Histol Histopathol (2017) 32: 987-1000

http://www.hh.um.es

Histology and Histopathology

From Cell Biology to Tissue Engineering

Review

Role of skeletal muscle in ear development

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Summary. The current paper is a continuation of our work described in Rot and Kablar, 2010. Here, we show lists of 10 up- and 87 down-regulated genes obtained by a cDNA microarray analysis that compared developing *Myf5-/-:Myod-/-* (and *Mrf4-/-*) petrous part of the temporal bone, containing middle and inner ear, to the control, at embryonic day 18.5. *Myf5-/-:Myod-/-* fetuses entirely lack skeletal myoblasts and muscles. They are unable to move their head, which interferes with the perception of angular acceleration. Previously, we showed that the inner ear areas most affected in *Myf5-/-:Myod-/-* fetuses were the vestibular cristae ampullaris, sensitive to angular acceleration. Our finding that the type I hair cells were absent in the mutants' cristae was further used here to identify a profile of genes specific to the lacking cell type. Microarrays followed by a detailed consultation of web-accessible mouse databases allowed us to identify 6 candidate genes with a possible role in the development of the inner ear sensory organs: *Actc1, Pgam2, Ldb3, Eno3, Hspb7* and *Smpx*. Additionally, we searched for human homologues of the candidate genes since a number of syndromes in humans have associated inner ear abnormalities. Mutations in one of our candidate genes, *Smpx*, have been reported as the cause of X-linked deafness in humans. Our current study suggests an epigenetic role that mechanical, and potentially other, stimuli originating from muscle, play in organogenesis, and offers an approach to finding novel genes responsible for altered inner ear phenotypes.

Key words: Mouse embryo, Inner ear, Crista ampullaris, Type I hair cell, Microarray

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DOI: 10.14670/HH-11-886

Introduction

The present review is part of our research program focusing on the role of skeletal muscle in the epigenetic shaping of tissues, organs and cell fates. We have been employing a unique experimental model system mouse embryo, in which two myogenic regulatory factors, *Myf5* and *Myod,* are eliminated. These compound-mutant *Myf5-/-:Myod-/-* mouse embryos and fetuses (also known as amyogenic) show a complete absence of one basic tissue type, the skeletal muscle (Rudnicki et al., 1993). They are only viable *in utero* and die at birth. (N.B., another myogenic regulatory factor, *Mrf4*, has been shown to be eliminated in these mutants as well; Kassar-Duchossoy et al., 2004. As visible later in the Tables of the current report, *Mrf4* has not been found to be implicated in the ear development.)

Our previous studies revealed that several organs fail to fully develop in the absence of skeletal muscle, such as skeleton (e.g., palate, sternum, mandible and clavicle), lungs, retina, and ear (reviewed in Kablar, 2011). Specifically, in the absence of skeletal musculature, lungs fail to grow appropriately resulting in pulmonary hypoplasia (Inanlou and Kablar, 2005), and

cholinergic amacrine cells in the retina apparently responsible for motion vision fail to differentiate (Kablar, 2003), palatal shelves fail to fuse resulting in cleft palate (Rot and Kablar, 2013), mandibular secondary cartilage cannot be maintained resulting in temporomandibular joint syndrome (Rot-Nikcevic et al., 2007) and micrognathia (Rot et al., 2014), and, lastly, vestibular sensory fields responsible for angular acceleration fail to properly develop (Rot and Kablar, 2010). The interaction between muscle activity and other tissues and organs is an example of epigenetic process in the original Waddingtonian sense (Waddington, 1942). The epigenetic interactions between heterogeneous tissues, such as muscles and bones, for example, integrate them into a functional system through complex networking of genes and their products that will result in a specific phenotype (Waddington, 1975).

In the current review, we aim to elucidate the role of muscle-originating (probably mostly mechanical) cues in the development of the mouse inner ear sensory fields. Our review analyzes processes occurring during inner ear development and discusses developmental mechanisms that can only be understood in terms of interactions that are above the genome level. We discuss the effects that the altered mechanical (and other) cues, resulting from the absence of the skeletal musculature, have on the inner ear development, and aim to identify novel molecular players that regulate development of the inner ear sensory epithelia.

Inner and middle ear morphology and development

The mammalian inner ear consists of the cochlea and the vestibule, responsible for hearing and balance, respectively. There are six different sensory fields in the inner ear: three cristae ampullaris in the three semicircular canals sensitive to angular acceleration (rotation), and two maculae within the saccule and the utricle (macula sacculi and macula utriculi), sensitive to linear acceleration (gravity). The sixth sensory field is the organ of Corti within cochlea, sensitive to sound vibrations. The sensory epithelia contain mechano-sensory hair cells, whose role as mechanoreceptors is to detect mechanical stimulus, either as head movements, gravity or sound waves, and transduce it into neuronal signals carried via the vestibulocochlear nerve. In cochlea, the movement of endolymph fluid bends the stereocilia, the mechanosensory organelles of the hair cells, against the tectorial membrane. In vestibule, the endolymph within the three semicircular ducts (i.e., the kinetic labyrinth) deflects the gelatinous cupula against the hair cells of the crista ampullaris during angular acceleration. Within utricle and saccule (i.e., the static labyrinth) the stereocilia and kinocilium of the hair cells are embedded in the otolithic membrane. The hair cells are deflected by movements of otoconia, the small crystals of calcium carbonate.

In addition to hair cells, each sensory organ contains the supporting cells that space the hair cells in a precise pattern.

The development of vestibular organs in mice starts at embryonic day (E) 8 with the formation of the otic placode. The proliferation of hair cell precursors starts at E10.5 (Fritzsch et al., 2002; Beisel et al., 2005). The anterior and posterior semicircular canals appear at E12, and the differentiation of the neuroepithelium starts at E12-13. Peak hair cell mitosis in crista ampullaris and maculae of saccules and utricles appears between E13-17. Hair cells differentiate into type 1 and type 2 between E16 and E18 (Kawamata and Igarashi, 1993). First afferent and efferent nerve endings appear at E17 and E18, respectively. At E18.5 the maculae of the saccule and utricle, and the semicircular canals, have developed. The coiling of cochlea is finished at E18.5, but, although hair cells are present, the hearing organ has not yet matured, and the animal cannot hear. The onset of hearing is around postnatal day 10-12 (reviewed in Romand, 1983).

The inner ear does not contain skeletal muscle. There are only two small skeletal muscles in the middle ear, the tensor tympani and the stapedius, that attach to the middle ear ossicles malleus and stapes, respectively. The stapedius and tensor tympani dampen sounds and protect the inner ear from the damaging effects of very loud sounds. These two small skeletal muscles in the middle ear help to transfer the sound vibrations via the chain of three middle ear ossicles to the oscillations of the fluids within the inner ear.

