RNA Microarray Analysis in Prenatal Mouse Cochlea Reveals Novel IGF-I Target Genes: Implication of MEF2 and FOXM1 Transcription Factors

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Abstract

Background: Insulin-like growth factor-I (IGF-I) provides pivotal cell survival and differentiation signals during inner ear development throughout evolution. Homozygous mutations of human IGF1 cause syndromic sensorineural deafness, decreased intrauterine and postnatal growth rates, and mental retardation. In the mouse, deficits in IGF-I result in profound hearing loss associated with reduced survival, differentiation and maturation of auditory neurons. Nevertheless, little is known about the molecular basis of IGF-I activity in hearing and deafness.

Methodology/Principal Findings: A combination of quantitative RT-PCR, subcellular fractionation and Western blotting, along with in situ hybridization studies show IGF-I and its high affinity receptor to be strongly expressed in the embryonic and postnatal mouse cochlea. The expression of both proteins decreases after birth and in the cochlea of E18.5 embryonic Igf1−/− null mice, the balance of the main IGF related signalling pathways is altered, with lower activation of Akt and ERK1/2 and stronger activation of p38 kinase. By comparing the IGF1−/− and IGF1+/+ transcriptomes in E18.5 mouse cochlea using RNA microchips and validating their results, we demonstrate the up-regulation of the FoxM1 transcription factor and the misexpression of the neural progenitor transcription factors Six6 and Mash1 associated with the loss of IGF-I. Parallel, in silico promoter analysis of the genes modulated in conjunction with the loss of IGF-I revealed the possible involvement of MEF2 in cochlear development. E18.5 IGF1−/+ mouse auditory ganglion neurons showed intense MEF2A and MEF2D nuclear staining and MEF2A was also evident in the organ of Corti. At P15, MEF2A and MEF2D expression were shown in neurons and sensory cells. In the absence of IGF-I, nuclear levels of MEF2 were diminished, indicating less transcriptional MEF2 activity. By contrast, there was an increase in the nuclear accumulation of FoxM1 and a corresponding decrease in the nuclear cyclin-dependent kinase inhibitor p27Kip1.

Conclusions/Significance: We have defined the spatiotemporal expression of elements involved in IGF signalling during inner ear development and reveal novel regulatory mechanisms that are modulated by IGF-I in promoting sensory cell and neural survival and differentiation. These data will help us to understand the molecular bases of human sensorineural deafness associated to deficits in IGF-I.

Introduction

Insulin-like growth factor I (IGF-I) is a member of the insulin family that regulates development and tissue homeostasis [1,2]. It acts primarily by binding with high affinity to the IGF-I tyrosine kinase receptor (IGF1R) and its activity is modulated by IGF-binding proteins (IGFBP) [3]. IGF factors, receptors and binding proteins form the IGF system. Peak expression of IGF-I in the nervous system occurs during late embryonic and neonatal periods, although relatively high expression is maintained in areas of high plasticity, such as the olfactory bulb and hippocampus [4]. Mutations in mice have shown that IGF-I modulates survival, proliferation and differentiation of all the neural lineages studied, and it promotes synaptogenesis and dendritic arborisation in projection neurons [4,5]. Activation of the IGF1R leads to phosphorylation of insulin-receptor-substrates and activation of the cytosolic serine-threonine
MAP kinases and Akt kinases that induce the translocation of transcription factors to the cell nucleus, thereby initiating specific gene expression programmes [6,7]. Deficits in IGF-I are associated with severe nervous system disorders, including neurodegenerative diseases, and treatment with IGF-I promotes neural cell repair and regeneration [8]. Homozygous mutations in human *IGF1* result in a wide range of disorders including intrauterine growth retardation, postnatal growth failure, microcephaly and mental retardation. They also cause severe bilateral sensorineural deafness [ORPHA73272; http://www.orpha.net; [9,10,11]].

Normal development of the inner ear depends on IGF-I signalling [12]. The auditory sensory epithelium is the organ of Corti, which is composed of linear rows of hair cells and supporting cells housed within the cochlea. The mouse inner ear develops from embryonic day E18 from the otic placode, a patch of ectoderm that invaginates and pinches off to form the otic vesicle from which all the sensory epithelial cells and sensory neurons are derived. By E15.5, the organ of Corti has acquired its full complement of cell types although it does not become functionally mature until the onset of hearing at postnatal day 12–14 [13]. *Igf1* null (*Igf1*−/−) mice are dwarfs that present organ-specific growth retardation and a 30% reduction in brain size. The impact on the nervous system includes loss of selective neuronal populations, hypomyelination and reduced peripheral conduction velocities [14,15]. As in man, IGF-I deficit in the mouse causes all-frequency bilateral sensorineural hearing loss and a delayed response to acoustic stimuli [16]. From postnatal day P5 cochlear development is severely impaired in mutant *Igf1*−/− mice, which develop a smaller cochlea with an immotile tectorial membrane. In addition, these animals suffer aberrant synaptogenesis, abnormal innervation of the sensory hair cells in the organ of Corti, poor myelination and a significant decrease in the number and size of auditory neurons [17,18]. The marked reduction reported in neural cell number at P20 is due to increased apoptotic cell death of both neurons and Schwann cells [17].

Here, we have explored the otic-specific targets of IGF-I signalling to further understand the function of this factor in the inner ear and how its deficit causes neurosensorial deafness. Comparative gene expression profiles from the cochlea of wild-type (*Igf1*+/+) and *Igf1*−/− mice at embryonic day E18.5 suggest that IGF-I modulates sensory cell differentiation and neural cell fate decisions during late otic development. The expression patterns of *Six6*, *Mshh* and *Igf1* are altered in the cochleae of *Igf1*−/− mice. Changes also occur in the expression, protein levels and nuclear localization of *FoxM1*, a forkhead box transcription factor that is ubiquitously expressed in proliferating cells and one of its targets the cyclin-dependent kinase inhibitor p27Kip. In *silico* analysis of the promoter regions of differentially expressed genes selected from the microarray analysis of null versus wild type cochleae at E18.5, pointed to the transcription factor myocyte enhancing factor 2 (MEF2) as a novel downstream target of cochlear IGF-I signalling. The nuclear expression of MEF2A and D was lower in the absence of IGF-I. Thus, for the first time we show that MEF2 and FoxM1 activities are modulated by IGF-I in the mouse cochlea. These results also provide novel clues to the molecular mechanisms underlying otic development and the causes of neurosensorial deafness associated with defects in IGF-I.

**Materials and Methods**

**Mouse Handling and Genotyping**

Heterozygous mice in which the *Igf1* gene underwent targeted disruption were bred, maintained and genotyped as described [14,17]. In brief, E18.5 *Igf1*−/− embryos on a C57BL/6J genetic background, which die at birth, were used for the DNA array study, whereas a hybrid MF1/129sv genetic background was used to increase survival in the resultant null mice as described [14,17]. Adult survival was around 20% of the newborn null *Igf1*−/− mice.

Both mouse strains showed similar cochlear gene and protein expression profiles when tested at E15.5 and E18.5. Mouse genotypes were identified using the REDExtract-N-Amp™Tissue PCR Kit (XNAT, Sigma) following the manufacturer’s instructions and with the following primer sets specific for the *Igf1* and neomycin genes (*Igf1* forward 5′-GTCGAAAGCCCACTTCTGGT-3′; *Igf1* reverse 5′-GAGCTATTCCTACCCATCTTG-3′; neomycin forward 5′-GCTTGTTGGAAGGCTTAT-3′; and neomycin reverse 5′-CCAGGTCCTTCAGCAATATCGAGG-3′). Hearing was tested in adult animals by recording auditory brainstem responses as described [16] (results not shown). Animals were humanely sacrificed and all procedures were in accordance with the European Council Directive (86/609/EEC) and the Bioethics Committee of the CSIC.

