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Serial Analysis of Gene Expression in the Chicken Otocyst

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ABSTRACT

The inner ear arises from multipotent placodal precursors that are gradually committed to the otic fate and further differentiate into all inner ear cell types, with the exception of a few immigrating neural crest-derived cells. The otocyst plays a pivotal role during inner ear development: otic progenitor cells sub-compartmentalize into non-sensory and prosensory domains, giving rise to individual vestibular and auditory organs and their associated ganglia. The genes and pathways underlying this progressive subdivision and differentiation process are not entirely known. The goal of this study was to identify a comprehensive set of genes expressed in the chicken otocyst using the serial analysis of gene expression (SAGE) method. Our analysis revealed several hundred transcriptional regulators, potential signaling proteins, and receptors. We identified a substantial collection of genes that were previously known in the context of inner ear development, but we also found many new candidate genes, such as *SOX4, SOX5, SOX7,* *SOX8, SOX11,* and *SOX18*, which previously were not known to be expressed in the developing inner ear. Despite its limitation of not being all-inclusive, the generated otocyst SAGE library is a practical bioinformatics tool to study otocyst gene expression and to identify candidate genes for developmental studies.

Keywords: gene array, inner ear development, otic vesicle, SAGE, Sox

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INTRODUCTION

The otic vesicle, or otocyst, is one of the earliest morphological manifestations of the vertebrate inner ear. It arises by invagination of the otic placode, which is an ectodermal thickening that develops near the developing hindbrain. This process happens in chicken embryos during the second and third days of embryonic development (E2–E3). In intermediate stages, the otic placode has folded inward to form a pouch that is also called otic pit. The otic pit subsequently pinches off from the surface ectoderm into the underlying mesenchyme, resulting in the formation of the otocyst.

It has been hypothesized that axis formation of the developing inner ear already happens during otocyst formation, where signals from the surrounding tissues result in the regionalization of the developing otic placode, pit, and otocyst (reviewed in Fekete 1996; Fekete and Wu 2002). Despite a complex patterning process that is already manifest at the otocyst stage by regionalized expression of specific markers (for a review, see Streit 2007), the otocyst itself is a remarkable structure because it contains all the necessary progenitor cells to form the major cell types of the inner ear. This autonomy was revealed by grafting otocysts into

other regions of the developing body (Swanson et al. 1990) as well as by determining that the otic lineage that is specified during otic induction has already reached a largely committed state when the otocyst is formed (Groves and Bronner-Fraser 2000).

Inner ear cell regeneration research has been utilizing the fact that stem cell-derived mammalian otic progenitor cells, defined by the expression of otocyst markers such as PAX2, PAX8, and DLX5, display a certain degree of commitment toward the otic lineage (Li et al. 2003a, b; Oshima et al. 2007, 2010). This was demonstrated by showing the potential of otic progenitors to differentiate into different cell types that express makers indicative of neurons, hair cells, and supporting cells. Based on these findings, it has been hypothesized that stem cell-derived otic progenitors are similar to otocyst cells (Brigande and Heller 2009; Diensthuber et al. 2009).

Here, we present the results of an unbiased interrogation of gene expression in the chicken otocyst. This analysis was spurred by the lack of comprehensive gene expression information at this important stage of development. We decided to utilize serial analysis of gene expression (SAGE), which is a quantitative method that can be used to identify known as well as new genes (Saha et al. 2002; Velculescu et al. 1995). Of the 39,326 seventeen-base pair sequence tags that we found, we evaluated 16,008 unique sequences that resulted in 4,153 unequivocally identified genes. Although our study lacked the sensitivity of more modern high-throughput deep sequencing methods, we consider the results as an important contribution because they provide a comprehensive summary of genes that are expressed at medium and high levels. Our analysis revealed potential signaling proteins and receptors as well as almost 300 transcriptional regulators that are expressed in the chicken otocyst. Some of these regulators have been previously known to play important roles during inner ear development, but we found additional candidate genes, such as several members of the Sox gene family, which have thus far

not been evaluated in the context of the developing inner ear.

METHODS

Tissue dissection, RNA preparation, and SAGE library preparation

Fertilized eggs (*Gallus gallus*) of the white leghorn strain (California Golden Eggs, Dinuba, CA) were incubated at 38°C for 72 h on a rocking platform in a humidified egg incubator (Sportsman 1202A, GQF Manufacturing, Savannah, GA). The embryos were removed from the eggs, washed in Hanks' balanced salt solution (HBSS; Invitrogen, Carlsbad, CA), and collected in HBSS. Staging was conducted according to Hamburger and Hamilton's (HH) guidelines (Hamburger and Hamilton 1992), and only HH stage 18–19 embryos with clearly identifiable closed otic vesicles and unpigmented eyes were used. Otic vesicles were microdissected using fine forceps and attention was given to remove as much of the surrounding periotic mesenchyme as possible (Fig. 1A). The vesicles were individually inspected and frozen in bulks of 10–20 in liquid nitrogen and stored at −80°C.

Total RNA was isolated using a commercial kit (RNeasy Mini Kit, Qiagen, Hilden, Germany). RNA integrity and quality was confirmed by gel electrophoresis and by visual assessment. Five micrograms total RNA, the combined yield of 200 otocysts, was used for SAGE library synthesis (I-SAGE Long kit, Invitrogen) starting with attaching polyA+ RNA to oligo(dT)-paramagnetic beads, reverse transcription, and second strand synthesis. The resulting cDNA was cleaved with *Nla*III, divided into two fractions, and bound to two different adapters containing a type IIS restriction nuclease recognition site. The adapters with adjacent 21-bp cDNA pieces were released from the oligo(dT)-magnetic beads using the type IIS restriction endonuclease *Mme*I. The two pools of released adapter-linked tags were ligated to 130-bp
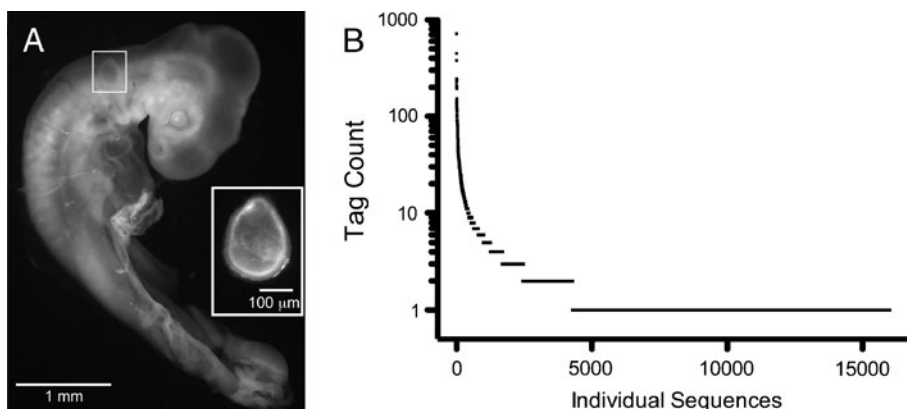

FIG. 1. SAGE library from the chicken otocyst. **A** HH stage 18–19 chicken embryo. The otocyst is indicated with a *white box. Inset*, otocyst after dissection. **B** SAGE tag frequency (tag count) plot for all tags identified.

ditags and amplified with 27 PCR cycles with specific primers to each adapter. The adapters were then cleaved with *Nla*III, 34-bp ditags were purified from the adapters by polyacrylamide gel electrophoresis, and concatenated. Concatemers were fractioned by size, gel-purified, and then cloned into pZErO-1 provided with the kit.