Inner, middle and external ear morphology in *Myf5-/-:Myod-/-* embryos

The external auditory meatus is absent in compound-mutants, indicating malformation or a complete collapse of the distal ear canal, and the auricle is buried under the epithelium (Hong et al., 2015). The amyogenic mutants show fusion sites between cervical vertebrae and are unable to tilt their head, which, in addition to the complete absence of the voluntary musculature, prevents them from perceiving angular acceleration. The stapedius and tensor tympani muscles are absent which makes the chain of the three middle ear ossicles, responsible for the transfer of sound waves, rigid (Rot-Nikcevic et al., 2006). Our detailed study of the inner ear in *Myf5-/-:Myod-/-* mouse embryos revealed disturbances in the development of cristae ampullaris, the vestibular sensory fields sensitive to angular acceleration. There was a complete absence of the vestibular tenascin-positive type 1 hair cells in cristae. Maculae sacculi, sensitive to linear acceleration due to gravity, were affected to a much lesser degree - the hair cells appeared normal, and the only difference found was in the decreased size of supporting cells in the mutant embryos. The cochlear sensory field, the organ of Corti, was not affected (Rot and Kablar, 2010).

Since we discovered disturbances in the vestibular sensory fields of compound-mutant mouse embryos, our subsequent step, described in this review, was to perform

microarray analyses and compare gene expression between the mutant and normal inner ear sensory fields. Our goal was to identify molecular players responsible for the abnormal inner ear phenotype, as well as to discuss the epigenetic role of mechanical (and possibly other muscle-originating) cues in the development of the inner ear phenotype.

The role of mechanical cues in the inner ear development

The skeletal muscle does not exert any direct mechanical cues in the inner ear, such as static or dynamic loading, but allows for the acoustic mechanical cues to reach the inner ear cochlea. Indirectly, the presence or absence of the skeletal muscle can affect the mechanical cues that reach the vestibule as well. In the absence of skeletal muscle, as seen in the amyogenic compound-mutant mouse embryos (Rot and Kablar, 2010), the embryos are not able to turn their heads, and therefore the angular acceleration cues are at least decreased, if not absent.

Due to the fused cervical vertebrae and mutant embryos' inability to tilt their head, the angular acceleration cues (to which cristae ampullaris are sensitive) were altered, i.e., either decreased or fully absent; the linear acceleration cues and the perception of gravity (to which maculae are sensitive) were not altered, since the embryos, while *in utero*, still experience linear accelerations induced by the movements of the mother (Ronca et al., 1993). The mechanical, i.e., acoustic, cues that reach cochlea were present and probably enhanced, since the stapedius muscle, normally responsible for dampening the sounds, was absent. Thus the normal cochlear morphology and hair cell differentiation.

The exposure to altered gravity has been shown to affect vestibular sensory epithelia with variable consequences depending on the period of development (reviewed in Jamon, 2014). Rats raised in hypergravity and rotational environment from E9-19 showed increased sensory innervation of the vestibulo-cerebellar fibers in the utricle and the posterior semicircular canal. It was suggested that this accelerated development and maturation of the utricle and the semicircular canal following rotational and hypergravity exposure may be directed through increased stimulation of the hair cells (Bruce et al., 2006). The opposite, i.e., a decreased field of innervation, was found in rats raised in microgravity during the same developmental period (Bruce and Fritzsch, 1997; Bruce, 2003; Bruce et al., 2006). Similarly, the embryonic development of hamsters under hypergravity modified the otolithic crystals of their maculae utriculi and a greater area of otolith consisted of smaller otoconia (Sondag et al., 1996).

In addition, the experiments with hair cells exposed to microgravity *in vitro* showed modifications of microtubule organization and loss of the morphological phenotype of type 1 hair cells (Gaboyard et al., 2002). The pear-shaped type 1 hair cells are considered to be the true sensory receptors, while cylinder-shaped type 2 hair cells function as amplifiers. Type 1 hair cells receive predominantly afferent innervation, while type 2 hair cells are mostly innervated by efferent fibers (Spoendlin, 1970). Combined, the above listed findings suggest that prenatal exposure to altered gravity influences development of the vestibular sensory epithelia, and that linear and rotational acceleration cues may act as epigenetic factors.

Molecular regulation of the inner ear development

Studies of mutant mouse models that exhibit inner ear defects were instrumental in the discovery of genes with a role in the development of the inner ear sensory fields. The loss-of-function experiments also helped reveal genes that have separate or overlapping roles.

The regulation of the mechanosensory hair cell fate specification and their differentiation involves the Notch signaling and basic helix-loop-helix genes (reviewed in Zine, 2003). *Atoh1* is a basic helix-loop-helix (bHLH) transcription factor and is necessary, and sufficient, for hair cell differentiation (Bermingham et al., 1999; Fritzsch et al., 2002). It is expressed as early as E12.5 (Chen et al., 2002). *Atoh1* is a component of the Notch-signaling pathway, and is expressed in the developing inner ear together with *Notch1* and its ligands, *Delta1, Jagged1* and *Jagged2* (Lewis et al., 1998; Lanford et al., 1999; Zine et al., 2000). The bHLH genes *Hes1* and *Hes5* are also required for the normal development of mammalian hair cells. They act as negative regulators of hair cell differentiation in both cochlea and vestibule, probably through negative regulation of *Atoh1*, and are crucial for the production of the correct number of hair cells (Zheng and Gao, 2000; Zine et al., 2001). Retinoblastoma gene, *Rb*, and the encoded protein, pRb, are also expressed in the inner ear hair cells. pRb is required for keeping differentiating hair cells in a postmitotic state, and *Rb* inactivation results in the hyperplastic inner ear sensory epithelia due to excessive hair cell formation (Mantela et al., 2005).

The development of each sensory organ is coupled with the development of its non-sensory component, and the molecular interaction between the two components is crucial for their normal formation (Pirvola et al., 2000; Pauley et al., 2003). Sensory hair cells and non-sensory supporting cells arise from the common progenitor. The progenitor cells differentiate into hair cells by default, which is generally inhibited by Notch signaling (Fekete and Wu, 2002; Kelley, 2006; Yamamoto et al., 2006). Myosin plays a crucial role in the function of stereocilia. The differentiation of hair cells involves myosin-dependent synthesis of actin filaments to form stereocilia bundles. Myosin genes *Myo3a, Myo6, Myo7a* and *Myo15a* are expressed in the mouse inner ear only in hair cells, and have been shown to have a role in bundle organization (reviewed in Hertzano and Avraham, 2005).