**Transcriptome Analysis by GeneChip Arrays**

E18.5 was selected because most *Igf1*−/− mice die in early postnatal development. E18.5 cochleae from two *Igf1*−/− and two *Igf1*+/+ embryos were dissected and pooled to obtain RNA. Three independent RNA pools of each genotype (6 mice) were isolated with Trizol (Invitrogen) following the manufacturer’s instructions. The purity of RNA was assessed with an Agilent Bioanalyzer 2100 (Agilent Technologies). Six additional microarrays were hybridized with whole lung RNA obtained from the same mice or their siblings and were included in the analysis to compare the expression profiles of different organs (GSE17157; JGP manuscript in preparation).

Cochlear complementary RNA (cRNA) for hybridization to MOE430A Genechips® (Affymetrix) was prepared by sequentially generating cDNA with the one-Cycle cDNA Synthesis Kit, which was purified and used as a template in the in vitro transcription reaction for cRNA amplification and biotin labelling. The cRNA was then hybridized to the GeneChip® arrays and scanned with a GeneChip® Scanner 3000 7G 4C (Affymetrix).

An initial analysis was performed with MAS5.0 (Affymetrix) and Robust Multiarray Average (RMA) software [19], which indicated a very high variability that was associated with the biological variability and non-specific hybridization. Because most of the tissue-specific genes are expressed at low levels at this developmental stage, the high background signal of common genes generated a very high noise-to-signal ratio. The software package PUMA (Propagating Uncertainty in Microarray Analysis) was then used to estimate the gene expression levels. This package, integrated in the Bioconductor project [http://www.bioconductor.org], uses novel probabilistic models to analyse affymetrix GeneChip array data. Specifically, the multi-mgMOS (multi-chip modified gamma model for oligonucleotide signal) model was used to extract gene expression levels and their estimated uncertainties [20]. The analysis of the Fold change (FC) was used in combination with the Probability of Positive Log Ratio (PPLR) algorithm from the PUMA package, to reduce the number of false positives [21]. PPLR associates probability values (between 0 and 1) to each log ratio, which represents the probability of the log Ratio being positive. This probability is a measure of the false positive detection of differential expression and it allows the selected Differentially Expressed (DE) genes to be ranked in order of the robustness of the prediction. We defined DE genes in *Igf1*−/− mice as those that presented a positive FC greater than one log 2 unit with an associated probability higher than 0.95. Conversely, in *Igf1*+/+
mice we defined DE genes as those that presented a negative FC less than -1 with an associated probability lower than 0.05.

Genes were classified by their ontology and by the biological processes in which they are implicated using the PANTHER Classifications Systems software (http://www.pantherdb.org) and FATIGO+ (http://babelomics.bioinfo.cipf.es/).

Transcription Factor Analysis

The promoter regions of up-regulated genes in the Igf1−/− mouse cochlea selected either by their DE ranking and/or their association with sensorial deficits in humans (H1SSna, Pgf15, Foxx1, Six6, Rho, Rp11 and Ush1c), were compared using the MEME software (http://meme.sdsc.edu/meme/intro.html) to identify common motifs. The promoter regions were selected using the Promoter database (http://biowww.bu.edu/zhb/promo/promoSet/) extracting 1.5 Kb upstream and 50 bp downstream of the transcription start site (TSS). Only motifs with a pair-wise correlation lower than 0.30 were selected and the selected motifs were searched for known transcription factor binding sites using TESS (http://www.chil.upenn.edu/cgi-bin/tess/). Only the transcription factors sites with the highest Log-likelihood score were selected. Similarly, down-regulated genes with a FC>1 were grouped and the common motifs in the promoter region were analysed using either the method indicated above or the FATIGO+ software with similar results.

Low Density Arrays and Quantitative RT-PCR

TaqMan® Low Density Arrays containing three replica probes for each of the twenty genes selected from the array data were hybridized with cDNA generated by reverse transcription (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems). cDNA was prepared from three to five different RNA pools corresponding to six to ten mice for each genotype. Each RNA pool was isolated as described above from the pooled cochleae from two embryos or mice for each genotype taken at the following times: E15.5, E18.5, P5, P15, P30, P60 and P90. PCR was performed on an Applied Biosystems 7900HT Fast Real-Time PCR System and the genes were selected on the basis of their FC, physiological interest and the availability of appropriate TaqMan® probes. In addition, probes to test the temporal expression of the IGF system factors, receptors and transport proteins were used. Euksaryotic 18S rRNA was chosen as an endogenous housekeeping control gene and the estimated gene expression was calculated as 2−ΔΔCT, multiplying this value by a factor of 106 to generate a clearer graphical representation. Alternatively, gene expression was analyzed by real time PCR using validated probes from TaqMan® Gene Expression Assays (https://products.appliedbiosystems.com/ab/en/US/adirect/ab/cmdl=ABGExKeywordSearch; Applied Biosystems). Probes used are listed in Table S1 and included those for Igf2, Ih2, Foxm1, Foxg1, Mash1, Mef2a, Mef2c and Mef2d. Assays were done following manufacturer’s instructions and using as reference the expression levels of 18S. The relative quantification values (RQ) were calculated by the 2−ΔΔCT method and data are presented as means of log16RQ.

In Situ Hybridization

In situ hybridization was performed essentially as described previously [22], with minor modifications. The cDNA used to generate the in situ hybridization probes are detailed in Table S2. Three E15.5, E18.5 and P5 mice per genotype were tested in parallel in three independent experiments. No signal was obtained with the control sense probes (data not shown). Sections were incubated overnight at 70°C with 1 μg/ml of the digoxigenin-labeled probes, and binding detected by overnight incubation with alkaline phosphatase-conjugated anti-digoxigenin antibody (1:5000, Roche), which was visualised with NBT (Nitro blue tetrazolium chloride)/BCIP (5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt; 1:50, Roche) or Fast Red (Roche) for fluorescence.

Cochlear Morphology and Immunohistochemistry

Selected E13.5, E18.5, P5 and P15 cochlear sections were examined by dual in situ hybridization and immunohistochemistry as described in [23] using the primary antibodies summarized in Table S3. Sections were then sequentially covered with the secondary antibody solution (1:100, biotin-conjugated anti-mouse IgG or biotin-conjugated anti-rabbit, Chemikon), and extravidin peroxidase (1:200, Sigma). Finally, antibody binding was visualised using DAB as the chromogen and the sections mounted in Mowiol for observation under a Nikon 90i microscope. When indicated, Alexa Fluor 488 goat anti-rabbit, Alexa Fluor 546 goat anti-rabbit, Alexa goat anti-mouse 488 or Alexa donkey anti-goat 488 dyes (1:400, Molecular Probes) were used as the secondary antibody. Three embryos or mice per genotype were tested in parallel in three independent experiments. Control experiments without primary antibody were carried out for each experiment and indicated that the staining pattern was specific for antigen recognition (data not shown).

