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Development/Plasticity/Repair

An RNA Interference-Based Screen of Transcription Factor Genes Identifies Pathways Necessary for Sensory Regeneration in the Avian Inner Ear

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Sensory hair cells of the inner ear are the mechanoelectric transducers of sound and head motion. In mammals, damage to sensory hair cells leads to hearing or balance deficits. Nonmammalian vertebrates such as birds can regenerate hair cells after injury. In a previous study, we characterized transcription factor gene expression during chicken hair cell regeneration. In those studies, a laser microbeam or ototoxic antibiotics were used to damage the sensory epithelia (SE). The current study focused on 27 genes that were upregulated in regenerating SEs compared to untreated SEs in the previous study. Those genes were knocked down by siRNA to determine their requirement for supporting cell proliferation and to measure resulting changes in the larger network of gene expression. We identified 11 genes necessary for proliferation and also identified novel interactive relationships between many of them. Defined components of the *WNT*, *PAX*, and *AP1* pathways were shown to be required for supporting cell proliferation. These pathways intersect on *WNT4*, which is also necessary for proliferation. Among the required genes, the CCAAT enhancer binding protein, *CEBPG*, acts downstream of Jun Kinase and *JUND* in the *AP1* pathway. The *WNT* coreceptor *LRP5* acts downstream of *CEBPG*, as does the transcription factor *BTAF1*. Both of these genes are also necessary for supporting cell proliferation. This is the first large-scale screen of its type and suggests an important intersection between the *AP1* pathway, the *PAX* pathway, and *WNT* signaling in the regulation of supporting cell proliferation during inner ear hair cell regeneration.

Introduction

The inner ear is comprised of the vestibular and auditory sensory organs. Within the vestibular system, the utricle senses linear acceleration and head orientation to maintain balance. The cochlea is the auditory organ and detects sound. The cochlea and the vestibular organs use a small population of sensory hair cell (HCs) as mechanoelectric transducers. Loss of inner ear hair cells is the most frequent cause of human deafness and balance disorders (Frolenkov et al., 2004). Sensory hair cells are surrounded by nonsensory supporting cells (SCs). Both cell types originate from the same lineage and together comprise the sensory epithelia (SEs). The mammalian inner ear lacks the ability to regenerate sensory hair cells

when damaged, but birds and other lower vertebrates are capable of regenerating sensory hair cells throughout their life (Corwin and Cotanche, 1988; Jørgensen and Mathiesen, 1988; Ryals and Rubel, 1988; Weisleder and Rubel, 1993).

The specific signaling pathways required for triggering sensory hair cell regeneration have yet to be identified. In this study, we characterized transcription factor (TF) genes that are differentially expressed during avian sensory HCs regeneration. These were identified in a gene expression study in which we measured changes in gene expression for >1500 TF genes across two different time courses of *in vitro* HC regeneration (Messina et al., 2004; Hawkins et al., 2007). One time course measured TF expression changes following laser microbeam injury. The second time course measured TF changes as the SEs regenerated after antibiotic ablation of the HCs (Warchol, 1999, 2001). These time courses were conducted on multiple pure SEs dissected from the cochlea and utricles of chickens. From this regeneration dataset, seven "known" pathways were identifiable: *TGF-β*, *PAX*, *NOTCH*, *WNT*, *NFκB*, *Insulin/IGF*, and *AP1*. A large number of TF changes were also identified for genes that have not yet been placed into established pathways. Together, this list of "known" and "unknown" TF genes was the starting point for the current study that focused upon testing their role in early regenerative proliferation. A major limitation in the restoration of sensory function in the human inner ear is the inability of the SCs to proliferate and differentiate into new sensory HCs in response to

damage. In the current study, we focus on the genetic pathways required for the earliest stages of the regenerative process, specifically sensory epithelium proliferation. We used siRNA knockdown and treatment with small molecule inhibitors to test 27 genes for their effects upon early stages of avian regenerative proliferation. We identified 11 components that are necessary for the early steps in the regenerative process and identified individual components and new pathway intersections within the *AP-1*, *PAX*, and *WNT* pathways that appear to be important effectors of SC proliferation.

Materials and Methods

Tissue dissections. Ten to twenty-one day posthatch White Leghorn chicks were killed via CO_2 asphyxiation and decapitated. Utricles were explanted, and after incubation for 1 h in 500 μg/ml thermolysin, the SEs were removed from the stromal tissue. A detailed description of culture methods has appeared previously (Warchol, 2002).

Laser ablation. Fragments of sensory epithelia were cultured for 7–10 d on laminin-coated wells (Mat-Tek) that contained 50 μl of Medium-199/10% FBS. Semiconfluent cultures were then lesioned via laser microsurgery (Hawkins et al., 2007). Laser-lesioned protocol was initially performed for *JNK*, *JunD*, *PAX2*, and *CEBPG* and replicated with the dissociated utricle sensory epithelia protocol. All subsequent siRNA treatments were performed with the dissociated utricle sensory epithelia protocol.

Dissociated utricle sensory epithelia. Utricle sensory epithelia were physically dissociated into small fragments, pooled, and plated at a final concentration of 0.5 utricles per well in 96-well cultures to ensure that total cell density is uniform between compared samples. Cultures were grown for 3 d and transfected before confluency with siRNAs (50 ng/well) or inhibitor in 0.1% DMSO (15 μM SP600125 *JNK* inhibitor) using previously described methods (Elbashir et al., 2002).