Recent studies of another group of proteins, the

Usher proteins, such as *cadherin23, protocadherin 15, myosin VIIa, harmonin, sans,* and *whirlin*, showed that most of them are localized in the stereocilia of the hair cells, and are crucial for the function of the mechanosensory hair cells of the inner ear (Ahmed et al., 2013).

An ongoing study in our laboratory has revealed that some calcium-binding and other proteins, such as calbindin, tyrosine-hydroxylase and nestin, were significantly down-regulated in the compound-mutant maculae and the organ of Corti, whereas calretinin and acetylcholine esterase were unaffected in those locations and in the cristae (B. Kablar, unpublished data).

Finally, a large number of known signaling molecules, such as FGFs, Wnts, BMPs, sonic hedgehog and retinoids, are known to regulate the differentiation of the inner ear (reviewed in Groves and Fekete, 2012). Since the expression of, or response to, some of these secreted molecules appear to occur in gradients, it was suggested that they might function as classical morphogens (Groves and Fekete, 2012).

Materials and methods

Compound-mutant (*Myf5-/-:Myod-/-*) fetuses were obtained by the interbreeding of heterozygous (*Myf5+/-:Myod+/-*) parents, as previously described by Rudnicki et al. (1993). All fetuses were collected by Cesarean section at E18.5 and genotyped by PCR using *Myf5* and *Myod* primers (Inanlou and Kablar, 2005). In addition, the presence or absence of skeletal muscle was confirmed by myosin-fast immunostaining.

Systematic Subtractive Microarray Analysis

The Systematic Subtractive Microarray Analysis (SSMAA) approach (Baguma-Nibasheka and Kablar, 2009a,b) was used to identify a profile of genes with a role in development of the inner ear sensory fields. The petrous part of the temporal bone, containing middle and inner ear, was dissected out using a stereomicroscope (as described in Rot and Kablar, 2013 for other bones and in Bohne and Harding, 2011 for the ear). The total RNA was isolated from the middle and inner ear of two wild-type and two *Myf5-/-:Myod-/-* embryos using the RNeasy™ kit from Qiagen, Mississauga, Ont., Canada, according to manufacturer's instructions. For each group (wild-type or *Myf5-/-:Myod-/-*), RNA from the tissue of two embryos was pooled to eliminate the individual differences. Fluorescent labeling of the fragments obtained from the pooled samples and their simultaneous hybridization to MOE340 GeneChip mouse genome arrays was performed at the Ottawa Genome Centre according to standard Affymetrix (Santa Clara, CA) protocols as in Seale et al., 2004. The hybridized chips were scanned and the results analyzed using the Affymetrix statistical expression algorithms to obtain the gene expression ratios and fold changes between the wild-type and compound-mutant fetal inner ear at E18.5. Considering that only one microarray experiment was performed, we lack the ability to analyze the significance of the differential gene expression, but the data within the context of the story told in the current report are made reliable in a different way. First, the muscle-dependent ear phenotype was very specific (lack of type I tenascin-positive hair cells), clearly established previously and described separately (Rot and Kablar, 2010). Second, our laboratory performed and published identical microarray experiments with tissue samples from different anatomical locations, such as lung (Baguma-Nibasheka et al., 2012), palate (Rot and Kablar, 2013), mandible (Rot et al., 2014), back and limb muscle (Baguma-Nibasheka et al., 2016), and found 100% reliability of microarray data after the RT-PCR confirmation (Baguma-Nibasheka et al., 2006; 2007; 2016). Third, based on our previous experience, we relied on web-based mouse and human databases (N.B., hence the reason for classifying our reports as "Reviews") and confirmed the expression and distribution patterns, knock-out phenotypes and human disease relationships for a number of genes found by the microarray analysis, further confirming the reliability of the microarray data. Therefore, an arbitrary cut-off value for $\log_2$(ratio) of |3.0| (i.e., $-3.0 \leq \log_2(\text{ratio}) \geq 3.0$), which translates to ≥8-fold differences, was chosen as a means of determining the up- and down-regulated probesets. By using this very high fold difference in the current report another level of microarray data reliability was added. We hypothesized that the genes differentially expressed between the normal and mutant inner ears would play a role in the vestibular sensory fields development, and, therefore, that the difference in their gene expression would be related to the abnormal inner ear phenotype.

Microarray analysis identified a total of 109 probesets, corresponding to 95 named genes, which met the cut-off criterion. *Myod* and *Myf5* were added to the table as positive controls (differentially expressed probably due to the middle ear muscle), for a total of 97 named genes differentially expressed between the compound-mutant and the wild-type ear tissues. Ten of these genes were up-regulated in the compound-mutant embryos (see Table 1) and 87 were down-regulated (Table 2).

The lack of *Myf5* and *Myod* expression and distribution in the inner ear (Kablar et al., 1997) suggests that their function is not required for the inner ear development. Therefore, any resulting differences in phenotype are due to the absence of mechanical (and other muscle-originating) cues (in the absence of the skeletal muscle), and not to the absence of these two genes.

The subsequent step was to search for a mouse mutant of one of the differentially expressed genes showing the same inner ear phenotype as seen in fetuses without skeletal muscle. This was done through the bioinformatics approach.

Results

The bioinformatics approach (Bard, 1999, 2002a,b) involved a detailed consultation of the web-accessible mouse databases, such as Mouse Genome Informatics (MGI), (http://www.infomatics.jax.org). Our search was aimed to elucidate: 1) whether any of the listed genes have already been shown to be expressed and/or distributed in the inner ear, and 2) whether any of the listed genes have been shown to cause abnormal inner ear development as revealed by the knockout phenotype.

The bioinformatics search allowed us to identify a set of 6 genes with a possible role in inner ear development (Table 3). These genes were down-regulated in the mutant inner ear, and have been shown to be expressed in the mouse inner ear. Our subsequent step was to search for knockout mouse embryos for 6 candidate genes, and a possible presence of the mutant

Table 1. Genes up-regulated in the middle and inner ear of E18.5 Myf5-/-:Myod-/- mutant mouse embryos, with greater than 8-fold alteration in expression ($\log_2$(ratio)≥3), sorted by function and expression.