Cochlear Protein Extraction and Analysis

Frozen cochleae from E15.5, E18.5, P5, P15, P60 and P90 mice were pooled and homogenized in 200 μl of ice-cold RIPA lysis buffer containing 0.01% of the P8340 protease and P5726 phosphatase inhibitor cocktails (Sigma) and heated to 95°C for 5 min. Cochlear extracts were cleared by centrifugation at 11,800 rpm for 5 min at 4°C, and the supernatant was stored at −70°C until use. Three to six different pools from each genotype were used. When indicated, NE-PER® Nuclear and Cytoplasmic Extraction Reagent (PIERCIE Biotechneology) was used to prepare the cytoplasmic and nuclear extracts from E18.5 and P15 cochleae as indicated by the manufacturers. The protein content of the samples was determined with the Coomassie® Plus Protein Assay Reagent Kit or Micro BCA Protein Assay Kit (PIERCE Biotechneology) using BSA as the standard.

Equal amounts of cochlear protein were subjected to SDS-PAGE on 8%, 10% or 15% polyacrylamide gels and the proteins were then transferred to PVDF membranes in a Bio-Rad Trans Blot apparatus according to the manufacturer’s instructions. After incubation with a blocking solution, the membranes were probed overnight at 4°C with the appropriate primary antibodies summarized in Table S3. All antibodies were diluted in blocking solution except those against Akt, P44/42 ERK and p38 MAPK, which were diluted in TBS-T containing 5% BSA. The membranes were then washed and incubated with the appropriate peroxidase conjugated secondary antibodies for 1 h at RT. Immunoreactive bands were visualized by ECL (GE Healthcare Amersham) and the bands were quantified by densitometry with NIH Image J software. Statistical significance was estimated by Student’s t-test after using Levene’s test to confirm the equality of variances.

Results

Spatiotemporal Pattern of Expression of the IGF System Elements and Modulation of Target Kinase Activities in the Igf1−/− Null Mouse

Previous studies have shown that cochlear structures are positive for IGF-I immunostaining, which was observed in the stria
vascularis, spiral limbus and sensory supporting cells, as well as in
subpopulations of auditory ganglion neurons at postnatal day P20 [17]. However, because IGF-I is a hormone secreted by the liver, it
was important to determine whether or not it was synthesised in the
cochlea. To address this question we performed in situ
hybridization for \( \text{Igf1} \) and \( \text{Igf1r} \) at stages E15.5, E18.5 and P5 (Fig. 1).

At stages E15.5 and E18.5, the auditory epithelium can be
divided into the greater epithelial ridge (GER), which includes the
single row of inner hair cells, and the lesser epithelial ridge (LER),
which includes the three rows of outer hair cells (Figs. 1A–B). By P5 (Fig. 1C), the structure of the organ of Corti more closely
resembles that of the adult. \( \text{Igf1} \) and \( \text{Igf1r} \) temporal expression patterns are shown in Fig. 1A–C painted in blue and red tones,
respectively. At E15.5, \( \text{Igf1} \) mRNA was strongly expressed in an
area corresponding to the future stria vascularis and more weakly
in those areas that will give rise to Reissner’s membrane, the spiral
limbus and the outer sulcus (Fig. 1D,G). The expression of Proxl was
used to define the LER (see Fig. 1M; [24]). At E18.5, \( \text{Igf1} \) was
still expressed strongly in the stria vascularis and spiral limbus,
while it was relatively weak in the outer sulcus and Reissner’s
membrane (Fig. 1E,H). The expression of \( \text{Igf1} \) was restricted by P5, when it was detected in the marginal cells of the stria vascularis and
it overlapped with the cells expressing the Kir4.1 potassium
channel (Fig. 1F,I,J). Weaker expression was observed in the inner
and outer sulcus.

\( \text{Igf1r} \) was ubiquitously expressed at E15.5 but it was stronger in
the GER, LER and auditory ganglion (Fig. 1J,M). The expression pattern remained similar at E18.5 (Fig. 1K,N), although it was
notably stronger in the apical turn of the auditory ganglion (Fig. 1K).
As with \( \text{Igf1} \), the expression of \( \text{Igf1r} \) was more restricted by stage P5 and interestingly, the pattern was complementary to
that of \( \text{Igf1} \) with this receptor being confined to the inner spiral sulcus, Hensen’s, Claudius cells and the basal cells of the stria
vascularis (Fig. 1L,O,O’). The pattern of expression of the \( \text{Igf1r} \) did
not show any change in the \( \text{Igf1}^{+/+} \) null mouse cochlea with
respect to the wild type cochlea at the stages studied (E15.5, E18.5,
P5 and P15; Fig. S1).

The temporal expression profiles of several genes of the IGF
system were studied in the cochlea of \( \text{Igf1}^{+/+} \) and \( \text{Igf1}^{-/-} \) mice by qRT-PCR. These included \( \text{Igf1} \), \( \text{Igf1r} \), Ins2, \( \text{Igf2} \), \( \text{Igfbp2} \) and \( \text{Igfbp3} \) at stages E15.5, E18.5, P5, P15, P30, P60 and P90. In \( \text{Igf1}^{-/-} \) mice the expression of \( \text{Igf1r} \) remained high during development, despite
the modest postnatal decrease, while as expected it was absent in the
\( \text{Igf1}^{+/+} \) cochlea (Fig. 2A). Ins2 was not detected in wild type or
mutant mice, at any of the time points studied (data not shown).
In contrast, \( \text{Igf2} \) expression remained high during development and
dropped after birth, the expression levels of \( \text{Igf2} \) did not show
statistically significant differences between wild type and mutant
mice at any of the time points studied (data not shown). Expression of \( \text{Igf1r} \) in the \( \text{Igf1}^{+/+} \) cochlea decreased dramatically from E15.5 to P5, and it increased with age thereafter (Fig. 2B). In the \( \text{Igf1}^{-/-} \) cochlea, \( \text{Igf1r} \) was expressed at higher levels than normal after
birth and it remained proportionally higher throughout the period
studied (Fig. 2B). IGFBP expression has been reported in the
cochlea of several species [25,26]. High levels of \( \text{Igfbp2} \) and \( \text{Igfbp3} \) expression were detected at E15.5, although this expression
diminished rapidly thereafter. There was slightly higher expression
of these binding proteins in the cochlea of \( \text{Igf1}^{-/-} \) null mice (Fig. 2C–D).

Upon IGF-I binding, its high affinity receptor IGF1R tyrosine
kinase activity is turned on and autophosphorylates receptor
residues that act as docking sites for adaptor proteins like the
insulin receptor substrate 2 (IRS2) [27], which in turn will activate
downstream signalling pathways. Fig. 2E and Fig. S2 show that
IGF1R is less phosphorylated in the \( \text{Igf1}^{-/-} \) null mouse cochlea than in the wild type. Interestingly, there was a slight (30% p<0.05) increase in the tyrosine phosphorylation ratio of the
IGF1R in the \( \text{Igf1}^{-/-} \) from E18.5 to P90 when compared with the
relative tyrosine phosphorylation ratio observed at E15.5; no
changes could be shown for IRS2 levels at the times studied (Fig. 2F and S2).

IGF-I signalling is mediated by a network of intracellular
mediators that include the phosphatidylinositol-3-kinase/Akt
pathway and the mitogen-activated kinase cascades. In the
E18.5 \( \text{Igf1}^{-/-} \) null mouse cochlea there were reductions in the relative levels of activated phospho-AktSer473 (31%, p<0.01) and
phospho-ERK1/2 (56%, p<0.05), whereas phospho-p38 MAPK
was strongly activated (261%, p<0.005) when compared with the
\( \text{Igf1}^{+/+} \) wild type mouse cochlea (Fig. 2E,G). Akt and ERK1/2
MAPK activation are essential for cell survival and proliferation,
whereas p38 MAPK forms part of the cellular response to
environmental stress, such as ultraviolet light, heat, osmotic shock
and inflammatory cytokines [28]. These data indicate that
the IGF-I deficit at E18.5 diminishes the activity of the pathways
that control cell proliferation and survival, whilst those involved in the
cellular response to stress are heightened.