siRNA generation. Double-stranded RNA (dsRNA) was generated by first PCR amplifying a portion of the gene of interest from chicken SE cDNA (supplemental Table S9, available at www.jneurosci.org as supplemental material). PCR products were amplified using gene-specific primers containing the 5′ T7 promoter sequence CTCTAATACGACTCACTATAGGG, under the following conditions: 100 ng of cDNA, 0.2 μM (final concentration) each primer, 10× Advantage Taq Buffer (BD Biosciences), and 5 U of Advantage Taq (BD Biosciences) in a final volume of 50 μl, incubated at 95°C for 2 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, and 68°C for 2 min. PCR products were verified by DNA sequencing. Promoter-containing PCR products were used as template DNA in *in vitro* transcription (IVT) reactions (Ambion). IVT reactions, including postreaction DNase treatment and precipitation, were performed according to the manufacturer's protocol for 12 h. Equal amounts (typically 3 μg each) of sense and antisense RNA strands were mixed and heated at 75°C for 10 min and brought to room temperature on the bench for 2 h. dsRNAs were treated with RNase ONE (50 U, Promega) for 45 min at 37°C. dsRNA was cleaned using RNA Purification Columns 1 (Gene Therapy Systems). siRNAs were generated using the Dicer enzyme (Gene Therapy Systems) following the manufacturer's protocol. Dicer-generated siRNA (d-siRNA) was checked on a 3% agarose gel for ~23 bp size. d-siRNA was cleaned up using RNA Purification Columns 2 (Gene Therapy Systems). Regions used to generate these target sequences were computationally compared to the chicken genome using custom PERL scripts and NCBI Blast. Only regions containing no more than 14 bp of nonspecific sequence overlap in 21 bp sliding windows were used for these siRNA treatments. PCR products were amplified using gene-specific primers containing the 5′ T7 promoter sequence. These were used as template DNA in IVT reactions (Ambion). Fifty nanograms of d-siRNA were transfected in each well of dissociated SE cultures or laser microbeam-ablated SE cultures using standard Lipofectamine 2000 (Invitrogen) protocols. siRNA treatments that inhibited sensory epithelium proliferation were independently replicated with chemically synthesized Dicer-substrate RNAs (DsiRNAs) obtained from Integrated DNA Technologies predesigned DsiRNA Library when available or custom designed (supplemental Table S10, available at www.

jneurosci.org as supplemental material) and transfected using standard Lipofectamine 2000 (Invitrogen) protocols. All significantly altered transcripts after siRNA treatments were computationally scanned for possible off-target sequence homologies with the siRNA products.

Confirmation of siRNA knockdown. Knockdown of target siRNAs were determined by expression profiling of each siRNA knockdown or by endpoint quantitative RT-PCR (*JunD*, *PAX5*, *BCL11A*, *TRIP15*, and *MYT1L*) when microarray expression values were not statistically significant due to dye effects on microarray probe performance. For microarray expression confirmation, knockdowns are defined as >1.3-fold decrease in expression with $p < 0.05$ across all replicates.

Proliferation index. Cells were assayed 48 h after transfection using previously published protocols (Warchol and Corwin, 1996). Quantification of cell proliferation was measured by calculating a proliferation index (defined as the number of BrdU+ cells/total cells). Cells from 10,000 μm^2 × 3 regions of a 96-well plate were combined to determine the proliferation index per well with a minimum of four wells per biological samples. Proliferation assays were replicated with independently dissociated sensory epithelia and siRNA transfections, with a minimum of two biological samples per treatment. Mean proliferation indexes were determined using ImageJ 1.36b software (http://rsb.info.nih.gov/ij/), and error bars were generated by calculating the SD of proliferation indexes across all wells and biological samples. Differences between control values in experimental groups most likely reflect minor changes in initial plating density (0.5 utricles/well) and BrdU labeling efficiency between different experimental groups. To control for these variations, each experimental group was compared to a control sample that was plated, transfected, labeled, and counted in parallel.

Exogenous WNT4 treatment. Chicken utricle sensory epithelia were physically dissociated and plated as previously described. Mouse WNT4 protein (R&D Systems) was initially added at 15, 50, and 100 ng/well in 100 μl of media. Mouse WNT5A protein (R&D Systems) was assayed at 100 ng/well in 100 μl of media. Cells were assayed 48 h after treatment using previously published protocols (Warchol and Corwin, 1996).

Microarray hybridizations and analysis. RNA isolation and cDNA synthesis was performed as previously described (Hawkins et al., 2003). Microarray comparisons were Lowess normalized, and genes with intensity below background as determined by control spots were removed. All comparative microarray hybridizations consisted of a minimum of two biological samples and four technical replicates for each biological sample, including dye switch experiments. A one-sample *t* test was used to determine statistically significant changes in gene expression ($p < 0.05$) across all replicates for each treatment. All microarray data for the current study has been deposited with NCBI GEO with accession number GSE16842.