Quality control was conducted in two steps. First, 20 colonies were picked, plasmid DNA was prepared, and the resulting plasmids were digested with *Nsi*I, which resulted in the release of the individual inserts. We obtained 20 distinct restriction patterns with a mean insert length of 671 bp (±425 bp (SD)). The smallest fragment was 200 bp and the largest was 1,700 bp. In a second step, we sequenced 24 inserts, obtaining 12,025 bp of raw data. These insert lengths slightly exceeded the expected ≈25 SAGE tags per clone predicted by the kit manufacturer. Three thousand eight hundred forty colonies were robotically picked and the plasmid concatemer inserts were directly sequenced (GeneWiz, South Plainfield, NJ).

Modifications from the manufacturer's protocol included that the gel electrophoreses for the 130- and 34-bp ditags were performed on 10% polyacrylamide gels (Novex TBE Gels, Invitrogen) and that DNA was isolated from polyacrylamide gels with QIAEXII beads (Qiagen). Concatemers were separated on a 2% agarose gel and purified before subcloning with a column-based gel extraction kit (QIAquick, Qiagen).

Data analysis

At the highest stringency settings for DNA sequence quality and tag extraction, 39,326 individual 17-bp tags were extracted from the 3,512 sequencing data files using SAGE2000 analysis software (version 4.5, Invitrogen). Only unequivocal sequences were used for library construction, which resulted in a library about three times smaller than predicted by the quality control samples. A potential reason for this shortfall might be that the sequence quality achieved with direct sequencing was lower than the sequencing results obtained with plasmid DNA, which was used for the quality control clones. For computerized mapping, we appended the *Nla*III restriction site (5′-CATG-3′) to the 5′ end of each tag. The resulting 21-bp tags were mapped using 22,290 *G. gallus* cDNA sequences available through the Ensembl 52 database (www.ensembl.org/info/data/ftp/), 19,307 *G. gallus* cDNAs available from the RefSeq database (ftp.ncbi.nih.gov), and 33,383 *G. gallus* sequences from the Unigene database (www.ncbi.nlm.nih.gov/unigene). Mapping was conducted using MAQ software (Li et al. 2008; maq.sourceforge.net) with all parameters set to default allowing for two mismatches in the sequence alignments. Ingenuity Pathway Analysis (IPA) software was

accessed via the Stanford University Bioinformatics Resource (cmgm.stanford.edu).

Reverse transcriptase PCR

Chicken otocyst RNA was isolated (Absolutely RNA Miniprep Kit, Stratagene/Agilent Technologies, La Jolla, CA) and treated with RNase-free DNase I (Roche Diagnostics, Mannheim, Germany). The RNA concentration was determined by spectrophotometric analysis using a NanoDrop (Thermo Fisher Scientific, Wilmington, DE). Total RNA extracts were then used for reverse transcription (RT) into cDNA (first strand) using SuperScript III Reverse Transcriptase (Invitrogen) and Oligo(dT)$_{18}$ primer (Invitrogen) with 350 ng of total RNA per 20 μl reaction. To prevent RNA degradation, 1 μl RiboLock RNase Inhibitor (Fermentas, Thermo Fisher Scientific, Glen Burnie, MD) was also included in each reaction. Control reactions were done without reverse transcriptase.

Oligonucleotide primer pairs were designed for each gene of interest using NCBI Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast) with NCBI Reference Sequences as template. For each gene tested, at least two primer pairs covering two non-overlapping 300- to 700-bp regions were used to confirm mRNA expression. A full list of primers tested can be found in Electronic supplementary materials (ESM) Table 1. PCR was performed using GoTaq Green Master Mix (Promega, Madison, WI) with 2 μl cDNA template and 1 μl 400 nM each of forward and reverse gene-specific primers. The following cycling conditions were employed: initial denaturation at 94° C (3 min); 30 cycles of denaturation at 94°C (30 s), annealing at 55°C (1 min), and elongation at 72°C (1 min); and a hold at 4°C. Aliquots of PCR products were electrophoresed in a 2.0% agarose gel, stained with SYBR Safe (Invitrogen) in 1X TAE buffer at 120 V for 35 min, and documented using UV trans-illumination and digital photography (Kodak Gel Logic 200 Imaging System).

In situ hybridization

The T7 promoter sequence (5′-TAATACGACTCAC TATAGGG-3′) was added to the 5′ end of the forward or reverse primer for the different Sox2 cDNAs to allow for conversion of the PCR product to sense and antisense cRNA probes for in situ hybridization. Of the PCR product, 500 ng was used to synthesize digoxigenin-labeled antisense probes (DIG RNA Labeling Kit, Roche Diagnostics), which were resuspended in 30 μl RNAse-free water. Embryos were dissected at HH stage 18–19 (E3) and HH stage 26–28 (E5), fixed overnight with 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4), transferred

into 30% sucrose in PBS for 24–36 h, and embedded in O.C.T compound (Tissue-Tek). Sections were cut with a cryomicrotome (CM3050 S, Leica), collected on ultrastick slides (precleaned Gold Seal, Rite-on, Micro Slides), dried at 37°C for 45 min, and stored frozen at −70°C. For hybridization, the sections were brought to room temperature and rehydrated in 100 µl diluted probe (1:100) in 50% formamide, 10% dextran sulfate, 1 mg/ml yeast RNA, 1x Denhardt's solution, 185 mM NaCl, 5.6 mM NaH$_2$PO$_4$, 5 mM Na$_2$HPO$_4$, 5 mM EDTA, and 15 mM Tris at pH 7.5. After coverslipping and overnight incubation at 65°C in a chamber humidified with 50% formamide in 150 mM NaCl, 15 mM trisodium citrate, pH 7 (1× SSC), the coverslips were removed in 5x SSC and the slides washed twice for 30 min each in 50% formamide and 0.1% Triton X-100 in 1x SSC at 65°C. Thereafter, the slides were washed for 15 min in 0.2x SSC and for 15 min in 150 mM NaCl and 100 mM Tris at pH 7.5 at room temperature. For antibody detection, the sections were blocked for 30 min in 0.5% blocking powder (Roche Diagnostics), 10% heat-inactivated goat serum, 100 mM NaCl, 0.1% Triton X-100, and 100 mM Tris at pH 7.5. The slides were then incubated for 2 h at room temperature in a blocking solution pre-incubated for 1 h with alkaline phosphatase-conjugated anti-digoxigenin Fab fragments (1:500, Roche Diagnostics). Unbound Fab fragments were removed by washing twice for 30 min each in 150 m NaCl and 100 mM Tris at pH 7.5. The sections were first incubated in detection buffer (100 mM NaCl, 50 mM MgCl$_2$, 100 mM Tris at pH 9.5) for 10 min. For detection, the sections were then covered with 200 µl of chromogen solution consisting of 20 µl NBT/BCIP stock solution (Roche Diagnostics) and 50 µl

Levamisol stock solution (20x concentrate, Invitrogen) in 1 ml detection buffer, coverslipped, and incubated overnight at room temperature in a humidified chamber. Coverslips were removed and color reaction was stopped in 1 mM EDTA and 10 mM Tris at pH 8.1. Slides were embedded in 50% glycerol in PBS and coverslipped. Analysis and photography was conducted on an Axiovert 25 microscope with an AxioCam MRC camera, using AxioVision software (V 4.6.3.0, Zeiss).