Gene	$\log_2$(ratio)	Gene name	Molecular function
Bpifa2	8.4	BPI fold containing family A, member 2	Lipid binding
Prol1	6.6	proline rich, lacrimal 1	Peptidase inhibition
BC048546	5.7	cDNA sequence BC048546	
Pip	6.4	prolactin induced protein	Protein binding
Car6	4.4	carbonic anhydrase 6	Catalytic activity
Lpo	4.2	lactoperoxidase	
Eif2s3y	3.1	eukaryotic translation initiation factor 2, subunit 3, structural gene Y-linked	
Muc19	3.2	mucin 19	Protein transport
Smgc	7.5	submandibular gland protein C	Not yet specified
Spt1	4.3	salivary protein 1	

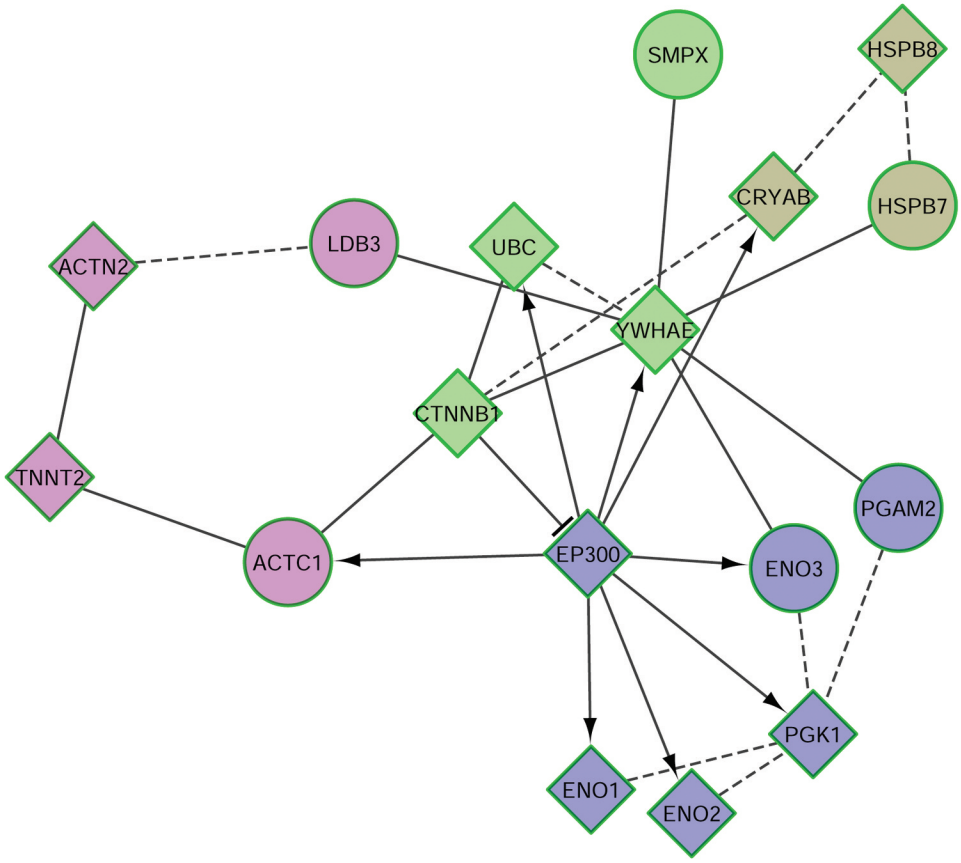

Fig. 1. Functional relationships ("internal") of the candidate molecules implicated in the mouse inner and middle ear development. Cytoscape 3.3.0 Reactome FI map generated from Myod, Myf5 (not shown), and the identified six (see the text and Table 3) candidates possibly linking skeletal muscle presence and the inner and middle ear development reveals the network of molecular interactions pictured. The nodules shown indicate a group of proteins primarily involved in: glycolysis and metabolism (blue; ENO3 and PGAM2), cellular signalling (green; SMPX), muscle contraction (purple; ACTC1 and LDB3), and molecular chaperone activity (olive; HSPB7). Note: EP300 is a highly-connected (1011 functional interactions) transcription co-activator that functions in regulating chromatin remodelling and is important in cellular proliferation and differentiation. CTNNB1 and YWHAE appear to be central to the function of this group of molecules.

Table 2. Genes down-regulated in the middle and inner ear of E18.5 Myf5-/-:Myod-/- mutant mouse embryos, with greater than 8-fold alteration in expression (log2(ratio)≤-3), sorted by function and expression.