Results

A high-throughput, quantitative measure of SE proliferation

The design of our previous TF gene expression study (Hawkins et al., 2007) is summarized in Figure 1*A*. TFs identified as being upregulated in that discovery set were moved into the current study, which is diagrammed in Figure 1*B*. This consisted of testing individual components for their effects on cell proliferation and on gene expression. We initially used laser lesioning to damage SEs and measured the effects of siRNA or small molecule inhibitors to stop regenerative proliferation (left side of Fig. 1*B*; Fig. 2). This slow and qualitative assay was replaced by a higher-throughput and quantitative assay, which used cultures of dissociated utricular SCs (right side of Fig. 1*B*). RNA interference (RNAi) and inhibitor treatments that blocked repair of a laser-lesioned SE also showed similar patterns of proliferative inhibition in our 96-well assays. This suggests that our higher-throughput assay system correctly identified a subset of genes that are necessary for regenerative proliferation in the intact SE. All quantitative proliferation results and expression profiling presented here were performed with the dissociated utricle SE protocol.
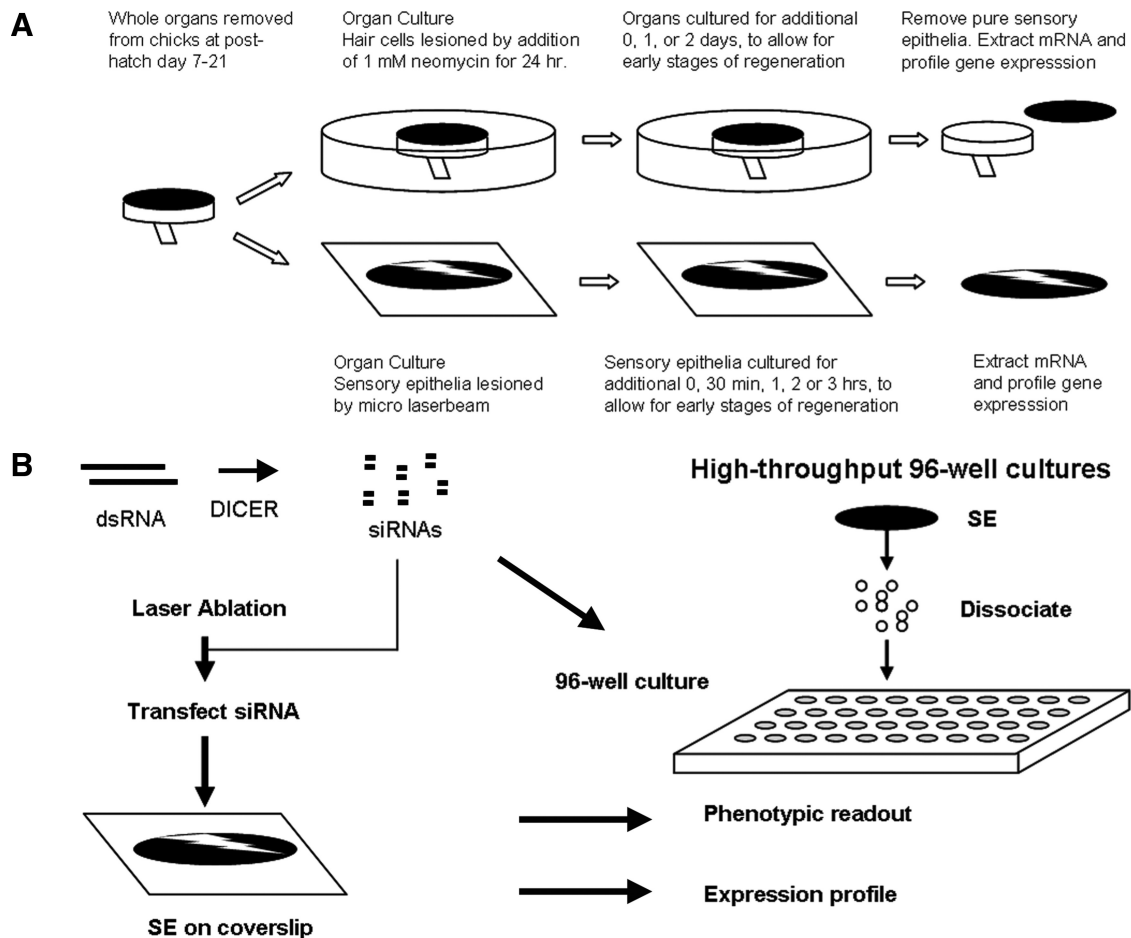
Figure 1. Experimental design. Flow diagram of experimental design scheme for time course profiling in the utricle and cochlea SE and RNAi profiling. **A**, Time course of laser and neomycin recovery. **B**, TFs revealed in the time course of recovery were targeted by siRNA to assess a proliferation phenotype and expression profiled to evaluate knockdown of the target gene and potential epistatic relationships between TFs.
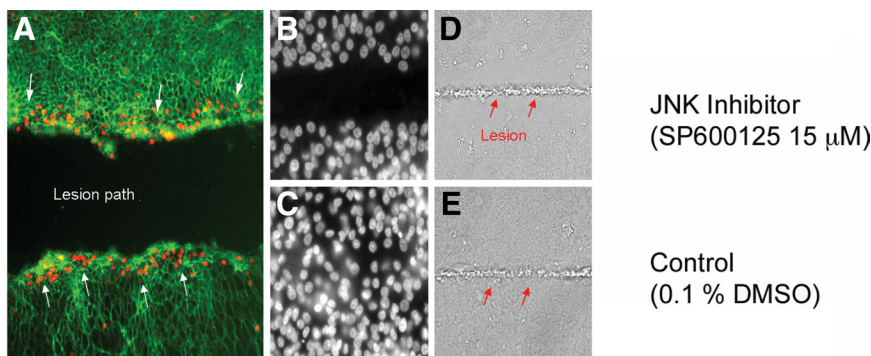

Figure 2. JNK signaling during SE regeneration. JNK signaling is evident at the leading edge of the lesion path in the SEs and necessary for proliferative regeneration. SE cultured on a glass coverslip was lesioned by microbeam laser ablation. **A**, Phosphorylated c-JUN was detected by a phosphorylation-specific antibody to the protein (red dots; white arrows). **B**, **C**, Following laser ablation, the cultured SE was treated with JNK inhibitor (SP600125, 15 μM) (**B**) or 0.1% DMSO (control) (**C**) and allowed to recover for 24 h; nuclei are shown by DAPI staining. **D**, **E**, The laser lesion path is visible by etching of the coverslip through the phase contrast (**D** and **E**, red arrows). Only the JNK inhibitor exhibited a failure to close the wound.

Genes necessary for regenerative proliferation

A complete list of siRNA and small molecule inhibitor treatments and their effects on SE proliferation is given in Table 1 (discussed below). All RNAi knockdowns were confirmed by microarray expression profiling or endpoint quantitative PCR. Cellular proliferation was assessed by BrdU labeling and is expressed as a "proliferation index," defined as the number of BrdU-labeled nuclei/total DAPI-labeled nuclei per microscopic field.

Our previous work suggested that HC regeneration is regulated (in part) by the activating protein 1 (*AP1*) complex that includes the *JUN* family of TFs (Hawkins et al., 2007). *JUN* proteins can be induced by a large number of signaling molecules, as well as by physical or chemical stress (Shaulian and Karin, 2002). Ten known components of the *AP1* pathway were differentially expressed during SE regeneration (Hawkins et al., 2007). To determine whether activation of *JUN* occurs after SE injury, we conducted immunohistochemical staining on laser-lesioned utricular SEs, using an antibody specific to the phosphorylated form of *c-JUN* (Fig. 2*A*). Phosphorylated *c-JUN* was detected at both leading edges of the laser lesion site. To test whether the initial activation of the *JUN* family of TFs is necessary for SE regeneration, we treated the laser-lesioned SE with a small molecule inhibitor (SP600125; 15 μM) of the *JUN* activator, c-*JUN*-N-terminal kinase (*JNK*) (Bennett et al., 2001; Heo et al., 2004; Assi et al., 2006). This led to a failure in regen-