To recap, the main elements of the IGF system are present
during the development of the cochlea and they are expressed in
specific spatiotemporal patterns. IGF-I deficit affects the expression
levels of the IGF system elements, and key IGF-I-activated
signalling pathways are profoundly altered. These data, together
with the reported morphological alterations and the profound
sensorineural deafness that the deficit in IGF-I causes in mice and
humans, prompted us to further study the molecular mechanisms
underlying IGF-I activity in the developing mouse cochlea.

Identification of Differentially-Expressed Genes in the
E18.5 \( \text{Igf1}^{-/-} \) Mouse Cochlea

To study the otic-specific gene targets of IGF-I, mRNA from whole cochlea of E18.5 \( \text{Igf1}^{+/+} \) and \( \text{Igf1}^{-/-} \) mice was hybridized with mouse ‘whole genome’ arrays (MOE430A) from Affymetrix.
The results were submitted to the Gene Expression Omnibus
(http://www.ncbi.nlm.nih.gov/geo/) with the accession number
GSE11821.

We used multi-mgMOS and the model developed by the
PUMA group to estimate gene expression levels with credibility
intervals that quantify the measured variance associated with the
estimated target concentration within a sample. This within-
sample variance is a significant source of uncertainty in
oligonucleotide arrays, especially for genes expressed at low levels.
Final targets were selected using the PPLR algorithm that reduces
the number of false positives. Genes that presented a Fold Change
higher than a log2 unit of 1 (FC<1 with P<0.05 and FC>1 with
P>0.95) were selected for further analysis. Following these criteria,
the expression of 167 genes was seen to be considerably lower and
64 genes were expressed more strongly in the absence of IGF-I.
These genes were then compared with the NCBI Mus musculus
gene database, and ordered by gene ontology and biological
processes with the programs PANTHER and FATIGO+. This
analysis identified the different biological processes and cellular
activities of the cochlear genes affected by IGF-I deficit (Fig. S3). A
further selection was carried out on the basis of biological function,
reported expression in the inner ear or association with human
deafness, as well as for technical parameters as the FC and the low-
variance between arrays. Table S4 shows selected genes
differentially expressed in the absence of IGF-I, which include
genes related to sensorial defects (Ush1c, Esrrb and Tub), ion
Figure 1. *Igf1* and *Igf1r* mRNA expression in the cochlea. (A–C) Cartoons of the organ of Corti at E15.5, E18.5 and P5 show the expression of *Igf1* (blue), *Igf1r* (orange and red) or both (purple). (D–O) *In situ* hybridization of *Igf1* (D–I,I') and *Igf1r* (J–O, O') in normal embryos at E15.5 (D,G,J,M), E18.5 (E,H,K,N) and in P5 mice (F,I,L,O). Dual immunostaining with anti-Kir4.1 was performed to identify the neural projections, the stria vascularis and pillar cells (F,I') and with anti-Prox1 to identify the pillar cells, Deiter's cells, and auditory neurons (J–O,O'). (D–I) *Igf1* expression was located in the stria vascularis (long black arrows), spiral limbus (black arrowheads), outer sulcus (green arrowheads) and Reissner's membrane (short black arrow). At P5 the *Igf1* expression in the stria vascularis was observed in the marginal cells (I'). (J,K,M,N) At E15.5 and E18.5, *Igf1r* was strongly expressed in the GER (red arrow) and weakly in the LER (red arrowhead). (L,O) At P5, *Igf1r* expression presented a complementary pattern to that of *Igf1* and was observed within the inner spiral sulcus (red arrows), Claudius and Hensen's cells (red arrowheads). *Igf1r* was also located in the AG (asterisk in J,K, K') and in the basal cells of the stria vascularis (O'). Three embryos per genotype were tested in parallel in three independent experiments. GER, greater epithelial ridge; IHC, inner hair cells; LER, lesser epithelial ridge; OHC, outer hair cells; PC, pillar cells; AG, auditory ganglion; SM, scale media; ST, scala tympani; SV, scala vestibuli; TM, tectorial membrane. Scale Bars: D,E,F, 150 μm (D,E,F,J,K,L); A,B,C, 50 μm; G,H,J, 50 μm (G,H,J,M,N,O); I', 10 μm; O', 20 μm and K', 30 μm.

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transporters (Cacna1f, Kcnd2, Kcnmb1 and Mlc1), the acetylcholine transporters Slc18a3 and Slc5a7 and the strial functional modulators Esrrb and Cldn18, among others that have been previously reported as cochlear-expressed genes.

To assess the organ specificity of the IGF-I target genes identified in this study, a parallel study was carried out on total RNA obtained from the lungs of Igf1/2 and wild type littermates (GSE17157; JGP, manuscript in preparation). The comparison of the differentially expressed genes obtained from both studies indicated that 94 genes were up-regulated in the lung, whereas only 64 genes were up-regulated in the cochlea. Interestingly none of these genes was common to the two tissues. In addition, 56 lung genes were down-regulated in the Igf1/2 mouse, in striking contrast to the weaker expression of 167 cochlear genes when compared to that in the wild type mouse cochlea. Only 3 genes were present in both databases: integrin alpha V (Itgav), solute carrier family 4 member 1 (anion exchanger - Slc4a1) and the ubiquitin specific peptidase 12 (Usp12).

Figure 2. Time-course of mRNA expression of IGF-system genes and the activation levels of signalling mediators in the E18.8 cochlea. (A–D) mRNA expression levels of Igf1, Igf1r, Igfbp2 and Igfbp3 were analyzed by qRT-PCR in Igf1+/+ (open circles) and Igf1−/− (closed circles) mice at E15.5 and E18.5 (n = 8), P5, P15, P30, P60 and P90 (n = 6). Eukaryotic 18S rRNA was used as the endogenous housekeeping control gene. The estimated gene expression was calculated as 2^(-ΔΔCt). (A) Igf1 expression was high in normal cochlea and absent in the null mice. (B) Igf1r expression in normal cochlea decreased dramatically from E15.5 to P5 and increased with age thereafter. In the Igf1−/− cochlea, Igf1r followed the same pattern but consistently presented higher levels at all time points studied. Igfbp2 (C) and Igfbp3 (D) mRNA levels were high at E15.5 but they dropped thereafter. Their profiles were slightly higher in the Igf1−/− cochlea. (E) IGF-I modulates IGF1R, ERK, Akt and p38 activation at E18.5. (F) Levels of phosphorylated-IGF1R and IRS2 in cochlear protein extracts from Igf1+/+ and Igf1−/− mice were studied by Western blotting at E15.5, E18.5, P5, P60 and P90. Data are presented as percentage of Igf1−/− null mouse protein levels compared to the Igf1+/+. (G) To determine the levels of phosphorylated Akt (Ser473), ERK and p38 MAPK, cochlear protein extracts from E18.5 Igf1+/+ and Igf1−/− mice were analysed by immunoblotting. Membranes were re-probed with β-Actin as a loading control, and for the non-phosphorylated forms of AKT and ERK1/2. Films were scanned, densitometry performed by using ImageJ software and the levels were normalised by giving a value of 100 to the Igf1+/+ mouse samples. Values are presented as mean±SEM of at least 3 different experiments involving at least 6 mice per condition for Akt, ERK and p38 MAPK. The statistical significance estimated by Student’s t-test was as follows ***p<0.005; **p<0.01; *p<0.05.