RESULTS

SAGE library of the chicken otocyst

Otocysts were dissected from HH stage 18–19 chicken embryos (Fig. 1A), total RNA was extracted, and subjected to a commercial long-SAGE protocol, resulting in a library of concatemerized tags. Individual clones of SAGE concatemers were sequenced, resulting in 39,326 seventeen-base pair tags with tag counts up to 718 for the most abundant tag; 3,292 tags were represented between two and five times, whereas the majority of tags (11,717) were only found once (Fig. 1B). Overall, we identified 16,008 unique sequence tags (ESM Table 2; NCBI Geo DataSet accession no. GSM651351).

Although the chicken genome has been fully sequenced (The Chicken Genome Consortium 2004), the annotation of chicken genes is far from complete, and consequently, gene annotation needed to be conducted by combining several database resources (Fig. 2). We started by mapping all 16,008 tags consisting of the 4-bp *Nla*III restriction site and each individual 17-bp SAGE tag using the MAQ software (Li et al. 2008). Three reference databases were used—Ensembl 52, RefSeq,
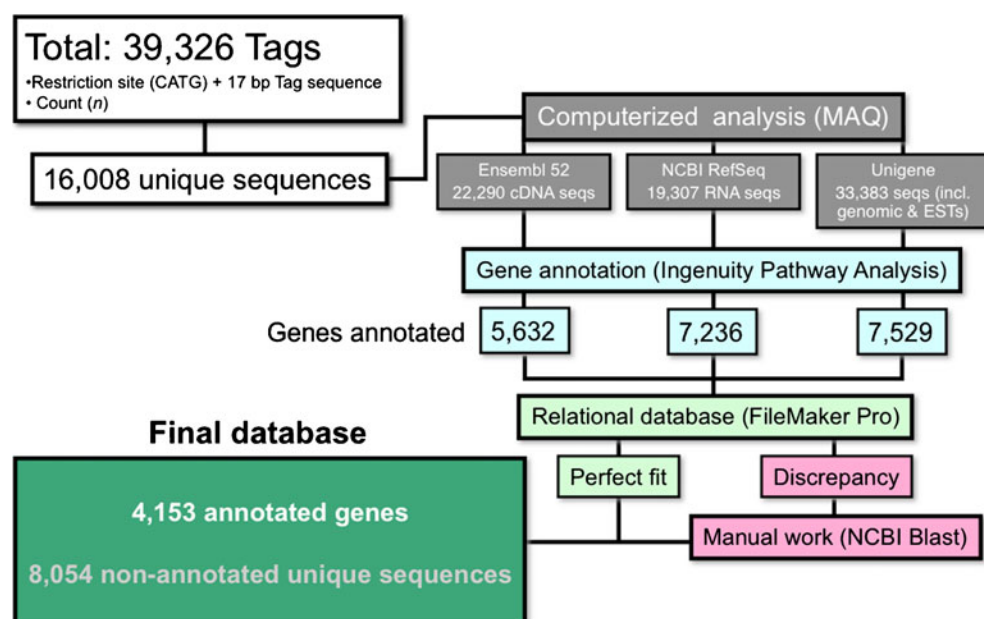

FIG. 2. Flowchart of SAGE tag analysis resulting in a library of 4,153 annotated genes expressed in the chicken otocyst.

and Unigene—resulting in 7,026, 8,682, and 13,405 matches, respectively. To compare the matches from the three different databases that were composed of assortments of unofficial and official gene names, we imported the results into the IPA software, which revealed official gene symbols as common identifiers. IPA software recognized 5,632, 7,326, and 7,529 gene identifiers from Ensembl, RefSeq, and Unigene database matches, respectively. In a next step, official gene symbols created in IPA were used to directly compare matches of different databases using a relational database. Only when all three different databases suggested a match for a given gene was the gene accepted for further analysis. In case of discrepancies in matches between different databases or in case of missing matches in some of the databases, we performed manual BLAST searches (blast.ncbi.nlm.nih.gov/Blast.cgi) with each tag. If a match was found, the resulting gene was added for further analysis. In 52 cases, a single tag corresponded to two different genes, and in one case, a single tag corresponded to five different genes. These 53 ambiguous tags (ESM Table 3) were removed from further analysis. After this step, the library consisted of 7,912 gene matches with unique tags. However, many genes were represented in the library with two or more different tags, which could be due to alternative poly-adenylation sites, internal priming, or alternative splicing near the 3′ end of the mRNA. These duplicates were combined, resulting in 4,180 genes that were re-imported into IPA for final analysis. At this final step, IPA recognized 4,153 genes, which in the final annotated SAGE library were associated with the aggregate count number of all the tags of every given gene (ESM Table 4).

Gene annotation reveals abundance of transcriptional regulators

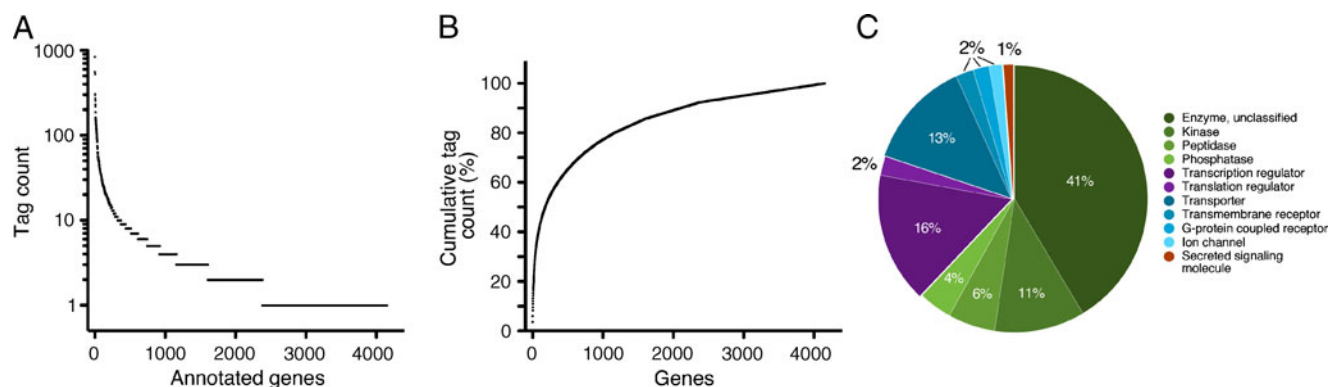Analysis of the relation between tag count and annotated gene number revealed that 50% of all tags in the otocyst were encoded by only 180 genes, whereas the majority of genes were represented by fewer than 10 tags (Fig. 3A, B). Not unexpectedly, the genes with the highest tag count were housekeeping genes involved in the maintenance of basal cellular functions (Table 1). The IPA database provided unequivocal gene family information for about half of the identified genes. Analysis of all unambiguous gene family annotation identifiers revealed that the majority of otocyst genes encoded unclassified enzymes as well as kinases, phosphatases, and peptidases. The second largest family of genes identified encoded transcriptional regulators, followed by transporters, transmembrane domain-containing receptors, and ion channels (Fig. 3C). Translational regulators, growth factors, cytokines, and other protein families comprised the rest.