Table 1. Effects of siRNA/inhibitor treatments on sensory epithelia proliferation

siRNA/inhibitor treatment	Inhibit proliferation	Average fold knockdown	Regeneration pathway/category
CEBPG	Yes	−3.92	AP-1 pathway
JNK inhibitor	Yes		
JUND	Yes	*	
BTAF1	Yes	−1.57	AP-1 siRNA commonalities
LRP5	Yes	−5.71	
RARA	Yes	−1.41	
PAX2	Yes	−1.61	Pax pathway
PAX3	No	−1.61	
PAX5	Yes	*	
PAX7	No	−1.72	
MYT1L	No	*	AP-1/Pax siRNA commonalities
WNT4	Yes	−2.27	
CUTL1	Yes	−1.86	Cell cycle
p27KIP	No	−2.93	
ID1	No	−1.48	
CBX3	No	−4.15	Polycomb complex
CBX4	No	−1.09	
EZH2	No	−1.87	
IGF inhibitor	No		Pathway inhibitors
MAPK inhibitor	Yes		
SHH inhibitor	No		
HRY	No	−1.30	Notch signaling
BCL11A	No	−1.35	Common to all tissues/damage
TRIP15	No	−1.12	
CTNNB1	No	−2.39	Common to cochlea and utricle
TIME	No	−1.16	Early regeneration
PPARGC1	No	−1.42	Neomycin specific

Proliferation phenotypes were quantified for each siRNA knockdown. Inhibition was determined as a significantly lower proliferation index than a GFP siRNA control ($p < 0.05$). Knockdowns of siRNA targets were confirmed by microarray analysis or (*) endpoint semiquantitative PCR.

Figure 3. Effects of siRNA treatments on SC proliferation. Proliferation phenotypes were quantified for each siRNA knockdown compared to a GFP control by calculating a proliferation index. BrdU-labeled proliferating cells were compared to the total number of DAPI-stained cells to calculate a percentage proliferation for genes differentially expressed during SE regeneration (**A**) and PAX genes that were upregulated during SE regeneration (**B**).

erative wound closure (Fig. 2B). Treatment for 24 h with 15 μM SP600125 reduced the proliferation levels of cultured SCs by 32% (relative to untreated controls; $p < 0.001$), while treatment for 48 h reduced proliferation by 44% ($p < 0.001$). In contrast, treatment with 10 μM SB203580, a small molecule inhibitor of p38 (a MAP kinase not implicated in our previous studies), had no effect on SC proliferation. These results demonstrate that functional *JNK* signaling is required for repair in the utricular SE.

Members of the *JUN* family of TFs are thought to be constitutively expressed (Brivanlou and Darnell, 2002) with their activity regulated by phosphorylation via *JNK*. However, our data suggest some degree of transcriptional regulation, since we observed increased expression of *JUN* family members (and in particular *JUND*) during regeneration (Hawkins et al., 2007). To determine whether reducing *JUND* levels inhibited SC proliferation, we used RNAi targeted to chicken *JUND*. These resulted in reduced SC proliferation 48 h after siRNA treatment (Fig. 3A), confirming that a functional *AP1* pathway is necessary for SC proliferation.

Seven components of *WNT* signaling are differentially expressed during SE regeneration, including β-catenin, a critical component of canonical *WNT* signaling (Hinck et al., 1994). We selected β-catenin for knockdown by siRNA (see more *WNT* components below). We also selected genes that did not necessarily fall within known pathways, but were upregulated during one or more time points of SE regeneration. One example is the CCAAT element binding protein (*CEBPG*), which was upregulated at specific time points in utricular regeneration (Hawkins et al., 2007). We also identified *BCL11A* (a zinc finger gene associated with hematopoietic malignancies) (Medina and Singh, 2005; Singh et al., 2005) and *TRIP15* (a component of the COP9 signa-
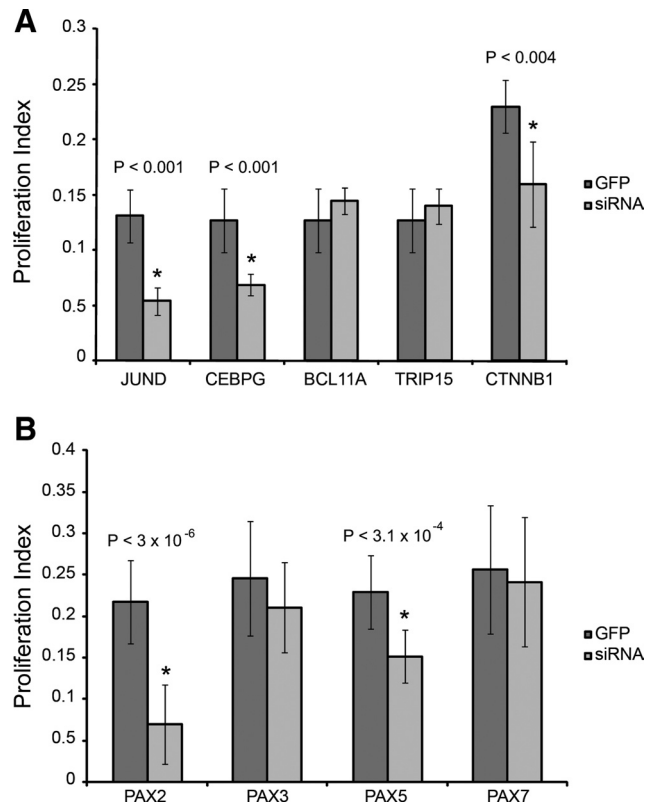
losome that regulates G_1–S transition) (Yang et al., 2002) as being differentially expressed across all four treatment/tissue combinations (Hawkins et al., 2007). Knockdowns of *CEBPG and β-catenin* significantly reduced SC proliferation, similar to *JUND* siRNA treatments (Fig. 3A). However, siRNA knockdowns of *BCL11A* and *TRIP15* failed to significantly affect SC proliferation.