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The changes in the expression of 15 genes were confirmed by qRT-PCR using TaqMan probes where available, or by in situ hybridization. qRT-PCR has proven to be an efficient method to verify DNA array results and the predicted differences were confirmed for 68% of the genes studied (see Table S3). At E18.5, qRT-PCR of total cochlear mRNA confirmed that Akr1c13, Fgf15, From1, Mash1, Rpy1h, Six6 and Ush1c transcripts were more strongly expressed in the cochleae of Igf1<sup>2/2</sup> mice [29,30,31,32]. In contrast, Foxg1, which is involved in the morphogenesis of the mammalian inner ear [33], did not present a differential expression in the null mice (see Table S3).

These data contribute to our understanding of the molecular basis of the delayed maturation of the sensory epithelium reported in IGF-I deficit [17], extending the actions of this factor and highlighting a relationship with the Usher syndrome molecules Ush1c [92], Rp1h [34] and Tab [35] whose mutations cause both deafness and blindness in men (ORPHA120433, ORPHA886).

Parallel in situ hybridization studies confirmed the aberrant cellular expression of Six6 and Mash1, and of the fibroblast growth factor Fgf15. These genes were expressed in the Igf1<sup>2/2</sup> cochlea despite being absent or expressed at very low levels in the Igf1<sup>+/+</sup> cochlea. Within the central nervous system, Six6 is expressed in the presumptive and differentiating neural retina, ventral optic stalk, olfactory placodes, hypothalamus, and pituitary gland [36]. Although Six6 mRNA is not normally expressed in the auditory nerve at E18.5 (Fig. 3A,B), transcripts were clearly detected in the

![Figure 3. Up-regulation of Six6, Mash1 and Fgf15 in the embryonic cochlea of the Igf1<sup>−/−</sup> mouse. In situ hybridization for Six6 (A, B, D, E), Mash1 (C, F) Fgf15 (G–J and N–Q), Fgf8 (K) and FgfR3 (L) transcripts was performed on cryostat sections from Igf1<sup>−/−</sup> and Igf1<sup>−/+</sup> E18.5 (A–F and G–M) and P5 (N–Q) cochleas. (M) Schematic drawing of the organ of Corti showing the different cell types at E18.5. Six6 and Mash1 expression was higher in the auditory nerve (AN) of E18.5 Igf1<sup>−/−</sup> cochlea. Fgf15 mRNA expression located in the border cells (BC) and in the inner phalangeal cells (IPC) of E18.5 Igf1<sup>−/−</sup> mice (arrows in L), was absent in Igf1<sup>−/+</sup> mice (G,H). Fgf8 (blue arrowhead in K) expression was detected in IHC in Igf1<sup>−/−</sup> and FgfR3 (blue staining in L) transcripts were also detected in Igf1<sup>−/−</sup> supporting cells. At P5, Fgf15 expression was observed in the IPC and BC in the basal turn of the cochlea of both Igf1<sup>−/−</sup> (arrows in N,O,O',P) and normal (arrowhead in Q) mice. Immunostaining for Prox1 (brown in A,B,D,E), MyosinVIIa (brown in H,I,J,K) identified supporting cells, inner and outer hair cells and pillar cells respectively. Three embryos per genotype were tested in parallel in three independent experiments. DC, Deiters cells; HC, hair cells; IHC, inner hair cells; OHC, outer hair cells; PC, pillar cells; SM, scala media; ST, scala tympani; SV, scala vestibuli; TM, tectorial membrane. Scale Bars: A, 100 μm (A,D,N); E, 100 μm (B, C, F, H); 50 μm (G); I, 20 μm (I,O,O'); and 30 μm (H,J,K,L,P,Q); M, 10 μm.

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Igf1<sup>−/−</sup> mouse (Fig. 3D,E, arrows in E). The proneural bHLH transcription factor Mash1 was also more strongly expressed in the auditory nerve (AN) of the E18.5 Igf1<sup>−/−</sup> mouse (Fig. 3C,F), overlapping the glial transition zone [37]. At P5, Mash1 expression was similar between genotypes and was associated with neuronal bodies amongst fibres that were strongly labelled for myelin basic protein, which may suggest that at this stage Mash1 positive cells are root neurones [30] (Fig. 3G). These results suggest that IGF-I participates in late neural cell fate decisions in the auditory ganglia.

Fibroblast growth factors and their receptors have key roles during inner ear development [39,40]. At E15.5 and E18.5, Fgf15 is not normally expressed in the organ of Corti of wild type mice but transcripts from this gene were found in the inner phalangeal cells and border cells of the Igf1<sup>−/−</sup> mice at E16.5 and E18.5, close to the inner hair cells in the basal turn of the cochlea (data not shown and Fig. 3G,J). These transcripts were associated with the specific markers p75 for pillar cells and myoVIIa for hair cells (Fig. 3L), see reference cartoon in M. At P5, Fgf15 expression in the border and inner phalangeal cells was very similar between genotypes (Fig. 3N-Q). Expression of Fgf8 (Fig. 3K) and Fgf3 (Fig. 3L) was unchanged in the Igf1<sup>−/−</sup> cochlea at E18.5.

The expression profiles of Akir1c3, Dnabj7, Fgf15, Foxf1, Foxm1, Kcnj2, Igf17, Mash1, Shbg, Retnla, R1ph, Six6, Slc19a2 and Ush1c at stages E15.5, E18.5, P5, P15, P30, P60 and P90 were studied in the cochleas of Igf1<sup>+/+</sup> and Igf1<sup>−/−</sup> mice by qRT-PCR. They were categorised in three groups: i) genes with profiles that differed during embryonic development, either increasing like Akir1c3, Fgf15, Foxm1 and Six6 (Fig. 4A) or decreasing like Dnabj7 (Fig. 4B), in the Igf1<sup>−/−</sup> cochlea; ii) genes with profiles that differed during the postnatal stages, increasing like Fibp, Shbg (Fig. 4C), Slc19a2 and R1ph or decreasing like Igf17 and Retnla (Fig. 4D) in the Igf1<sup>−/−</sup> cochlea; iii) genes that were affected by the absence of Igf1 throughout embryonic and postnatal development, such as Mash1, Ush1c (Fig. 4E) and Kcnj2 (Fig. 4F). One example from each category is shown (Fig. 4).

FoxM1 and MEF2 Levels and Intracellular Localization Are Differentially Regulated in the Igf1<sup>−/−</sup> Mouse Cochlea

In the mouse embryo, FoxM1 is associated with cell cycle control and DNA repair in neural progenitors and its expression decreases after differentiation [41]. FoxM1 prevents nuclear localization of the cyclin-dependent kinase inhibitor p27Kip1 and is essential for cytokinesis [42,43]. In the E18.5 Igf1<sup>−/−</sup> mouse cochlea, Foxm1 expression levels were up-regulated (+1.7 fold log<sub>2</sub> change using Genechip Arrays and +1.2 with qRT-PCR) with respect to the wild type. To further explore the possible functional consequences of this altered Foxm1 expression, cytoplasmic and nuclear protein extracts from the whole cochlea of E18.5 and P15 mice were analyzed. At E18.5, nuclear FoxM1 protein levels were 154% higher (p<0.05) in