Overall, we identified 299 genes that encode transcriptional regulators (ESM Table 5) which can be categorized into transcription factors containing zinc-coordinating DNA-binding domains (11%), helix-loop-helix domains (13%), basic domains (15%), ß-scaffold factors with minor groove contacts (16%), and others (45%). Fifty-one transcriptional regulators were previously known to be expressed in the developing inner ear. Known examples for each respective category are *GATA2* and *GATA3* (Lillevali et al. 2007) for zinc-coordinating DNA-binding domains, *PAX2* and *FOXG1* (Herbrand et al. 1998; Li et al. 2004; Pauley et al. 2006) for helix-loop-helix domains, *NEUROG1* and *NEUROD1* (Liu et al. 2000; Ma et al. 2000) for basic domains, and *SOX10* and *NOTCH1* (Lewis et al. 1998; Stone and Rubel 1999; Watanabe et al. 2000) for ß-scaffold factors with minor groove contacts. Two hundred forty-eight transcriptional regulators were previously unknown in the context of early inner ear development.

Secreted proteins and transmembrane proteins

Genes that encode growth factors, cytokines, and other secreted proteins are the second group of

FIG. 3. SAGE library after annotation. **A** Tag count frequency plot for all 4,153 annotated genes. **B** Cumulative tag count analysis reveals that 180 of the most abundantly expressed annotated genes represent 50% of all identified tags. **C** Family representation of all unambiguously identified annotated genes.

TABLE 1

Genes with the highest expression based on SAGE tag count

Gene symbol	Count	Family	Gene name
COX1	840	Enzyme	Cytochrome c oxidase I
ARIH1	560	Enzyme	Ariadne homolog, ubiquitin-conjugating enzyme E2 binding protein, 1
COX2	526	Enzyme	Cytochrome c oxidase II
COX3	307	Enzyme	Cytochrome c oxidase III
ATP6	298	Transporter	ATP synthase 6, ATPase subunit 6
NPM1	273	Transcription regulator	Nucleophosmin (nucleolar phosphoprotein B23, numatrin)
ND4	257	Enzyme	NADH dehydrogenase, subunit 4 (complex I)
GAPDH	254	Enzyme	Glyceraldehyde-3-phosphate dehydrogenase
RPL13	230	Ribosomal structure	Ribosomal protein L13
RPL10A	218	Ribosomal structure	Ribosomal protein L10a
RPL4	187	Ribosomal structure	Ribosomal protein L4
TUBA4A	162	Cytoskeletal structure	Tubulin, α4a
RPL23	161	Ribosomal structure	Ribosomal protein L23
RPS27A	156	Ribosomal structure	Ribosomal protein S27a
MDK	154	Growth factor	Midkine (neurite growth-promoting factor 2)
ND5	151	Enzyme	NADH dehydrogenase, subunit 5 (complex I)
RPS3	146	Ribosomal structure	Ribosomal protein S3
EEF1A1	142	Translation regulator	Eukaryotic translation elongation factor 1 α1
ATP5B	139	Transporter	ATP synthase, H+ transporting, mitochondrial F1 complex, β polypeptide
RPS29	136	Ribosomal structure	Ribosomal protein S29
RPS27L	131	Ribosomal structure	Ribosomal protein S27-like
ACTB	124	Cytoskeletal structure	Actin, β
RPS15	120	Ribosomal structure	Ribosomal protein S15
TUBB2A	118	Cytoskeletal structure	Tubulin, β 2A
RPLP1	116	Ribosomal structure	Ribosomal protein, large, P1
RPS3A	112	Ribosomal structure	Ribosomal protein S3A
RPL21	107	Ribosomal structure	Ribosomal protein L21
RPL36	102	Ribosomal structure	Ribosomal protein L36
RPL35	96	Ribosomal structure	Ribosomal protein L35
HNRNPA3	94	Nucleic acid binding	Heterogeneous nuclear ribonucleoprotein A3

developmentally interesting otocyst genes (ESM Table 6). Of the 172 genes that we identified in this group, several were previously known in inner ear development and include *BMP7, FGF10, FGF19, FRZB, TGFß2, NETRIN1, SLIT1, WNT3*, and *WNT5A* (Abraira et al. 2008; Alsina et al. 2004; Battisti and Fekete 2008; Hollyday et al. 1995; Liu et al. 2008; Oh et al. 1996; Okano et al. 2005; Sanchez-Calderon et al. 2007; Sienknecht and Fekete 2009). Transcripts encoding the secreted signaling protein midkine (MDK) were by far the most abundantly expressed mRNA that we detected. Midkine has been previously reported in the postnatal mouse cochlea, and it has been shown that the protein is involved in regulating the expression of the tectorial membrane component ß-tectorin (Jia et al. 2001; Zou et al. 2006), but early developmental roles in the inner ear have not been reported. Other proteins, such as opticin (OPTC), have previously been shown in the otic vesicle, but their function in inner ear development remains unknown (Frolova et al. 2004). Several genes emerged in our screen as novel candidates for roles in inner ear development, such as olfactomedin-like 2A, 2B, 3 (*OLFML2A, OLFML2B, OOLFML3*), which belong to a class of

proteins implicated in a variety of developmental processes (reviewed by Tomarev and Nakaya 2009), or neudesin (*NENF*), which may play roles in neuronal differentiation and development (Kimura et al. 2006). We identified various TGFß antagonists such as twisted gastrulation protein homolog 1 (*TWSG1*) or follistatin-like 3 (*FSTL3*). Lastly, we identified various secreted proteins of unknown function during development, but with previous implications in cancer or other cell growth- and death-related processes; examples for these proteins are AGR3, CLU, EGFL7, and HDGF.