The cyclin-dependent kinase (*CDK*) inhibitor $p27^{Kip1}$ is required for the transition in SCs from quiescence to the proliferative state (Chen and Segil, 1999; Löwenheim et al., 1999). The gene expression level of this *CDK* inhibitor within our regenerative time courses decreases 1 h after laser lesioning (Hawkins et al., 2007). Likewise, *CUTL1* (a CCAAT displacement protein), a known $p27^{Kip1}$ repressor (Ledford et al., 2002), is differentially expressed across the time course. To determine whether $p27^{Kip1}$ and *CUTL1* regulation are important regulators of SC proliferation, we used siRNAs targeted to each and measured the effects on cell proliferation. We reasoned that inhibition of *CUTL1* would lead to a release of $p27^{Kip1}$ repression and a consequent decrease in proliferation. In agreement with this model, *CUTL1* siRNA treatments inhibited SC proliferation and gene expression of $p27^{Kip1}$ increased in these treatments (1.68-fold change, $p < 0.0176$). However, siRNA knockdowns of $p27^{Kip1}$ had no apparent effect on proliferation (Table 1). This might be attributable to the already very high rate of ongoing cell division in these cultures (Kelley, 2006). However, as noted below, some other treatments can indeed result in SC hyperproliferation. Overall, these data are consistent with the known roles of *CUTL1* and $p27^{Kip1}$ in the regulation of the transition from quiescence to the proliferative state. siRNA knockdowns of *ID-1* upregulate $p27^{Kip1}$ and inhibit
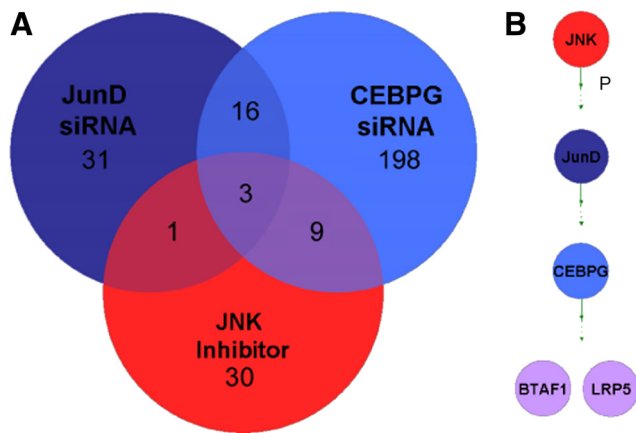
Figure 4. Analysis of overlapping expression profiles and novel epistatic relationships between genes that are necessary for SC proliferation. siRNA and inhibitor treatments were expression profiled to identify downstream effectors of SC proliferation. **A**, Numbers indicate genes differentially expressed in three treatments that each individually inhibit SC proliferation. Three genes are commonly downregulated, one of which is *CEBPG*. **B**, Novel epistatic relationships can be inferred from TF expression profiling siRNA and inhibitor treatments. *CEBPG* can be placed downstream of JNK and *JUND* and the other commonly downregulated genes, *BTAF1* and *LRP5*.

proliferation of mammalian tumors (Ling et al., 2002; Tam et al., 2008). However, our knockdowns of *ID-1* had no effect on SC proliferation. Negative RNAi results of this type are open to the caveat that none of our knockdowns completely abrogated transcript levels. Thus, some small level of transcript (10–20%) and protein product (not measured) may still be present and may be sufficient to maintain proliferation.

Our prior study revealed a cascade of 18 TF genes induced by *PAX* gene expression (Hawkins et al., 2007). Five *PAX* genes (*PAX2*, *PAX3*, *PAX5*, *PAX7*, and *PAX8*) were upregulated during cochlear regeneration (Hawkins et al., 2007). To determine whether *PAX* genes are necessary for utricular SC proliferation, we used RNAi to knockdown these genes. A chick ortholog for *PAX8* could not be unequivocally identified, and it was therefore not targeted for knockdown. Approximately 10% of the chicken genome is missing from the published or web-accessible DNA sequence (International Chicken Genome Sequencing Consortium, 2004). This includes many genes that lack clear orthologs such as *PAX8*, but are likely present in the chick genome. While most invertebrate genomes posses only a single *PAX2/5/8* gene, early in vertebrate evolution the closely related subclass of paired-box family of TFs *PAX2*, *PAX5*, and *PAX8* were produced by gene duplication (Noll, 1993; Mansouri et al., 1996; Czerny et al., 1997; Wada et al., 1998; Kozmik et al., 1999). From the four individual siRNA knockdowns, two (*PAX2* and *PAX5*) inhibited SC proliferation, while knockdowns of *PAX3* and *PAX7* did not have significant effects on proliferation (Fig. 3B).

Downstream effectors of sensory epithelium proliferation
We conducted TF microarray expression profiling on all samples that were treated with siRNAs or inhibitors. This served the dual purpose of confirming the knockdown of the target gene and identifying additional genes that showed consistent expression changes in response to the treatment. We next identified overlapping expression changes between treatments. One example of such an intersection is shown in Figure 4A, which illustrates the TF expression changes for three treatments, all of which individually inhibit SC proliferation: *JNK* inhibitor, *JUND* RNAi, and *CEBPG* RNAi. While there are numerous expression changes that
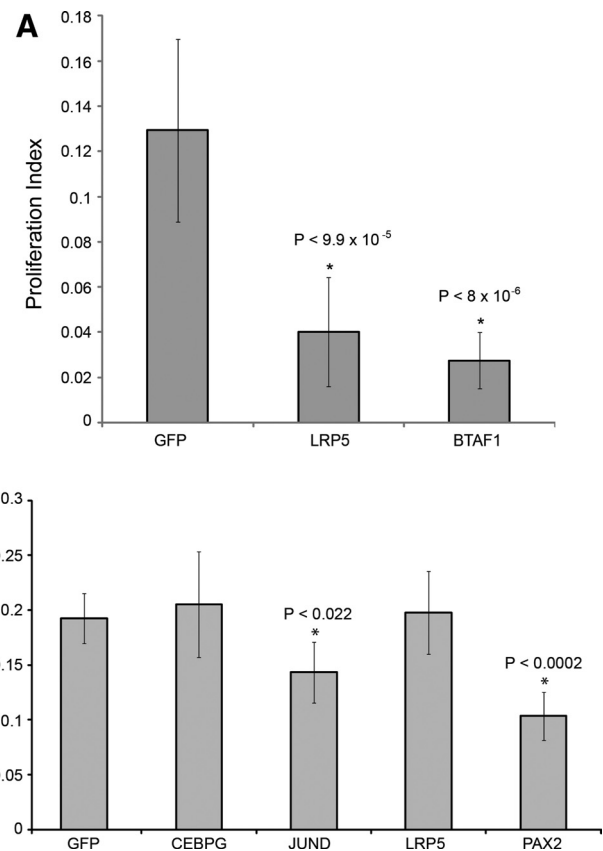