![Figure 4. Time-course of IGF-I target gene mRNA expression.](PLoS ONE | www.plosone.org 8 January 2010 | Volume 5 | Issue 1 | e8699)
We have studied the molecular mechanisms by which IGF-I regulates cochlear development and maturation by analyzing the following parameters in the Igf1+/+ wild type and Igf1−/− null mouse cochlea: i) the spatiotemporal expression of IGF-system factors, receptors and binding proteins; ii) the activation of the main IGF-I signalling kinases Akt, ERK and p38; iii) total cochlear transcriptome changes caused by IGF-I-deficit by using mRNA arrays; and iv) transcription factors associated with neuronal cell cycle regulation modulated by IGF-I availability. We have found novel regulatory genes for cochlear development whose normal expression and activation depends on IGF-I. Severe syndromic deafness in man is associated with null mutations in Igf1 [9,10,11] and also with low levels of IGF-I [47]. Accordingly, the Igf1−/− mouse shows poor growth rates, high mortality, profound sensorineural deafness and late postnatal morphological alterations in the cochlea [16]. We have shown previously that the absence of IGF-I causes poor myelination and delayed maturation of auditory neurones that suffer apoptosis during the early postnatal mouse development [5]. At birth, however, the Igf1−/− mouse cochlea is the normal size with the expected complement of cell types in the organ of Corti. At the molecular level, signs of delayed differentiation were obvious, but the molecular clues underlying this cochlear-specific phenotype were not clear. Here we show that IGF-I deficit could be compensated, at least in part, by increased expression of its high affinity receptor, which can also be activated by other insulin family factors, whose gene expression levels were unchanged. Typical IGF-I intracellular target kinases were also examined in the cochlea, and interestingly a 25% reduction in the activated forms of prosurvival Akt kinase and proliferation-associated ERK1/2 were found, with a dramatic increase in the levels of the stress kinase p38. Further analysis to uncover IGF-I targets in the cochlea was carried out by using gene microarrays to do a comparative analysis of the expression profiles of the developing cochlea in Igf1+/+ and Igf1−/− mice.

Here, we have identified 231 genes that are differentially expressed in the cochlea of the Igf1−/− mouse. A subset of these genes was further studied by using a combination of complementary approaches to further understand IGF-I actions in the inner ear. To our knowledge, this is the first time that a comparative gene expression profile has been carried out in an Igf1−/− mouse tissue. Fig. 8 schematically shows the localization of the differentially expressed genes in Igf1−/− cochleae that are known to be important for inner ear development or to be linked to inherited deafness (9%) of total), including: (1) deafness genes, (2) olfactory bulb specific neurons and (3) Usher's Syndrome 1C. Interestingly, this syndrome includes mutations in the hair cell-specific protein harmonin and mutations in this gene cause Usher’s Syndrome 1C [32].

Over-expression of IGF-I causes profound alterations in the vascularisation of the mouse inner ear [48]. A subset of the genes we found differentially expressed in the Igf1−/− cochlea had not been described previously. We have identified 231 genes that are differentially expressed in the cochlea of the Igf1−/− mouse. A subset of these genes was further studied by using a combination of complementary approaches to further understand IGF-I actions in the inner ear. To our knowledge, this is the first time that a comparative gene expression profile has been carried out in an Igf1−/− mouse tissue. Fig. 8 schematically shows the localization of the differentially expressed genes in Igf1−/− cochleae that are known to be important for inner ear development or to be linked to inherited deafness (9%) of total). Among these were: (1) deafness genes, (2) olfactory bulb specific neurons and (3) Usher's Syndrome 1C. Interestingly, this syndrome includes mutations in the hair cell-specific protein harmonin and mutations in this gene cause Usher’s Syndrome 1C [32].

Interestingly, the syndrome includes retinal degeneration, which is also associated with mutations in Rph1 [34], a gene expressed at higher levels in the Igf1−/− cochlea. Other expression of IGF-I causes profound alterations in the vascularisation of the mouse inner ear [48] but to our knowledge there are no reports of eye defects associated with IGF-I deficiency. In contrast, IGF-I deficit in the mouse severely impairs normal development of the olfactory bulb [49]. 91% of the genes we found differentially expressed in the Igf1−/− cochlea had not been described previously in the inner ear. For example, Fgf15, the ortholog of human and chicken Fgf19, presented an expression pattern suggestive of a novel contribution to cell fate specification within the sensory epithelium. This raises the question of the specific role of this member of the
Figure 5. IGF-I deficiency modifies FoxM1 and p27Kip1 levels and intracellular localization. (A) Cytoplasmic and nuclear fractions of protein extracts obtained from at least 12 different E18.5 or P15 Igf1+/+ or Igf1−/− mouse cochleas in at least six different experiments were immunoblotted to detect the presence of FoxM1 and p27Kip1. Blots were reprobed with β-actin (cytoplasmic fraction) or histone H3 (nucleus) as loading controls. The specific bands were measured by densitometry to determine the average expression with ImageJ software. Results were normalized by assigning a value of 100 to the cytoplasmic Igf1+/+ and represented graphically in (B). (C) Relative quantification value (RQ) of Foxm1 expression in the Igf1−/− cochlea compared to Igf1+/+, estimated by qRT-PCR at E15.5, E18.5-P5 and P15-P90. Data are presented as log_{10}RQ average. (D–M) Localization of immunostaining for FoxM1 in the P15 Igf1+/+ (D–H) and Igf1−/− (I–M) mouse cochlea. The expression was located in the AG, the stria vascularis and the organ of Corti (white arrows). Statistical significance estimated with the Student’s t-test was: ***p<0.005; **p<0.01; *p<0.05, of mutant versus wild type mice data. Open and closed bars: Igf1+/+ and Igf1−/− mice, respectively. Cyt, cytoplasm; Nuc, nucleus; AG, auditory ganglia, IHC, inner hair cell; OC, organ of Corti; OHC, outer hair cell; SM, scala media. Scale bars: D, 100 μm (D,J); E, 20 μm (E,F,J,X) and G, 20 μm (G, H, L, M). doi:10.1371/journal.pone.0008699.g005
FGF family during inner ear development and of its possible regulation by IGF-I [12].

Several transporters fundamental for the traffic of synaptic vesicles were also expressed differentially in the Igf1−−/ mouse cochlea, which is consistent with previous observations of aberrant synapsis at the inner hair cells [17]. For example, the thiamine transporter, Slc19a2 [32,50], the choline and acetylcholine transporters, Slc5a7 and Slc18a3 [51], and the membrane protein Vamp1 were normally

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**Figure 6. IGF-I deficiency modifies MEF2 levels and intracellular localization.** (A) Cytoplasmic and nuclear fractions of protein extracts obtained from E18.5 and P15 normal or Igf1−−/− mouse cochleae (n=21, from at least 7 different experiments) were immunoblotted to detect the presence of MEF2A and MEF2D. Blots were re-probed with β-Actin (cytoplasmic fraction) or histone H3 (nucleus) as loading controls. The specific bands were measured by densitometry (Image) software to determine the average expression. Results were normalized respect to β-actin or histone, a value of 100 was assigned to the scanned intensity of cytoplasmic forms in Igf1++/+ and represented graphically in (B,C). (D) Mef2a, Mef2c and Mef2d expression was measured by qRT-PCR at E15.5, E18.5-P5 and P15-P90 data points in the Igf1−−/− mouse cochleas and compared with the Igf1++/+. Data are presented as the mean of log10RQ. Statistical significance estimated with Student’s t-test was: ***p<0.005; **p<0.01; *p<0.05. Open bars: Igf1++/+ mouse; Closed bars: Igf1−−/− mouse. Cyt, cytoplasm; Nuc, nucleus; β-act, β-actine; H3, histone 3.

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expressed in the inner hair cells of the mouse but their levels were consistently lower in the Igf1<sup>−/−</sup> cochlea. In contrast, Mlc1, which encodes a protein located in the afferent fibers of the inner hair cells [52], was expressed at higher levels. These data support the idea that IGF-I is a key molecule for the maturation of the auditory neurons and the refinement of the synaptic connections at the inner hair cells.