Our analysis of transmembrane-spanning proteins revealed high transcript expression levels of many tight junction and cell adhesion proteins such as claudin 1 (CLDN1), CLDN3, and CLDN17; integrins α3 (ITGA3) and α6 (ITGA6); integrins ß1, ß2, ß3, and ß5 (ITGB1, ITGB2, ITGB3, ITGB5); neurexin 1 (NRXN1); as well as cell adhesion molecule 1 (CADM1) and epithelial cell adhesion molecule TACSTD1, among others. One of the most abundant genes identified in this category encodes protein tyrosine kinase 7 (PTK7), a protein implicated in the regulation of planar cell polarity, convergent extension, and Wnt signaling (Lu et al. 2004; Puppo et al. 2011; Yen et al. 2009). Another interesting

gene in this regard encodes the Ig superfamily protein protogenin (PRTG), which has been shown to play a role in suppressing premature neural differentiation and whose roles in other tissues might similarly be in controlling the timing of transitions between early progenitor state and differentiation (Ito et al. 2011; Wong et al. 2010). Probably the most interesting group of genes that we identified encodes receptors for signaling proteins because they might reveal information about the developmental processes happening in the otocyst. These include genes that encode receptors for ligands that are already known for playing roles in otic development such as *FGFR1, FZD1, FZD2, FZD3, FZD4, FZD7, NGFR,* and *NOTCH1,* which have previously been shown to be expressed in the vertebrate otocyst (Adam et al. 1998; Pirvola et al. 2002; Sienknecht and Fekete 2009; Stevens et al. 2003; von Bartheld et al. 1991; Wright and Mansour 2003). *BMPR1, BMPR2, LGFR1, SMO1, PTCH1, DISP1,* and *TGFBR2* are genes that were presumed to be expressed in the otocyst because their ligands, such as BMPs and other TGFß family members, IGF, as well as hedgehog signaling proteins, have been shown to be expressed and active during inner ear development (Bok et al. 2005; Frenz et al. 1991, 1992; Liu et al. 2002; Oh et al. 1996; Riccomagno et al. 2002; Yamashita and Oesterle 1995). Other identified genes include receptors for somatostatin (*SSTR1*), interleukin 11 (*IL11RA*), endothelin (*EDNRB*), and tumor necrosis factors (*TNFRSF1A, TNFRSF6B, TNFRSF19*) and orphan receptors such as lathrophilin 3 (*LPNHN3*; Sudhof 2001).

Other potentially interesting transcripts encoded transmembrane proteins involved in cell recognition and adhesion that play roles in axonal guidance and cell migration such as the semaphorins SEMA4B, SEMA5B, SEMA6D, SEMA7A and some components of their receptor complex such as Plexins A1 and B2 (PLXNA1, PLXNB2; Perrot et al. 2002). Additional genes with similar roles include ephrin B1 (*EFNB1*) and ephrin receptors (*EPHA4, EPHA5, EPHB3*), netrin G1 and the netrin receptor *UNC5B,* the Slit receptors *ROBO1* and *ROBO2,* as well as the Slit-like transmembrane protein *SLITRK6.* The possible roles of some of these genes in axon guidance and cell migration has been discussed in the context of the inner ear (Fekete and Campero 2007; Webber and Raz 2006), and their expression patterns and potential function are the focus of intensive research (Battisti and Fekete 2008; Katayama et al. 2009; Matilainen et al. 2007).

As initial validation of mRNA expression, we conducted RT-PCR on otic vesicle RNA template with oligonucleotide primer pairs specific for selected genes representing transcriptional regulators, signaling proteins, receptors, and other genes of interest (Fig. 4 and Table 2). Transcripts for all selected genes were detectable, and although the RT-PCR was not conducted in quantitative fashion, we observed a general trend where strong amplification products corresponded to genes with higher SAGE tag counts.

Known and novel Sox genes expressed in the otocyst

One of the most strongly represented groups of transcription factors in the chicken otocyst SAGE library were the Sox genes. Previous reports show the expression of *SOX1, SOX2, SOX3, SOX6, SOX9, SOX10,* and *SOX21* in the chicken otocyst, or in the otic vesicle of various species including African clawed frog, zebrafish, and mouse (Barrionuevo et al. 2008; Liu et al. 2003; Neves et al. 2007; Uchikawa et al. 1999; Watanabe et al. 2000). Clearly highlighting the limitation of SAGE, showing that about 40,000 tags are far from exhaustive, is the fact that tags for *SOX2, SOX3,* and *SOX6* were not represented in our SAGE library. Nevertheless, we found six Sox genes that previously were not known to be expressed in the developing inner ear, which include *SOX4, SOX5, SOX7, SOX8, SOX11,* and *SOX18.*

We verified the expression of the Sox genes by RT-PCR (Fig. 4) and by in situ hybridization at the otocyst stage (Fig. 5). *SOX8* and *SOX11* were abundantly expressed throughout the otocyst and also in the adjacent hindbrain. *SOX4* mRNA appeared to be more regionalized with stronger expression in the ventromedial regions of the otocyst, indicative of a potential subsequent expression in the prosensory domains. *SOX5* transcripts were weakly detectable throughout the otocyst, with a possible stronger expression in the dorsolateral part of the otocyst. *SOX7* and *SOX18* mRNA was detectable throughout the otocyst, perhaps with a slightly stronger expression in the ventral portion.

At E5, when the basilar papilla and vestibular compartments of the chicken inner ear are clearly defined and prosensory domains have been formed, only *SOX11* appeared to be specifically associated with the prosensory domains of the basilar papilla and vestibular maculae (Fig. 6). *SOX7* was no longer detectable, whereas *SOX4* and *SOX5* transcripts were detectable in the roof of the basilar papilla, presumably in the premordial tegmentum vasculosum. *SOX8* expression was strong throughout the inner ear as well as in the adjacent hindbrain (not shown).