Figure 5. Analysis of siRNA treatments in chick SC and RPE proliferation. Percentage proliferation was quantified for siRNA treatments in chick SC for genes commonly downregulated in treatments that inhibit SC proliferation (downstream of the *AP-1* pathway and *CEBPG*) (**A**) and chick RPE for genes that inhibited chick SC proliferation (**B**).

are unique to each treatment or shared between pairs of treatments, we identified three genes that are commonly downregulated in all three treatments (fold change >1.3, $p < 0.05$) (supplemental Tables S1, S2, S3, available at www.jneurosci.org as supplemental material). One of these shared genes is *CEBPG*, suggesting that *CEBPG* acts downstream of *JUND* and *JNK* in this pathway. In addition, the low-density lipoprotein receptor-related protein 5 gene (*LRP5*, a coreceptor of *WNT* signaling) and the B-TFIID TF-associated RNA polymerase (*BTAF1*) were commonly downregulated in all three treatments, suggesting that these two genes probably act downstream of *CEBPG* in the JUN cascade (Fig. 4B). To determine whether these commonly downregulated genes are also required for SC proliferation, we conducted siRNA knockdowns of *LRP5* and *BTAF1*. Both significantly inhibit SE proliferation (Fig. 5A).

siRNA effects on proliferation that are specific to the inner ear
To determine whether genes that regulate SC proliferation are also involved in the proliferation/repair of other types of epithelia, we performed RNAi knockdowns in cultures of chick retinal pigmented epithelium (RPE) (Fig. 5B) and measured proliferative indexes. Since *JUND* is the most broadly expressed TF of the *AP1* pathway, it was not surprising to observe that siRNA knockdown of *JUND* also inhibited proliferation of chick RPE. Knockdowns of the widely expressed TF *PAX2* also inhibited proliferation of RPE, suggesting that *JUND* and *PAX2* may serve common roles in both the ear and the eye. However, siRNA

Table 2. Genes commonly differentially expressed in treatments that inhibit sensory epithelia proliferation

	Downstream of Ap-1 pathway				Pax pathway			
Gene	CEBPG siRNA	p value	LRP5 siRNA	p value	PAX2 siRNA	p value	PAX5 siRNA	p value
MYT1L	−4.27	7×10^{-3}	−4.05	7.00×10^{-3}	−1.51	2.00×10^{-3}	−1.61	1.40×10^{-2}
WNT4	5.41	1.70×10^{-2}	4.16	3.90×10^{-2}	1.34	4.70×10^{-2}	1.37	7.00×10^{-3}

Expression profiles for siRNA knockdowns that inhibited sensory epithelia proliferation were compared to identify specific commonalities downstream of the Ap-1 and PAX pathways. MYT1L and WNT4 were commonly differentially expressed (average fold change >1.3, $p < 0.05$) in all four siRNA treatments that inhibit proliferation.

knockdowns of *CEBPG* and *LRP5* had no effect on RPE proliferation, suggesting they may be uniquely required for SC proliferation in the inner ear.

Pathways and pathway intersections
To identify known pathways downstream of *CEBPG* and *LRP5*, we used MetaCore Analysis software (Ekins et al., 2006) to compare gene expression profiles derived from *CEBPG* and *LRP5* siRNA knockdowns in dissociated utricular cultures (supplemental Tables S3, S4, S5, available at www.jneurosci.org as supplemental material). MetaCore Analysis is a web base tool that identifies components of known pathways enriched in our datasets. *p* values are generated to determine the probability that genes are found by chance. One of the highest scoring pathways in both *CEBPG* and *LRP5* siRNA knockdowns was the *NOTCH* signaling pathway ($p < 8.89 \times 10^{-13}$ and $p < 7.48 \times 10^{-4}$, respectively). *NOTCH* signaling is known to regulate the differentiation of HCs and nonsensory SCs during inner ear development and HC regeneration (Adam et al., 1998; Lanford et al., 1999; Cotanche and Kaiser, 2010). We also identified enrichment of *TGF-β* signaling ($p < 6.18 \times 10^{-8}$ and $p < 1.58 \times 10^{-7}$) and *WNT* signaling ($p < 1.01 \times 10^{-5}$ and $p < 2.62 \times 10^{-5}$). Components of both pathways are differentially expressed during SE regeneration (Hawkins et al., 2007). Three specific components of *WNT* signaling (*WNT4*, *WNT9B*, and *WNT16*) were commonly upregulated in both siRNA treatments (>2-fold change, $p < 0.05$) (supplemental Tables S3, S5, available at www.jneurosci.org as supplemental material).