Alterations in ion homeostasis and transport were also associated with IGF-I deficit because Kcnd2, Kif17, Kcnmb1 and Cacna1f showed lower levels in Igf1<sup>−/−</sup> cochleae. Kcnmb1 is known to be expressed in the cochlea but the null mouse has no obvious cochlear phenotype or hearing impairment [55]. In contrast, the presence of the calcium channel Cacna1f had not been described previously in the mouse cochlea, but mutations in man and mouse cause retinal neurotransmission disorders [56,57]. In addition, differentially regulated genes included Claudin 18 a tight

**Figure 7. MEF2A and MEF2D immunolocalization in the cochlea of Igf1<sup>+/+</sup> and Igf1<sup>−/−</sup> mice.** MEF2A expression in the cochlea of E18.5 (A,B,C) and P15 (L,J,K,M) Igf1<sup>+/+</sup> (A,B,I,J) and Igf1<sup>−/−</sup> (C,K) mice. At E18.5, MEF2A strongly stained the nuclei in the Igf1<sup>+/+</sup> auditory ganglion (arrowheads in B), whereas fewer nuclei appeared labelled in the Igf1<sup>−/−</sup> ganglia (arrowheads in C) where the staining appeared more cytoplasmatic. At P15, labelling was similar in the neurones (J,K), Deiter's cells, pillar cells and in the IHC (L,M) of both genotypes. MEF2D expression at E18.5 was shown in the auditory ganglia (E,F) and organ of Corti (G,H). MEF2D expression was less nuclear in the Igf1<sup>−/−</sup> (F, arrowheads pointing to unlabelled nuclei, H) than in the wild type mouse (E,G). At P15, MEF2D expression was observed in the nuclei of auditory neurons (N) and in the IHC (P,P') in the Igf1<sup>+/+</sup> but not in the Igf1<sup>−/−</sup> mouse (O,Q). AG, auditory ganglion; IHC, inner hair cells; OHC, outer hair cells; SM, scale media; ST, scala tympani; SV, scala vestibule. Scale bars: A, 100 μm; B, 25 μm (B,C,E,F); D, 75 μm; I, 100 μm; G, 20 μm (G,H); J, 20 μm (J,K); L, 20 μm (L,M); N, 20 μm (N,O); P, 20 μm (P,P',Q).

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The K<sup>+</sup> channel Kv4.2, which is expressed in neurons that innervate apical hair cells, which regulates dendritic excitability [53] and which is transported to the dendrites by the neuronal kinesin Kif17 [54]. The calcium-activated potassium channel Kcnd2 is known to be expressed in the cochlea but the null mouse has no obvious cochlear phenotype or hearing impairment [55]. In contrast, the presence of the calcium channel Cacna1f had not been described previously in the mouse cochlea, but mutations in man and mouse cause retinal neurotransmission disorders [56,57]. In addition, differentially regulated genes included Claudin 18 a tight
junction protein expressed in the stria vascularis [2] and the estrogen related receptor Esrrb, whose mutations in man cause autosomal-recessive non syndromic hearing impairment and that is expressed and controls the development of the strial marginal cells [20,21]. These data taken together suggest that ion homeostasis and vesicular transport are impaired in the deaf Igf1\(^{2/2}\) mouse.

Neuronal fate specification is mastered by transcription factors like SIX6 and Mash1, which are typically expressed in the central nervous system. SIX6 is a member of the Six/sine oculis family and it is known to be expressed in the developing and adult retina, in the optic nerve, and in the hypothalamic and pituitary regions [58,59]. Mash1 is a proneural transcription factor of the basic helix-loop-helix family, which participates in the commitment of neural progenitors, promotion of cell cycle exit and neuronal migration, and in the final specification of neuronal identities in the brain [60,61]. Interestingly, both were increased in the Igf1\(^{2/2}\) mouse embryonic cochlea, Mash1 transcripts were visible in the central part of the auditory nerve at the glial transition zone [37], where Atoh1, another member of the bHLH transcription factor family [62], has been shown to play a central role in root neurons survival and on the functional maintenance of the peripheral and central auditory pathway [63]. Taken together these data suggest that these bHLH transcription factors are key players for the differentiation and survival of the neurons at the interface between the peripheral and central nervous systems.

IGF-I promotes a faster transition of the otic neural progenitors to a mature neuronal state in the developing chicken inner ear [12,64]. These data taken together suggest that IGF-I represses the expression of SIX6 and Mash1 during normal inner ear development either directly or indirectly to facilitate neuronal differentiation.

It is known that IGF-I is a key factor for cell cycle progression and DNA repair and several cell types in the Igf1\(^{-/-}\) mouse, including cochlear neurones, are smaller and more immature than those of the wild type mouse [17,18,65]. Here we show that in the cochlea, IGF-I deficit causes an increase in IGF1R expression levels although there is a net reduction in the ratio of tyrosine phosphorylation, an increase in the activated phospho-p38 stress kinase, and a decrease in the levels of the active phosphorylated forms of the kinases ERK1/2 and Akt, the main intracellular executors of IGF-I actions, indicating that the balance between cell proliferation, survival and differentiation is altered. However, the complexity of the regulation of cellular processes was evidenced by the contrasting increase in the expression levels of the forkhead transcription factor FoxM1. FoxM1 is essential for mitotic progression and for the transcriptional response during DNA damage/checkpoint signalling [42,66,67]. Its presence in the developing cochlea had not been reported previously. Its increased activation in the Igf1\(^{-/-}\) cochlea was confirmed by its nuclear localization and by the inhibition of one of its downstream targets, the cyclin-dependent kinase inhibitor p27\(^{kip1}\). In contrast, transcripts for other cell cycle proteins, such as INCENP, were expressed at lower levels, suggesting problems in chromosome segregation [68]. In early postnatal cochleae from Igf1\(^{-/-}\) mice there is increased neuronal apoptosis and delayed neuronal maturation, but there is no evidence for altered cellular proliferation or cell damage [17]. These data suggest that FoxM1

Figure 8. Differentially expressed genes in the IGF-I-deficient cochlea. Names of selected differentially expressed genes (red) are shown on a schematic drawing of the adult scala media. BC, border cells; BS, basal cells; BM, basilar membrane; CC, Claudius’s cells; DC, Deiter’s cells; HC, Hensen’s cells; IC, intermediate cells; IDC, interdental cells; IHC, inner hair cells; IPC, inner phalangeal cells; IS, inner sulcus; Li, spiral limbus; MC, marginal cells; OHC, outer hair cells; PC, pillar cells; RM, Reisner’s membrane; AG, auditory ganglion; SL, spiral ligament; SM, scala media; ST, scala tympani; SV, scala vestibuli; TM, tectorial membrane.

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activation compensates for the unbalanced progression through the cell cycle caused by IGF-I deficit.

Further insight into IGF-I cochlear targets was obtained by in silico analysis of the promoters of IGF-I-modulated genes that unveiled a potential role for MEF2 and its modulation by IGF-I in the cochlea. MEF2 is essential for myogenic and neural differentiation [45,69]. In both cell types, IGF-I activates MEF2 by decreasing its degradation rate and by preventing its translocation from the nucleus to the cytoplasm [46,69]. MEF2D is expressed in sensory neurons during development and it is regulated by a Ttk-dependent ERK5 pathway that promotes neuronal survival [70]. MEF2 is activated by the Raf/MAPK cascade [46] and also by p38 MAPK [71]. Here we show that MEF2A and D but not C are highly expressed in the nuclei of embryonic mouse auditory ganglion neurons and that nuclear MEF2 protein levels are lower in the developing Igf1−/− cochlea. These data reinforce the conclusion that MEF2A and D are key targets for otic IGF-I action and that they may have a fundamental role during cochlear development. To our knowledge, this is the first time that MEF2 transcription factors expression has been reported in the auditory ganglia.