DISCUSSION

The chicken embryo is one of the major animal models used to study inner ear induction and development. In the past decades, many genes have been found that are expressed by cells of the otocyst, and the specific roles of some of these genes have been
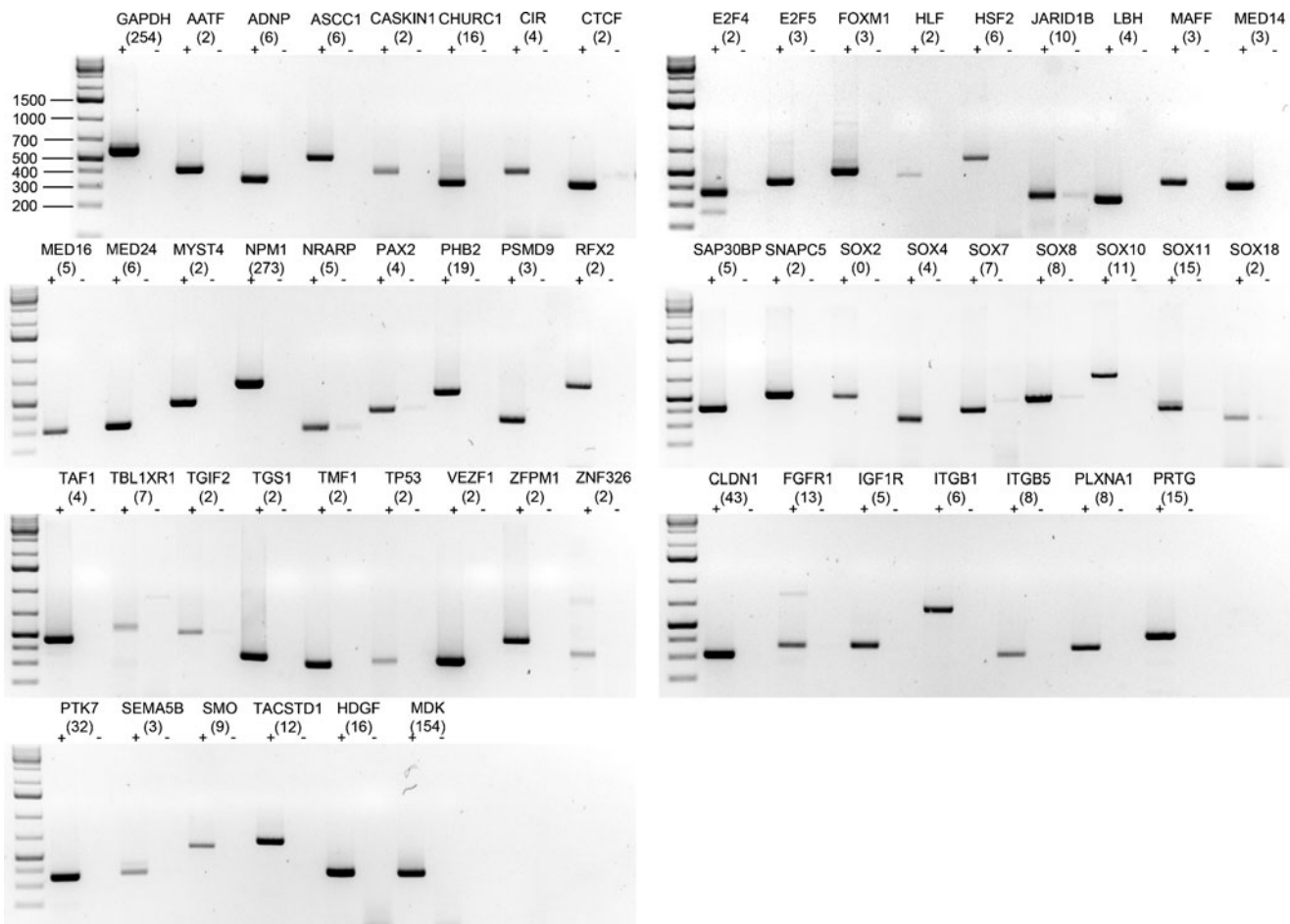
FIG. 4. Shown are agarose gels stained with ethidium bromide to visualize RT-PCR fragments for genes expressed in the chicken otocyst (indicated with "+"). Control reactions without reverse transcription are labeled with "−". Faint bands in the "−" control lanes are likely the result of residual genomic DNA contamination. The corresponding gene names are listed in Table 2. Predicted PCR product sizes are listed in ESM Table 1. SAGE tag count numbers are indicated in *parentheses*.

elucidated. Nevertheless, no comprehensive study has been conducted on gene expression in the chicken otocyst. We hypothesized that the existing collection of otocyst markers and genes is just the tip of an iceberg, and we consequently decided to investigate, using a high-throughput method, gene expression in this clearly defined transient structure. Unlike the mouse and human genomes, the chicken genome is comparably poorly annotated, which complicated the analysis strategy. We refrained from using gene arrays whose preselected genes are constrained by these shortcomings. Additionally, at the onset of this study, no comprehensive chicken gene arrays were commercially available and next-generation sequencing techniques, likewise, were not yet developed. We decided to employ SAGE, which is a relatively unbiased method, based on sequencing of short tags that are directly adjacent to a *Nla*III restriction site in the 3′ region of any given mRNA (Velculescu et al. 1995). The *Nla*III recognition sequence is 4-bp long (5′-CATG-3′) and theoretically occurs once in every 256 bp. Using long-SAGE (Saha et al. 2002), which

employs 17-mer tags instead of 10-mer tags, which were used in initial SAGE protocols, we were able to utilize a specificity of 4^{21}. Indeed, we only found 53 ambiguous tags, which either occur more than once in the transcriptome or were associated with more than one gene as a result of annotation ambiguities.

Our analysis is not based on comparative or subtractive studies, and consequently, many genes identified are widely expressed. Nevertheless, the results of our study do not preclude the use of bioinformatic tools to extract subtractive or otherwise user-defined datasets, and the reader is encouraged to use our dataset as needed. A recent very elegant gene array study focusing on FGF-based otic induction in mouse embryos is an example of the powerful specificity that can be achieved by selecting proper tissues for comparison (Urness et al. 2010). In this specific case, wild-type mouse otic placode tissue was compared with tissue from the prospective otic placode of $Fgf3^{-/-}$;$Fgf10^{-/-}$ mice in which otic development fails to be initiated. This study revealed several transcriptional regulator genes that depend on FGF-based otic induction, including *Hmx2*, *Hmx3*, *Foxg1*,