To determine whether potential pathway intersections can be discovered within our siRNA data, we compared gene expression profiles of four siRNA treatments that individually inhibited SC proliferation: *CEBPG*, *LRP5*, *PAX2*, and *PAX5* siRNA. We identified two genes that are commonly upregulated or downregulated across all four siRNA treatments (>1.3-fold change, $p < 0.05$); these were the *WNT* gene family member (*WNT4*) and myelin TF 1-like (*MYT1L*) (Table 2, supplemental Tables S3, S5, S6, S7, available at www.jneurosci.org as supplemental material). To determine whether *WNT4* and *MYT1L* are also necessary for SC proliferation, we used RNAi to knockdown each in cultured SEs. Knockdowns of *MYT1L* had no effect, but knockdown of *WNT4* significantly reduced SC proliferation (Fig. 6). Upregulation of *WNT4* occurred in six of eight treatments that reduced SC proliferation ($p < 0.02$) (supplemental Fig. S1, available at www. jneurosci.org as supplemental material), supporting a critical role for *WNT4* during SC proliferation. Interestingly, five known components of *NOTCH* signaling (see below) were differentially expressed (>1.5-fold change and $p < 0.05$) in *WNT4* siRNA knockdowns (supplemental Table S8, available at www. jneurosci.org as supplemental material). Progenitor cells of the sensory epithelia acquire either the HC or SC fate by lateral inhibition through the *NOTCH* signaling cascade. Progenitor HCs express elevated levels of the *NOTCH* ligand, *DELTA*, causing neighboring cells to increase expression of *NOTCH* (Adam et al., 1998; Morrison et al., 1999). Increased levels of *NOTCH* induce
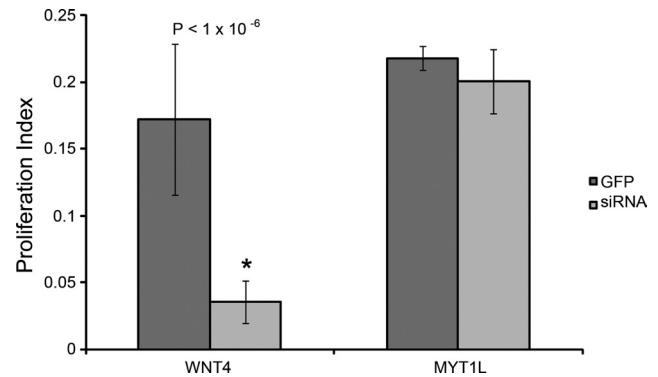
Figure 6. *WNT4* and *MYT1L* siRNA phenotypes. *WNT4* siRNA knockdowns inhibited SC proliferation compared to a GFP control, while *MYT1L* siRNA did not have a significant effect on proliferation.

Hairy and Enhancer of Split related genes, negatively regulating *DELTA* and inhibiting sensory HC fate (Zheng et al., 2000; Zine et al., 2001). In our *WNT4* siRNA knockdowns, we identified downregulation of *NOTCH1*, *NOTCH2*, and *HEY2* and upregulation of *DELTA1* and *DELTA3*, suggesting that *WNT4* may be involved in regulating *NOTCH* signaling. Recent reports have suggested a closely linked relationship between *WNT* and *NOTCH* ("*WNTCH*") signaling (Hayward et al., 2008). These reports suggest a model in which *WNT* signaling establishes a prepatterned group of cells capable of specific differentiation states, individual cell fates then being further refined by *NOTCH* signaling.

Exogenous Wnt4 increases proliferation
After establishing that *WNT4* is necessary for SE proliferation, we next interrogated the effects of exogenous *WNT4* protein on SE proliferation. To determine whether exogenous *WNT4* would result in increased proliferation, we examined dissociated chick utricle SEs after incubation in *WNT4*-supplemented media. SEs cultured in *WNT4* resulted in hyperproliferation compared to cultures grown in media supplemented with heat-inactivated *WNT4* and unsupplemented media (Fig. 7A). Interestingly, cultures supplemented with the *WNT* ligand *WNT5A* also resulted in SE hyperproliferation (Fig. 7B), suggesting that the activation of *WNT* signaling is a critical step during SE proliferation and is not solely limited to the expression of *WNT4*.

Discussion
Our data suggest that, while the *AP1* and *PAX* pathways have unique downstream components, both pathways intersect at *WNT4*. Moreover, expression of *WNT4* is also necessary for SC proliferation, pointing to a critical role for *WNT* signaling in the initiation of regeneration in the avian ear. *WNT4* levels increase in response to siRNA treatments that inhibit SC proliferation. If increased *WNT4* expression is inhibiting proliferation, then this would suggest that siRNA knockdowns of *WNT4* might lead to normal or even hyperproliferation. However, we found that
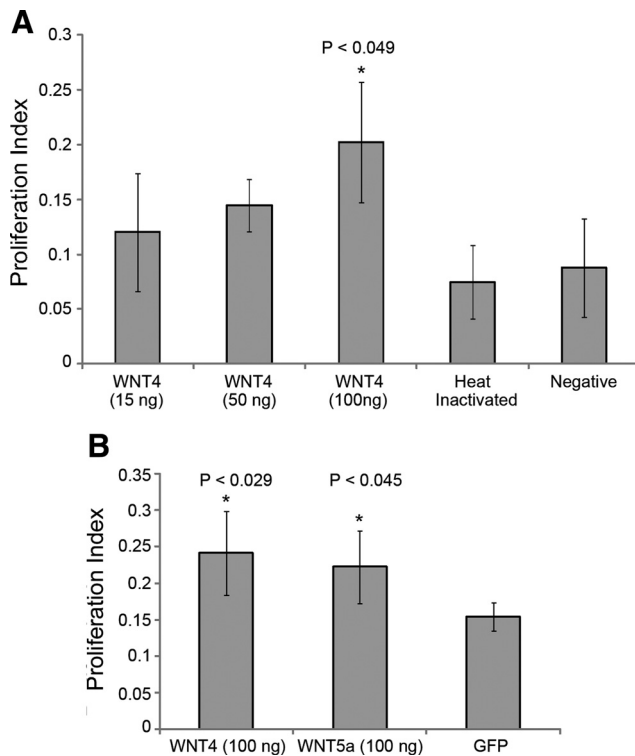
Figure 7. Exogenous WNT4 expression phenotypes. ***A***, Percentage proliferation was quantified for treatment with 15, 50, or 100 ng/well of exogenous WNT4 protein. Cells treated with 100 ng/well of exogenous WNT4 protein hyperproliferated compared to heat-inactivated WNT4 and cells cultured in unsupplemented media. ***B***, Treatment with exogenous WNT5a also caused hyperproliferation; however, *CEBPG* siRNA in combination with exogenous WNT4 treatment inhibited proliferation similarly to *CEBPG* siRNA treatment alone.