In summary, we show that Igf1 and Igf1r are expressed in the developing mouse cochlea with complementary cellular patterns. The stria vascularis apparently provides an intra-cochlear source of IGF-I. Analysis of IGF-I-deficient cochlea showed that the signalling levels of Akt and ERK1/2 were lower and that p38 activation was significantly higher. Transcriptional profiling of the Igf1−/− cochlea identified potential novel IGF-I targets, including factors like Six6, Mash1 and Fgf15. Finally, the transcription factors FoxM1, Mef2a and Mef2d are expressed in the developing inner ear and their sub-cellular localisation is modulated by IGF-I availability. The results presented here offer new insight into the mechanisms by which IGF-I support sensory cell and neuronal survival and differentiation in the auditory receptor, and reveal novel regulatory mechanisms of the cell cycle during cochlear development.

Supporting Information

Figure S1 Spatiotemporal expression patterns of Igf1r mRNA and IGF1R protein in the Igf1−/+ and Igf1−/− mouse cochlea. (A–F) shows that the mRNA expression of Igfr1 was identical in the two genotypes. (A,B,C) P5 Igf1+/+ and (D,E) P5 Igf1−/− mice. IGF1R protein was shown at the organ of Corti and in the auditory ganglion at E15.5 (G,H) and E18.5 (I,I′,J,J′) with similar cellular localization between genotypes. At P15, the expression was located mainly in the neurons of the auditory ganglion (K,L), no cellular localization between genotypes. At P15, the expression was identical in the mouse cochleas grouped according to their functional category. The 231 genes differentially expressed (closed bars), were classified in situ hybridization of Mash1 and 3A10 labelling. (L–S) Double in situ hybridization of Mash1 and 3A10 (red; L,P,O,S) and immunohistochemistry of myelin basic protein (green; MBP; M,Q,O,S). Cell nuclei were stained with DAPI (blue; N,R,O,S). Mash1 expression was perinuclear and was associated at P5 to the soma of cells, probably root neurones, embedded in auditory axons (white arrows; L–S). Three embryos per genotype were tested in parallel in three independent experiments. AN, auditory nerve; SM, scala media; ST, scala tympani; SV, scala vestibuli. Scale bars: H, 150 μm (A, H); K, 30 μm (B,G,I–K); L, 35 μm (L–O); P, 20 μm (P–S). Figure S2 Summary of inventoried TaqMan probes used for qRT-PCR. Applied Biosystems: https://products.appliedbiosystems.com/ab/en/US/adiract/ab.cmd?ABGEKeywordSearch. Found at: doi:10.1371/journal.pone.0008699.s002 (0.05 MB TIF) Figure S3 Clustering of the differentially expressed genes in Igf1−/+ cochleas grouped according to their functional category. The 231 genes differentially expressed (closed bars), were classified by their biological annotation compared with all Mus musculus genome annotations in the NCBI (open bars). The statistical analysis of the biological processes included a Bonferroni correction for multiple testing and processes were selected at p<0.05. The differentially expressed genes are implicated in the following biological processes: signal transduction, developmental process, ligand-mediated signalling, cell communication, immunity and defence, cytokine and chemokine mediated signalling, muscle contraction, lipid, fatty acid and steroid metabolism, granulocyte-mediated immunity and homeostasis. Found at: doi:10.1371/journal.pone.0008699.s003 (0.20 MB TIF) Figure S4 Mash1 expression in the Igf1−/+ and Igf1−/− P5 mouse auditory nerve. (A–K) Mash1 in situ hybridization was performed on cryostat sections in Igf1+/+ (A–G) and Igf1−/− (H–K) cochleas at P5. Axons in the auditory nerve (AN) were recognised by 3A10 immunostaining (red; C,D,F,G,J,K). At P5, Mash1 expression in the auditory nerve did not show differences between genotypes (B–G, I–K). B, E and I are higher magnification images of the boxed areas in A and H, respectively. D,G,K are merge images of Mash1 and 3A10 labelling. (L–S) Double in situ hybridization of Mash1 (red; L,P,O,S) and immunohistochemistry of myelin basic protein (green; MBP; M,Q,O,S). Cell nuclei were stained with DAPI (blue; N,R,O,S). Mash1 expression was perinuclear and was associated at P5 to the soma of cells, probably root neurones, embedded in auditory axons (white arrows; L–S). Three embryos per genotype were tested in parallel in three independent experiments. AN, auditory nerve; SM, scala media; ST, scala tympani; SV, scala vestibuli. Scale bars: H, 150 μm (A, H); K, 30 μm (B,G,I–K); L, 35 μm (L–O); P, 20 μm (P–S). Found at: doi:10.1371/journal.pone.0008699.s004 (7.24 MB TIF) Table S1 Summary of inventoried TaqMan probes used for qRT-PCR. Applied Biosystems: https://products.appliedbiosystems.com/ab/en/US/adiract/ab.cmd?ABGEKeywordSearch. Found at: doi:10.1371/journal.pone.0008699.s005 (0.06 MB DOC) Table S2 Summary of cDNAs used to generate the in situ hybridization probes. Prior to the in situ hybridization, all clones were sequenced (ABI 3130XL Applied Biosystems). At least 3 embryos per genotype were tested in parallel in three independent experiments. No signal was obtained with the sense probe (data not shown) * Igf1r probe was the generous gift of Prof. Flora de Pablo (CIB, CSIC, Madrid) Reference 1. Lopez-Rios J, Gallardo ME, Rodriguez de Cordoba S, Bovolenta P (1999) Six9 (Optx2), a new member of the six gene family of transcription factors, is expressed at early stages of vertebrate ocular and pituitary development. Mech Dev 83: 155–159. 2. Guillemonet F, Joyner AL (1993) Dynamic expression of the murine Achaete-Scute homologue Mash-1 in the developing nervous system. Mech Dev 42: 171–185. 3. 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Table S3 Summary of antibodies used for immunohistochemistry and Western blotting. For IHC, at least 3 embryos per genotype were tested in parallel in three independent experiments. Control experiments without primary antibody were carried out for each reaction and indicated that the staining pattern was specific for antigen recognition (data not shown).

| Antibody type | Rabbit polyclonal | Mouse monoclonal | Goat polyclonal |
|---------------|-------------------|------------------|----------------|
| Specificity   | RbP, rabbit polyclonal | MouM mouse monoclonal | GP, goat polyclonal |

Abbreviations: DSHB, Developmental Studies Hybridoma Bank; IHC, immunohistochemistry; WB, western blotting.

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Table S4 Differentially expressed genes in the E18.5 cochlea of the Igf1+/− mutant mouse ranked by gene ontology and biological process annotations. Genes were selected according to reported inner ear expression, their relation with deafness, biological function determined with the PANTHER and FATIGO programmes, as well as fold-change and low-variance calculated with multi-mgMOS software. nf, not described previously in the literature; nd, not determined by real-time PCR.

Average fold change from microarray experiments. Fold change determined by real-time PCR, nf, not determined; nd, not verified. References:

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Author Contributions

Conceived and designed the experiments: HS-C LRRdR JGP IVN. Performed the experiments: HS-C LRRdR JGP. Analyzed the data: HS-C LRRdR MM MH IVN. Contributed reagents/materials/analysis tools: MM MH JGP IVN. Wrote the paper: HS-C LRRdR MH JGP IVN.

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