Gene	log_2(ratio)	Gene name	Molecular function
Myh8	-6.7	myosin, heavy polypeptide 8, skeletal muscle, perinatal	Structural, Cytoskeletal, and Motor Activity
Myh3	-6.7	myosin, heavy polypeptide 3, skeletal muscle, embryonic	
Actc1	-6.7	actin, alpha, cardiac muscle 1	
Myot	-6.3	myotilin	
Acta1	-6.3	actin, alpha 1, skeletal muscle	
Myoz2	-6.2	myozenin 2	
Ttn	-5.8	titin	
Tnnt3	-5.8	troponin T3, skeletal, fast	
Neb	-5.7	nebulin	
Myoz1	-5.7	myozenin 1	
Myl1	-5.4	myosin, light polypeptide 1	
Tnni2	-5.1	troponin I, skeletal, fast 2	
Mylpf	-5.0	myosin light chain, phosphorylatable, fast skeletal muscle	
Des	-4.7	desmin	
Myh1	-4.6	myosin, heavy polypeptide 1, skeletal muscle, adult	
Myom2	-4.4	myomesin 2	
Myl4	-4.4	myosin, light polypeptide 4	
Pdlim3	-4.3	PDZ and LIM domain 3	
Ldb3	-4.3	LIM domain binding 3	
Tmod4	-4.1	tropomodulin 4	
Tnnc1	-4.0	troponin C, cardiac/slow skeletal	
Mypn	-4.0	myopalladin	
Mybpc1	-4.0	myosin binding protein C, slow-type	
Tpm2	-3.9	tropomyosin 2, beta	
Smpx	-3.9	small muscle protein, X-linked	
Actn3	-3.7	actinin alpha 3	
Mybpc2	-3.6	myosin binding protein C, fast-type	
Xirp2	-3.5	xin actin-binding repeat containing 2	
Myom1	-3.5	myomesin 1	
Myo18b	-3.5	myosin XVIIIb	
Lmod3	-3.5	leiomodin 3 (fetal)	
Myh7	-3.4	myosin, heavy polypeptide 7, cardiac muscle, beta	
Tmem8c	-3.4	transmembrane protein 8C	
Tnni1	-3.3	troponin I, skeletal, slow 1	
Myh2	-3.3	myosin, heavy polypeptide 2, skeletal muscle, adult	
Tnnt2	-3.2	troponin T2, cardiac	
Myl2	-3.2	myosin, light polypeptide 2, regulatory, cardiac, slow	
Lmod2	-3.2	leiomodin 2 (cardiac)	
Synpo2l	-3.1	synaptopodin 2-like	
Myom3	-3.1	myomesin family, member 3	
Nexn	-3.0	nexilin	
Klhl31	-4.5	kelch-like 31 (Drosophila)	Transcription Factor Activity
Myf6	-4.0	myogenic factor 6	
Myf5	-0.8	myogenic factor 5	
Myod	-0.7	myogenic differentiation	
Atp1b4	-7.2	ATPase, (Na+)/K+ transporting, beta 4 polypeptide	Receptor or Channel and Transport Activity
Atp2a1	-6.3	ATPase, Ca++ transporting, cardiac muscle, fast twitch 1	
Ryr1	-3.8	ryanodine receptor 1, skeletal muscle	
Sypl2	-3.7	synaptophysin-like 2	
Cacna1s	-3.5	calcium channel, voltage-dependent, L type, alpha 1S subunit	
Chrna1	-3.2	cholinergic receptor, nicotinic, alpha polypeptide 1 (muscle)	
Actn2	-5.9	actinin alpha 2	Signal Transduction Activity
Casq1	-5.6	calsequestrin 1	
Kbtbd10	-5.4	kelch repeat and BTB (POZ) domain containing 10	
Casq2	-5.4	calsequestrin 2	
Arhgap36	-5.1	Rho GTPase activating protein 36	
Asb12	-4.9	ankyrin repeat and SOCS box-containing 12	
Trdn	-4.7	triadin	
Hfe2	-4.1	hemochromatosis type 2 (juvenile) (human homolog)	
Smyd1	-3.4	SET and MYND domain containing 1	
Lipf	-5.6	lipase, gastric	Catalytic Activity
Ckm	-5.6	creatine kinase, muscle	
Ube2c	-5.5	ubiquitin-conjugating enzyme E2C /// troponin C2, fast	
Apobec2	-5.0	apolipoprotein B mRNA editing enzyme, catalytic polypeptide 2	
Pgam2	-4.9	phosphoglycerate mutase 2	
Pygm	-4.5	muscle glycogen phosphorylase	
Mylk4	-4.5	myosin light chain kinase family, member 4	
Eno3	-4.2	enolase 3, beta muscle	
Ckmt2	-4.1	creatine kinase, mitochondrial 2	
Ampd1	-4.0	adenosine monophosphate deaminase 1	
Ppp1r3a	-3.8	protein phosphatase 1, regulatory (inhibitor) subunit 3A	
Tecrl	-3.7	trans-2,3-enoyl-CoA reductase-like	
Habp2	-3.6	hyaluronic acid binding protein 2	
Srl	-3.4	sarcalumenin	
Mettl21e	-3.4	methyltransferase like 21E	
Cox6a2	-3.1	cytochrome c oxidase, subunit VI a, polypeptide 2	
Fitm1	-4.2	fat storage-inducing transmembrane protein 1	Other Metabolic and Housekeeping Activity
Hspb7	-4.1	heat shock protein family, member 7 (cardiovascular)	
Sacs	-3.8	sacsin	
Itgb1bp2	-3.7	integrin beta 1 binding protein 2	
Rps13	-3.6	ribosomal protein S13	
Csrp3	-3.5	cysteine and glycine-rich protein 3	
Mustn1	-3.4	musculoskeletal, embryonic nuclear protein 1	
Gh	-3.3	growth hormone	
Hspb1	-3.0	heat shock protein 1	
Fsd2	-3.9	fibronectin type III and SPRY domain containing 2	Not yet specified
Tmem182	-3.4	transmembrane protein 182	

inner ear phenotype. For three of our candidate genes, *Actc1*, *Ldb3* (or cypher) and *Smpx* (or Chisel), knockout mice have been generated. The research studies with *Actc1* null mice did not include inner ear investigation (J. L. Lessard, Department of Pediatrics, Cincinnati Children's Hospital Medical Center, Cincinnati, U.S.A.; M. Buckingham, Department of Developmental Biology, Pasteur Institute, Paris, France; personal communications). Similarly, the studies with *Smpx* knockout mice did not look at inner ear development, but it was noted that the Smpx null mice did not show the rotatory behavior that would indicate possible deafness (R. P. Harvey, The Victor Chang Cardiac Research Institute, Darlinghurst, Australia; personal communication). In the knockout mouse embryos for the third of our candidate genes, *Ldb3* (cypher), the inner ear has been analyzed. The study only looked at the cochlea and revealed that *Ldb3* was expressed in the cochlear hair cells in the organ of Corti. However, when the *Ldb3* gene was knocked out, the hair cells in the organ of Corti were not affected. These findings indicate that *Ldb3* has a role in hair cell development (hence the expression) but is not essential for it. Unfortunately, the vestibule and its sensory fields have not been investigated in these *Ldb3* knockout embryos, and the material is no longer available (J. Chen, Department of Medicine, University of California San Diego, La Jolla, California, U.S.A. and X. Liang, School of Medicine, Tongji University, Shanghai, China; personal communication).

We searched whether any of our 6 candidate genes have homologues in humans, and human diseases associated with them. This inquiry provided an interesting discovery: *SMPX*, the human homologue of our candidate gene *Smpx*, has been associated with hearing loss. The study of a German population with a non-syndromic hearing loss (Huebner et al., 2011) detected a mutation in the small muscle protein, X-linked (*SMPX*) of affected individuals. Similarly, a study of Dutch families with a hearing loss (Schraders et al., 2011) also identified mutations in *SMPX*.

We have also identified the up- and down-regulated genes (Tables 4, 5) in the compound-mutant mouse inner ear for which knockout mouse models have been previously generated but without any reports regarding abnormal inner ear morphology. Future analyses of these knockouts would establish a role of each molecule in ear development. If future analyses of their knockouts that specifically look at the inner ear morphology confirm

Table 3. Genes down-regulated in the middle and inner ear of E18.5 Myf5-/-:Myod-/- mutant mouse embryos, with greater than 8-fold alteration in expression (log2(ratio)≤-3), with a potential role in inner and/or middle ear development, sorted by expression.