TABLE 2

Index for gene names shown in Figure 4

| Gene symbol	Gene name	Tag count
Housekeeping genes		
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	254
Transcriptional regulators		
AATF	Apoptosis antagonizing transcription factor	2
ADNP	Activity-dependent neuroprotector homeobox	6
ASCC1	Activating signal cointegrator 1 complex subunit 1	6
CASKIN1	CASK interacting protein 1	2
CHURC1	Churchill domain containing 1	16
CIR	CBF1 interacting corepressor	4
CTCF	CCCTC-binding factor (zinc finger protein)	2
E2F4	E2F transcription factor 4, p107/p130-binding	2
E2F5	E2F transcription factor 5, p130-binding	3
FOXM1	Forkhead box M1	3
HLF	Hepatic leukemia factor	2
HSF2	Heat shock transcription factor 2	6
JARID1B	Jumonji, AT rich interactive domain 1B	10
LBH	Limb bud and heart development homolog (mouse)	4
MAFF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)	3
MED14	Mediator complex subunit 14	3
MED16	Mediator complex subunit 16	5
MED24	Mediator complex subunit 24	6
MYST4	MYST histone acetyltransferase (monocytic leukemia) 4	2
NPM1	Nucleophosmin (nucleolar phosphoprotein B23, numatrin)	273
NRARP	NOTCH-regulated ankyrin repeat protein	5
PAX2	Paired box 2	4
PHB2	Prohibitin 2	19
PSMD9	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 9	3
RFX2	Regulatory factor X, 2 (influences HLA class II expression)	2
SAP30BP	SAP30 binding protein	5
SNAPC5	Small nuclear RNA activating complex, polypeptide 5, 19 kDa	2
SOX2	SRY (sex determining region Y)-box 2	0
SOX4	SRY (sex determining region Y)-box 4	4
SOX7	SRY (sex determining region Y)-box 7	7
SOX8	SRY (sex determining region Y)-box 8	8
SOX10	SRY (sex determining region Y)-box 10	11
SOX11	SRY (sex determining region Y)-box 11	15
SOX18	SRY (sex determining region Y)-box 18	2
TAF1	TAF1 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 250 kDa	4
TBL1XR1	Transducin (beta)-like 1 X-linked receptor 1	7
TGIF2	TGFB-induced factor homeobox 2	2
TGS1	Trimethylguanosine synthase homolog (S. cerevisiae)	2
TMF1	TATA element modulatory factor 1	2
TP53	Tumor protein p53	15
VEZF1	Vascular endothelial zinc finger 1	2
ZFPM1	Zinc finger protein, multitype 1	2
ZNF326	Zinc finger protein 326	2
Transmembrane proteins		
CLDN1	Claudin 1	43
FGFR1	Fibroblast growth factor receptor 1	13
IGF1R	Insulin-like growth factor 1 receptor	5
ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	6
ITGB5	Integrin, beta 5	8
PLXNA1	Plexin A1	8
PRTG	Protogenin homolog (Gallus gallus)	15
PTK7	PTK7 protein tyrosine kinase 7	32
SEMA5B	Sema domain, seven thrombospondin repeats (type 1 and type 1-like), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 5B	3
SMO	Smoothened homolog (Drosophila)	9
TACSTD1	Epithelial cell adhesion molecule	12
Growth factors		
HDGF	Hepatoma-derived growth factor (high-mobility group protein 1-like)	16
MDK	Midkine (neurite growth-promoting factor 2)	154
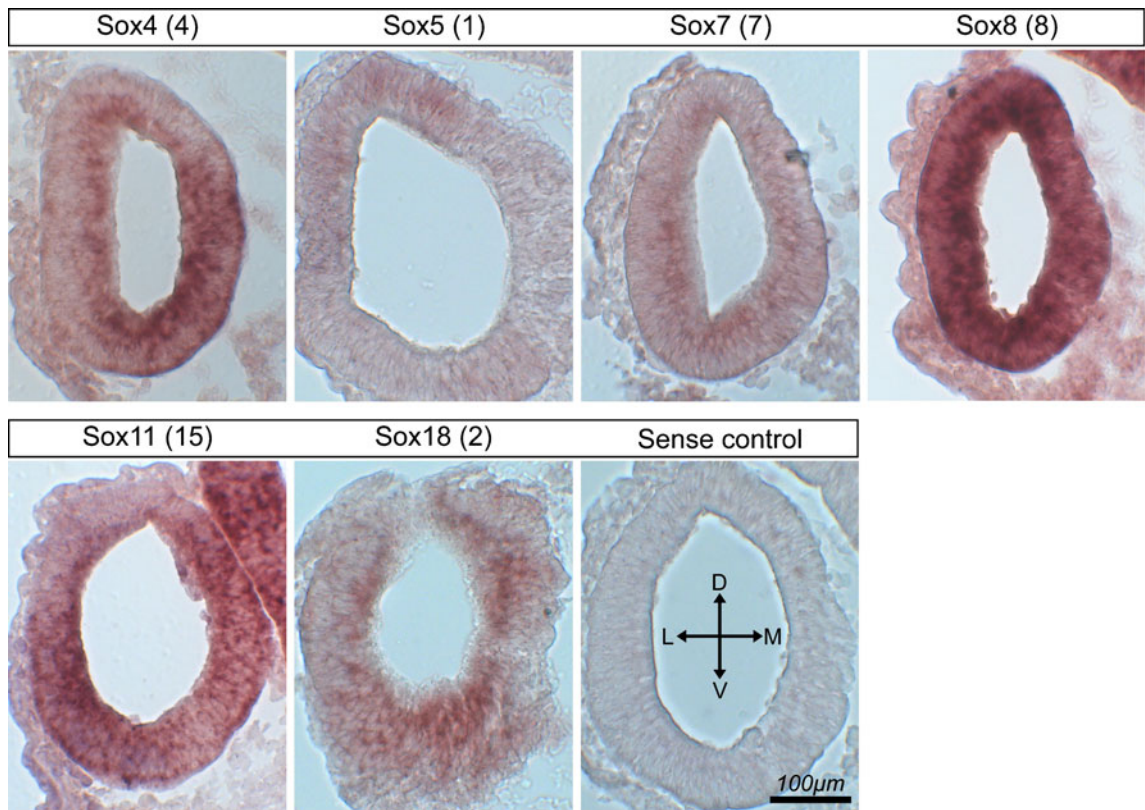
FIG. 5. In situ hybridization analysis of Sox gene expression in sections of the chicken otocyst. *Numbers in parentheses* indicate the SAGE tag count for each individual gene. Sense controls were negative for all probes used; a representative control is shown.

and *Sox9*, which we also found in our dataset. Other studies that focused on the identification of otocyst genes used differential display of chicken otocyst RNA against RNA from surrounding tissues (Gong et al.
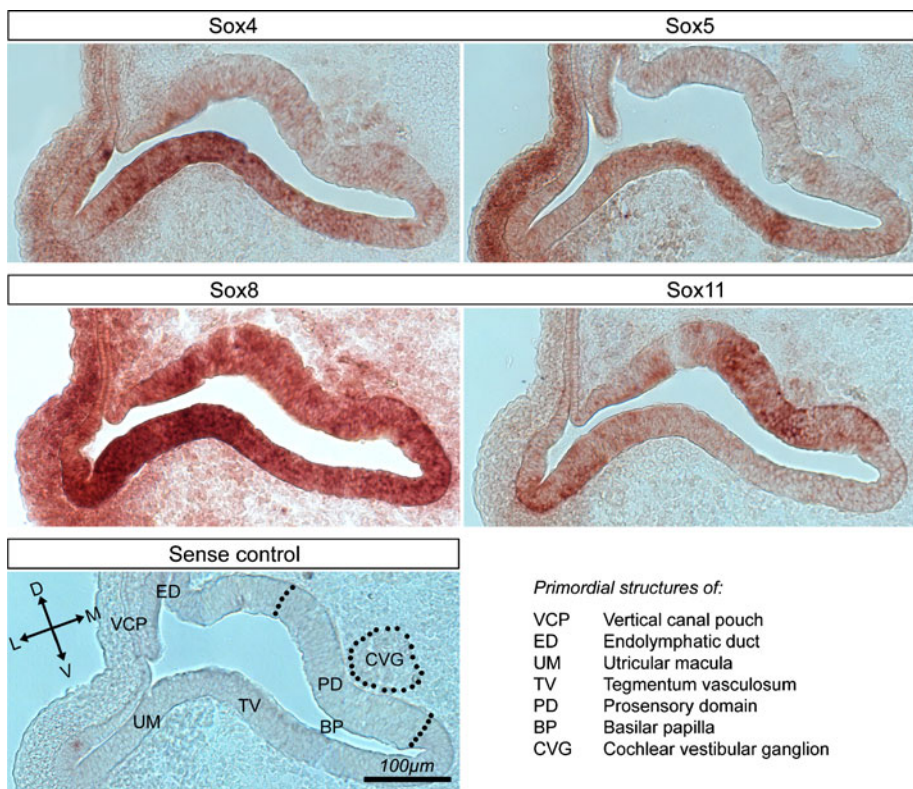

FIG. 6. In situ hybridization analysis of Sox gene expression in cross-sections of the E5 chicken inner ear. Sense controls were negative for all probes used; a representative control is shown.

Primordial structures of:

VCP	Vertical canal pouch
ED	Endolymphatic duct
UM	Utricular macula
TV	Tegmentum vasculosum
PD	Prosensory domain
BP	Basilar papilla
CVG	Cochlear vestibular ganglion

1997) and on cDNA subtraction of mouse otocyst minus liver cDNA (Powles et al. 2004). The differential display study identified only a small number of unknown genes, and the collection of 280 specific transcripts found in the mouse otocyst cannot be directly compared with our data because the dataset was only partially annotated and has not been deposited in a format usable for in silicio comparison, for example via the NCBI Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/).

