al., 2015). LHFPL5/TMHS is linked to deafness, is appropriately located in hair cells to be part of the transduction complex, and interacts with TMIE and PCDH15. Thus, by the same logic as applied to TMC1/2, LHFPL5/TMHS is a candidate for a pore-forming subunit of the transduction channel, but direct evidence supporting this model is lacking.

5. "TMC1/2 are essential for mechanotransduction."

TMIE is also essential (Zhao et al., 2014). TMIE binds to PCDH15 and to LHFPL5/TMHS, is encoded by a gene linked to deafness, and is appropriately localized in hair cells to be part of the transduction channel complex.

Studies in invertebrates suggest that TMC proteins have pleiotropic functions and might not be linked specifically to mechanosensory phenomena. No doubt, the study of the mechanotransduction channel in hair cells will remain a hot-button issue for some time.

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