Gene	Gene name	$\log_2$(ratio)	Molecular function	Gene expression	Knockout phenotypes
Actc1	actin, alpha, cardiac muscle 1	-6.7	APTase activity, ATP binding, myosin binding	sensory organs in inner ear, cochlea, labyrinth, middle ear (E14.5) (Visel et al., 2004)	Embryonic and postnatal lethality; reduced body size and heart muscle defects (Kumar et al., 1997). No ear phenotype reported.
Pgam2	Phosphoglycerate mutase 2	-4.9	cofactor binding, phosphoglycerate mutase activity	sensory organs in inner ear, cochlea, labyrinth, middle ear (E14.5) (Visel et al., 2004)	Not reported
Ldb3	LIM domain binding 3	-4.3	cytoskeletal protein binding, muscle alpha-actinin binding	sensory organs in inner ear, cochlea, labyrinth (E14.5) (Visel et al., 2004)	Mutants die within few days after birth from muscle abnormalities; exhibit myopathy, dysphagia, heart vascular congestion, cyanosis, respiratory distress (Zhou et al., 2001). No ear phenotype reported.
Eno3	enolase 3, beta muscle	-4.2	protein heterodimerization activity, glycolytic process, lyase activity	sensory organs in inner ear, cochlea, labyrinth, middle ear (E14.5) (Visel et al., 2004)	Not reported
Hspb7	heat shock protein family, member 7 (cardiovascular)	-4.1	filamin binding ubiquitin binding	sensory organs in inner ear, cochlea, labyrinth, middle ear (E14.5) (Visel et al., 2004)	Not reported
Smpx	small muscle protein, X-linked	-3.9	contractile fiber, costamere, muscle tendon junction	sensory organs in inner ear, labyrinth (E14.5) (Visel et al., 2004)	Null mice do not exhibit defects in heart or skeletal muscle morphology or development (Palmer et al., 2001). No ear phenotype reported.

Table 4. Genes up-regulated in the middle and inner ear of E18.5 *Myf5-/-:Myod-/-* mutant mouse embryos, with greater than 8-fold alteration in expression (log2(ratio)≥3), with knockout mouse models.

Gene	Comments on deletion mutants
Pip	Enlarged submandibular lymph nodes, enlarged medulla of the thymus, and abnormal prostate gland dorsolateral lobe morphology (Blanchard et al., 2009).
Car6	Greater number of lymphoid follicles in the small intestinal Peyer's patches (Pan et al., 2011).
Muc19	Sublingual gland mucous cell differentiation arrest (Hayashi et al., 1988).

Table 5. Genes down-regulated in the middle and inner ear of E18.5 Myf5-/-:Myod-/- mutant mouse embryos, with greater than 8-fold alteration in expression (log2(ratio)≤-3), with knockout mouse models.

Gene	Comments on deletion mutants
Actc1	Embryonic lethality; survivors to birth die within the first 2 weeks and display reduced body size, with heart muscle defects (Kumar et al., 1997).
Myot	Normal lifespan and fertility, and no abnormal phenotype detected (Moza et al., 2007).
Atp2a1	Respiratory distress, progressive cyanosis, and death within 2 hours after birth, the lung tissues and diaphragm muscle showing aberrant morphology (Pan et al., 2003).
Acta1	Scoliosis, reduced body weight/size, atrophy of brown adipose tissue, depleted glycogen stores, muscle weakness, and death by postnatal day 10 (Crawford et al., 2002).
Myoz2	Excess of skeletal muscle fibers; cardiac hypertrophy when chronically stressed (Frey et al., 2004).
Ttn	Embryogenesis defects; vascular, cardiac and skeletal muscle defects causing growth retardation, muscle weakness, abnormal posture, and death between embryonic day 11.5 and 8 weeks of age (Lane, 1985; Weinert et al., 2006).
Tnnt3	Neonatal lethality, decreased fetal weight, liver and kidney hemorrhage and thin diaphragm, growth retardation with mild skeleton defects (Ju et al., 2013).
Neb	Growth retardation, kyphosis, abnormal/stiff gait, progressive muscle weakness, and death within 3 weeks (Witt et al., 2006).
Myoz1	Reduced body weight and fast-twitch muscle mass; enhanced muscle regeneration after cardiotoxin injury (Frey et al., 2008).
Ckm	Abnormal function and energy utilization of skeletal and cardiac muscle (Van Deursen et al., 1993).
Casq1	Structural alterations of the Ca2+ release units, an increased number of mitochondria, and impaired calcium handling in skeletal muscle (Paolini et al., 2007).
Myl1	Ataxia, impaired locomotor coordination, decreased muscle spindle numbers (Hardy et al., 2007).
Casq2	Impaired calcium regulation in cardiac myocytes, leading to an arrhythmogenic syndrome called catecholaminergic polymorphic ventricular tachycardia (Song et al., 2007).
Mylpf	Completely lacking skeletal muscle, all die immediately after birth, presumably due to respiratory failure (Wang et al., 2007).
Apobec2	Growth retardation; myopathy with increased proportion of slow muscle fibers (Sato et al., 2010).
Trdn	Viable and fertile but with impaired skeletal muscle function (Oddoux et al., 2009).
Des	Defects of cardiac, skeletal, and smooth muscle; calcification, progressive degeneration, and necrosis of the myocardium (Weisleder et al., 2004).
Myh1	Kyphosis, reduced growth, muscular weakness, and abnormal kinetics of muscle contraction and relaxation (Acakpo-Satchivi et al, 1997).
Myom2	Reduced growth, muscular weakness, kyphosis, and abnormal kinetics of muscle contraction and relaxation (Sartorius et al., 1998).
Pdlim3	Partial prenatal lethality; ventricular dilation and dysplasia, hypotrabeculation, and cardiomyopathy in surviving adults (Pashmforoush et al., 2001).
Ldb3	Myopathy, dysphagia, heart vascular congestion, dilated heart ventricles, cyanosis, respiratory distress, and death within a few days after birth (Zhou et al., 2001).
Hfe2	Decreased hepcidin expression, severe iron overload, and male sterility (Niederkofler et al., 2005).
Ckmt2	Decreased body weight, hypertrophic dilated left ventricles, impaired skeletal muscle contractility (Nahrendorf et al., 2005).
Myf6	Abnormal rib and muscle morphology, death from respiratory failure within minutes (Rawls et al., 1998).
Smpx	No apparent defects in heart or skeletal muscle morphology or development (Palmer et al., 2001).
Ryr1	Skeletal abnormalities, fragmented muscle fibers, and perinatal death from respiratory failure (Takeshima et al., 1994).
Ppp1r3a	Obesity, glucose intolerance, insulin resistance, and reduced levels of skeletal muscle glycogen (Delibegovic et al., 2003).
Sypl2	Viable, fertile, and with normal motor coordination, but exhibit reduced body weight, abnormal skeletal muscle membranes and irregular skeletal muscle contractility (Nishi et al., 1999).
Itgb1bp2	Cardiac contractile dysfunction and dilated cardiomyopathy under pressure overload (Brancaccio et al., 2003).
Actn3	Increased mitochondria density and a shift from anaerobic to aerobic metabolism in fast muscle fiber (Macarthur et al., 2007).
Habp2	Decreased lethality but increased liver fibrosis, inflammation and injury following bile duct ligation (Borkham-Kamphorst et al., 2013).
Xirp2	Abnormal heart shape, ventricular septal defects, a failure of mature intercalated disc formation, severe growth retardation, and postnatal lethality (Wang et al., 2010).
Myo18b	Embryonic lethality during organogenesis, with internal hemorrhage, pericardial effusion, enlargement of the right atrium, and cardiac myofibril abnormalities (Ajima et al., 2008).
Csrp3	Heart ventricle dilation, hypertrophy and fibrosis, decreased contractility, and premature death (Arber et al., 1997).
Cacna1s	Failure of myoblast differentiation by embryonic day 13, skeletal anomalies, shortened head and jaw, cleft palate and perinatal death (Pai, 1965).
Tmem8c	Early postnatal lethality, paralysis, kyphosis and defective myoblast fusion and survival leading to the absence of differentiated muscle in the trunk, limb and head (Millay et al., 2013).
Srl	Impaired calcium store functions in skeletal and cardiac muscle cells, resulting in slow contraction and relaxation phases (Yoshida et al., 2005).
Smyd1	Enlarged heart, and developmental abnormalities of the right ventricle; embryonic death at day 10.5 (Gottlieb et al., 2002).
Gh	Dwarfism, increased percentage of body fat, elevated plasma ghrelin levels, pituitary hypoplasia, small liver, delayed sexual maturation, and reduced fertility (Meyer et al., 2004).
Tnnt2	Abnormal heart development, cardiomyopathy, and embryonic lethality during and prior to organogenesis (Ahmad et al., 2008).
Chrna1	Neonatal lethality, kyphosis, carpoptosis, abnormal endplate potential, increased motor neuron number, and abnormal neuromuscular synapse morphology (An et al., 2010).
Cox6a2	Cardiac dysfunction due to abnormal ventricular filling or diastolic dysfunction under maximal cardiac load (Radford et al., 2002).
Hspb1	Viable and fertile with no obvious abnormalities (Huang et al., 2007).
Myf5	Delayed appearance of myotomal cells in somites, impaired rib development, inability to breathe, and lethality at birth (Braun et al., 1992).
Myod	Normal muscle development, viable (Rudnicki et al., 1992).