An obvious limitation of the SAGE method is the number of tags which results in libraries that are reasonable large, but that are far from exhaustive, particularly when dealing with complex tissues consisting of different cell types. Analysis of our chicken otocyst dataset clearly revealed this limitation. For example, known and easily detectable otocyst genes such as *SOX2*, *PAX8*, and *FOXI3* (Groves and Bronner-Fraser 2000; Ohyama and Groves 2004; Uchikawa et al. 1999; Wood and Episkopou 1999) were not represented in our library, and 45% of all annotated tags were only represented once. The consequence of this limitation is probably a major reason why the SAGE method appears to be a transient technology that is in the process of being replaced with much more comprehensive and massive parallel next-generation sequencing methods capable of generating datasets of

tens of millions of tags with a single run. Likewise, microarray and cross-species comparison methods are becoming increasingly more accessible to study gene expression in avian species and have already been successfully used in recent years (Hawkins et al. 2007).

Despite these limitations, we were able to identify a large number of genes that previously were not known in the context of early inner ear development. Particularly transcriptional regulators were well represented in the library, and we were able to validate the expression of SOX4, SOX5, SOX7, SOX8, SOX11, and SOX18, all of which were previously not known to be expressed in the early developing inner ear. Analysis of the interaction networks of these transcription factors revealed some relation to various signaling pathways, including FGF, TGFß/BMP, EGF, and IGF signaling (Fig. 7). Furthermore, we showed for the first time the expression of SoxC group genes (*SOX4* and *SOX11*) as well as SoxF genes (*SOX18*) in the developing otocyst. We are just beginning to understand the interplay of different signaling pathways and the particular roles of Sox transcription factors in specific developmental processes. Previous studies with mouse Sox genes that are expressed in the otocyst show involvement in placode invagination for *Sox9* (Barrionuevo et al. 2008) as well as prosensory specification for *Sox2* (Kiernan et al. 2005). The
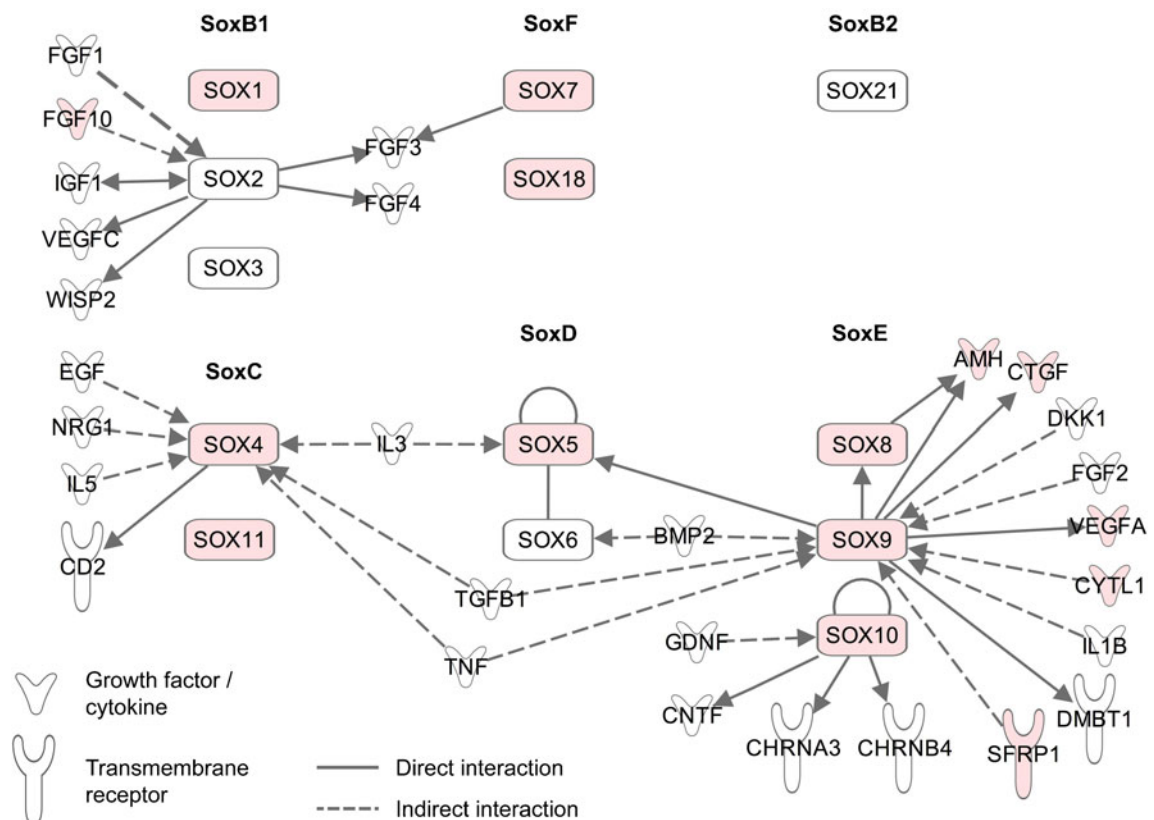

FIG. 7. IPA software analysis of direct and indirect interactions of inner ear Sox genes with growth factors and transmembrane receptors. Genes present in the annotated chicken otocyst SAGE library are labeled in *pink*.

overlapping expression of many Sox genes combined with a potential functional redundancy will make it difficult to study individual Sox gene function in the otocyst because single knockouts might lack a phenotype. Phylogeny, neofunctionalization, and redundancies within the Sox gene family have recently been comprehensively reviewed, highlighting the importance of this group of genes in many developmental processes (Guth and Wegner 2008).

Our analysis also revealed many other potentially important genes that have not previously been considered in the context of inner ear development, and some have just recently been investigated. We found a number of secreted proteins that are novel candidates for signaling functions in the developing otocyst. Transmembrane proteins consisted of members of previously known families of proteins that are essentially involved in inner ear development such as receptors for FGFs, BMPs, and WNTs, as well as NOTCH1, among others. Interestingly, we found a relatively large group of proteins belonging to families that have been implicated in axonal guidance and cell migration; some of these proteins have previously been shown to be expressed in the otocyst and other developmental stages of the inner ear (Battisti and Fekete 2008; Matilainen et al. 2007). The expression and function of Slit-like transmembrane protein SLITRK6, for example, has recently been analyzed in mouse inner ear development (Katayama et al. 2009). Slitrk6 is strongly expressed in the prosensory and sensory patches of the auditory and vestibular organs; the innervation density of these organs was reduced or abolished in $Slitrk6^{-/-}$ mice.

In summary, we have used the SAGE method to assemble a list of sequence tags that can be associated with gene expression in the chicken otocyst. Although not all-inclusive, this SAGE library is a practical bioinformatics tool to study otocyst gene expression. For user-defined analyses, the library is available in electronic formats that can be directly queried online such as NCBI GEO, or it can be imported into commercial or public domain bioinformatic software packages such as IPA. We used the Sox gene family as an example to highlight the depth as well as the limitations of the library and to demonstrate that the collection of otocyst SAGE tags is a useful tool for molecular and developmental studies of early inner ear development.

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