WNT4 siRNAs inhibited SC proliferation and exogenous WNT4 results in hyperproliferation. This suggests that while inhibition of the *AP1* pathway and downstream components results in altered WNT signaling regulation, both *CEBPG* and *WNT* signaling are necessary for SE proliferation. Further investigation of this complex circuit will be necessary to resolve this apparent conundrum. It is interesting to note that *WNT4* has been described in both the canonical and noncanonical WNT signaling pathways (Lyons et al., 2004; Miyakoshi et al., 2008) and appears to be capable of sequestering β-catenin at the cell surface (Chang et al., 2007). These apparently dual functions may be the basis for the complexity of the results when WNT4 levels are experimentally manipulated.

WNT4 is thought to act as an autoinducer of mesenchyme to epithelial transition during mouse kidney development (Stark et al., 1994). *WNT4* null mice die within 24 h of birth due to kidney failure, precluding subsequent analysis of hearing or balance disorders (Stark et al., 1994). It is first detected in the developing chicken otocyst at E4, in the prospective nonsensory cochlear duct (Sienknecht and Fekete, 2009), and later at E5, forming a border between the sensory primordia and nonsensory lateral wall (Stevens et al., 2003; Sienknecht and Fekete, 2008), suggesting that *WNT4* may play an important role in forming sensory/nonsensory boundaries in the developing inner ear. During a regenerative time course after neomycin treatment, *WNT4* expression increased at 72 h after the removal of neomycin (2.26-fold change, $p < 5.77 \times 10^{-4}$). This is at a time when the earliest known markers of HC regenerated via mitosis are first detected (Stone and Cotanche, 2007), suggesting that *WNT4* may be in-

volved in the early stages of SC to HC differentiation. *PAX2* has been shown to regulate *WNT4* expression during kidney development (Torban et al., 2006) and our microarray data suggest that *PAX2*, along with *PAX5*, *CEBPG*, and *LRP5*, may function as important regulators of *WNT4* in the inner ear. Thus, the *AP1* and *PAX* pathways both appear to affect *WNT* signaling during SE regeneration.

CUTL1 is a known downstream transcriptional target of *TGF-β* signaling (Michl et al., 2005; Hawkins et al., 2007) is upregulated during regeneration (Hawkins et al., 2007) and, as shown here, is necessary for SC proliferation. This gene was also downregulated in two treatments that inhibited SC proliferation (*WNT4* and *BTAF1* knockdowns; −1.90-fold and −1.38-fold changes, respectively) (supplemental Tables S8, S9, available at www.jneurosci.org as supplemental material), suggesting that regulation of *CUTL1* may be important during SC proliferation. *CUTL1* represses $p27^{Kip1}$, which is first detected in the sensory primordia of the developing mouse cochlea from E12 to E14, a time when proliferation is ending and HC differentiation is beginning (Chen and Segil, 1999). Expression of $p27^{Kip1}$ in the adult inner ear identifies cochlear SCs and participates in the inhibition of cell cycle entry in these cells (White et al., 2006). $p27^{Kip1}$ homozygous knock-out mice develop with an excess number of HCs and SCs (Chen and Segil, 1999; Löwenheim et al., 1999; Kanzaki et al., 2006). This suggests that $p27^{Kip1}$ plays an important role in maintaining mitotically inactive sensory epithelium cells in mammals. $p27^{Kip}$ is rapidly downregulated following injury to the avian utricle (Hawkins et al., 2007), possibly because of the increased expression of its repressor, *CUTL1*. Removal from the cell cycle plays an important role in maintaining functionally active SEs. This may be an important factor in the lack of regenerative capabilities in mammalian SEs.

JUN family TFs also play important roles in regulating cell cycle entry, proliferation, and differentiation. For example, *c-JUN* can remove p53-mediated inhibition of cell cycle entry (Shaulian et al., 2000) and *JUND* regulates mouse lymphocyte proliferation (Meixner et al., 2004). Members of the *JUN* family of TFs interact with *FOS* to activate *Cyclin D1* and increase cell proliferation (Shaulian and Karin, 2002). Ten components of the *AP1* complex, including *FOS*, were differentially expressed in one or more of our regenerative time points (Hawkins et al., 2007). Our data place *CEBPG* downstream in the *AP1* pathway during sensory regeneration. This is further supported by the recent observation that *CEBPG* interacts with *FOS* to activate the *IL-4* gene in Jurkat cells (Davydov et al., 1995). *CEBPG* belongs to the highly conserved CCAAT/enhancer binding protein (*C/EBP*) family of TFs. Members of this family act as master regulators of numerous processes, including differentiation, inflammatory response, and liver regeneration (Ramji and Foka, 2002). It is possible that *CEBPG* interacts with *FOS* or other members of the *AP1* complex to regulate proliferation during avian utricular regeneration.

Our results indicate that *LRP5* also acts downstream in the *AP1* pathway during sensory regeneration. The *LRP5* protein can function as a coreceptor in *WNT* signaling (Logan and Nusse, 2004), which connects another component of *WNT* signaling into this pathway. We have previously identified the *WNT* signaling components β-catenin and the *TCF/LEF* TFs, *TCF7L1* and *TCF7L2*, as being differentially expressed during HC regeneration (Hawkins et al., 2007). In the present study, three additional *WNT* signaling components, *WNT4*, *WNT9B*, and *WNT16*, were differentially expressed in siRNA treatments for *CEBPG* and *LRP5*. Canonical *WNT* signaling is transduced through the frizzled family of receptors and *LRP5/LRP6* coreceptors, leading to

activation of the β-catenin signaling cascade (Clevers, 2006). In our previous study, we determined that β-catenin is up-regulated during HC regeneration, and in the current study, we demonstrate that siRNA knockdowns of β-catenin inhibit SE proliferation.

This study represents the first large-scale characterization of genes that are necessary for regenerative proliferation of avian SCs. It is also the first study of pathway analysis and identification in this system. As larger transcriptome datasets are generated, the types of methods described here should be applicable to identifying critical proliferative and differentiation genes and pathways in the regenerating SEs of the avian inner ear. In the longer term, these observations can be compared, contrasted, and applied to the mitotically arrested mammalian inner ear SEs with a view to replenishing damaged HC.

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