normal ear phenotype, it would mean that these genes have no function in ear development, or that they are redundant, and/or a part of the secondary pathway (Davis, 1999; Bard, 2010).

Lastly, we searched mouse databases for genes involved in specific phenotypes under the umbrella term "abnormal inner ear morphology". This included phenotypes such as inner ear hypoplasia, abnormal inner ear development, abnormal hair cell morphology and abnormal hair cell number. The search turned out a total of 682 genotypes, none of which was found in our list of up- and down-regulated genes in the mutant mouse inner ear.

An examination of gene interactions (using the Reactome FI plugin in Cytoscape 3.3.0.) showed functional relationships of the candidate molecules with other molecular players (see Figs. 1, 2). For example, a review of various databases revealed that five of the six candidate molecules interact with YWHAE directly, and one of them indirectly. YWHAE is a member of the 14-3-3 family of proteins that mediate signal transduction by binding to phosphoserine-containing proteins.

Discussion

The inner ear abnormalities identified in the amyogenic mouse embryos were only present in the vestibule and not in the cochlea. Within the vestibule, the areas sensitive to angular acceleration, the cristae ampullaris in the semicircular canals, were most

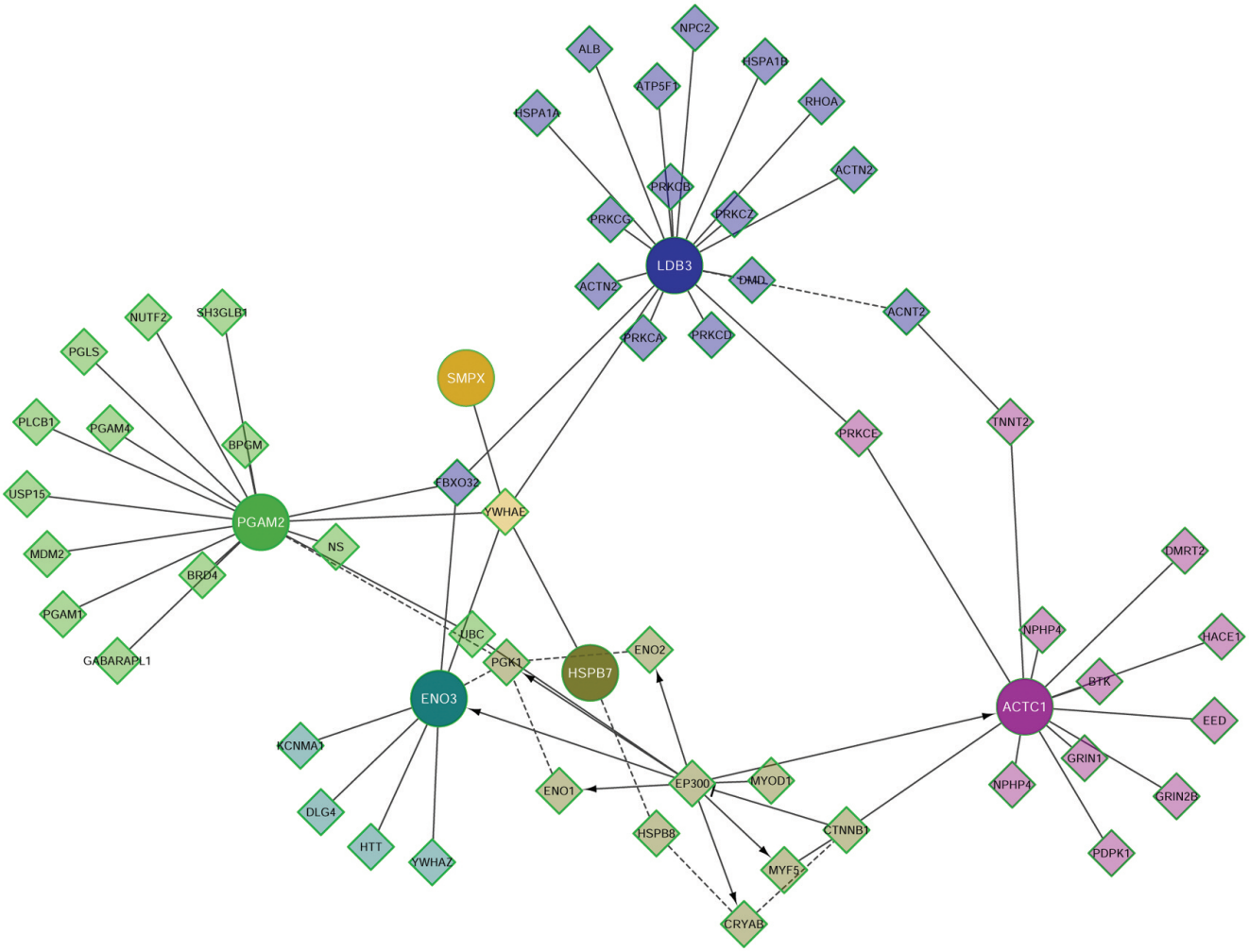

Fig. 2. Functional relationships ("external") of the candidate molecules implicated with other molecular players. By querying various databases it is possible to identify an interaction with YWHAE, which turns out to be highly connected to many of the other candidate molecules in Fig. 1. Merging protein-protein interaction information with the functional interaction graph (i.e., the Fig. 1) it is possible to produce the current figure which clusters a number of participating molecules around the six candidate molecules, and links YWHAE to five molecules directly (LDB3, SMPX, PGAM2, ENO3, HSPB7) and to one molecule (ACTC1) indirectly (i.e., via the olive nodule, molecular chaperone activity).

affected. These altered morphologies might be due to the changes in the mechanical cues that reach different sensory fields. Unfortunately, in the *in vivo* model system it is not possible to relate the causes and consequences with great certainty. In the case of cochlea, the mechanical (acoustic) stimulus was present, and probably enhanced, even though the mouse fetuses do not hear at this developmental stage. The gravitational force, or the linear acceleration, to which utricle and saccule in the static labyrinth are sensitive, was probably unchanged. Only the angular acceleration, to which the kinetic labyrinth is sensitive, was altered. Within cristae ampullaris in the semicircular canals, both hair cells and supporting cells were significantly smaller in compound-mutant embryos, and mutant cristae ampullaris completely lacked the tenascin-positive type 1 hair cells. Therefore, mechanical stimulus appears to be crucial for normal differentiation of the sensory epithelia, and in the absence of mechanical stimuli certain groups of hair cells fail to differentiate. The discovery that one hair cell type I (tenascin-positive) failed to differentiate in the mutant cristae ampullaris prompted the search for a profile of genes associated with the missing cell type. A set of 6 candidate genes with a possible role in the mouse inner ear development was identified. Further analyses of their knockouts is needed to confirm, or rule out, their role in hair cell differentiation. In our recent publication (Baguma-Nibasheka et al., 2016) we propose a number or ways of using the data and we mention various databases, software, and systems biology approaches.

Our search of mouse databases revealed a large number of genes associated with abnormal inner ear morphologies, but, interestingly, none of the genes up- or down-regulated in the compound-mutant inner ear has been previously linked to inner ear abnormalities. This is an indication that SSMAA is an appropriate and valuable approach to identifying the molecules that regulate the inner ear development.

Mouse-to-human translation and future directions

Previously we proposed several approaches to mouse-to-human translation (Baguma-Nibasheka et al., 2012; Rot and Kablar, 2013; Rot et al., 2014; Baguma-Nibasheka et al., 2016). Here we give a concrete example. *SMPX*, the human homologue of our candidate gene *Smpx*, has been associated with hearing loss. Small muscle protein, X-linked (*SMPX*) mutations have been reported in humans with deafness (Huebner et al., 2011; Schraders et al., 2011). Although *SMPX* has been shown to be highly expressed in muscle cells, the patients did not show signs of muscular dysfunction (Huebner et al., 2011). The vestibular function was also normal (Huebner et al., 2011). Similarly, in mice, SMPX (previously called Chisel, Csl) protein has been detected in all skeletal muscles (Palmer et al., 2001; Kemp et al., 2001), but the targeted disruption of *Smpx* showed no overt developmental phenotype in skeletal muscle, suggesting a genetic or functional redundancy (Palmer et al., 2001). Breeding of the *Smpx* knockout mouse line has been discontinued (R.P. Harvey, personal communication) and it was not possible to obtain knockout embryos for further analyses of the mouse inner ear.

In their interesting study approach, Yoon et al. (2011) searched for genes that were expressed in the embryonic mouse inner ear tissue in a similar pattern as *Atoh1*, a well-known factor required for hair cell differentiation. They discovered that *Smpx* showed the highest fold change in both saccule and utricle during inner ear development, and was specifically expressed in the hair cells of the vestibular organs, including saccule, utricle, and all three cristae at E15.5. *Smpx* expression was demonstrated in the cochlear hair cells at later stages, as well as in other cell types (e.g., Böttcher cells, pillar cells, root cells) in the mouse cochlea (Huebner et al., 2011; Yoon et al., 2011). This is consistent with the fact that the differentiation of vestibular hair cells occurs earlier than that of cochlear hair cells (Chen et al., 2002; Lumpkin et al., 2003).

Smpx encodes a protein associated with actin (Kemp et al., 2001) and is a regulator of cytoskeletal dynamics (Schindeler et al., 2005). Many genes associated with deafness encode actin or actin-binding proteins, motor proteins of the myosin family, or proteins associated with cytoskeleton (Petit and Richardson, 2009; Dror and Avraham, 2009). *Smpx* expression in skeletal muscle was observed to be highly up-regulated in response to passive stretch *in vivo* (Kemp et al., 2001). Huebner et al. (2011) argued that the fact that SMPX is associated with cytoskeleton and is responsive to mechanical force, combined with the detection of *Smpx* in the mouse hair cells, could indicate that SMPX plays a role in the maintenance of stereocilia that are permanently exposed to physical forces. They also suggested that the stress response of mechanically challenged inner ear cells might critically depend on SMPX function. It has been hypothesized (Schraders et al., 2011) that *Smpx* functions in the development and/or maintenance of the sensory hair cells based on: 1) its association with costameres (Palmer et al., 2001), i.e., the protein complexes that tether molecules which control mechanoreception and cytoskeletal remodeling, and 2) its links with integrin signaling (Schindeler et al., 2005) which are essential for normal hair-bundle development. The above listed findings establish Smpx as one of the molecular players with a crucial role in inner ear development.

More than sixty protein-coding genes have been linked to hereditary hearing loss in humans (reviewed in Friedman et al., 2007) and most are single mutations in a single gene. However, 75% of the genes that have been linked with inner ear dysfunction in mice have not been linked to hereditary hearing loss in humans yet (Van Camp and Smith, 2000). The International Mouse Phenotyping Consortium's (http://www.mousephenotype.org/) ongoing project intends to create a

comprehensive catalogue of the mouse genome, and produce knockouts for every gene in the mouse genome. This will allow us to generate mouse models of human diseases and identify genes that are critical for inner ear development, as well as mutations in specific genes that result in human ear pathologies.

Acknowledgements. We thank Heather E. Angka for her expertise with the experimental procedures and Dr. Bruce Greenfield for critical reading of the manuscript. This work was funded by an operating grant from NSERC and two infrastructure grants from Canada Foundation for Innovation (CFI) and Dalhousie Medical Research Foundation (DMRF) to BK.

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Accepted March 8, 2017