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Downstream Targets of *GATA3* in the Vestibular Sensory Organs of the Inner Ear

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Abstract

Haploinsufficiency for the transcription factor *GATA3* leads to hearing loss in humans. It is expressed throughout the auditory sensory epithelium (SE). In the vestibular organs, *GATA3* is limited to the striola reversal zone of the utricle. Stereocilia orientation shifts 180° at this region which contains morphologically-distinct type I hair cells. The striola is conserved in all amniotes, its function is unknown and *GATA3* is the only known marker of the reversal zone. To identify downstream targets of *GATA3* that might point to striolar function we measured gene expression differences between striolar and extra-striolar SE. These were compared with profiles after *GATA3* RNAi and *GATA3* over-expression. We identified four genes (*BMP2*, *FKHL18*, *LMO4* and *MBNL2*) that consistently varied with *GATA3*. Two of these (*LMO4* and *MBNL2*) were shown to be direct targets of *GATA3* by ChIP. Our results suggest that *GATA3* impacts *WNT* signaling in this region of the sensory macula.

Keywords

Transcription Factors; Inner Ear; Utricle

Introduction

The vertebrate inner ear originates from the otic placode, a thickening of surface ectoderm that forms above the hindbrain early in embryonic development. In the mouse embryo, the zinc finger transcription factor *GATA3* is expressed throughout this placode, beginning at E8-E9.5, and is required for otic cup invagination and closure. The resulting structure, the otic vesicle, eventually develops into all the structures of the inner ear including the vestibular (balance) and cochlear (auditory) organs (Grace Lawoko-Kerali, 2002; Lilleväli et al., 2006). In humans, mutations that cause haploinsufficiency for *GATA3* result in hypoparathyroidism, sensorineural deafness and renal anomaly syndrome (HDR) (Van Esch et al., 2000). Homozygous knockout of the *GATA3* gene in mice results in embryonic lethality by E11, due to multiple organ abnormalities, massive internal bleeding, a complete inhibition of T-cell differentiation (Pandolfi et al., 1995) and abnormal brain morphology. Heterozygote knockouts are viable, but have a progressive degeneration of cochlear sensory hair cells and corresponding hearing loss (van der Wees et al., 2004), similar to that observed in HDR in humans. Notably, both *GATA3* heterozygous and homozygous knock out mice exhibit misrouted axonal projections to the inner ear (Karis et al., 2001) and

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elsewhere in the nervous system (Nardelli et al., 1999; Lundfald et al., 2007) suggesting a role for *GATA3* in neural development.

GATA3 has been most extensively studied in the development and differentiation of the mammalian hematopoietic system. During differentiation of T lymphocytes from hematopoietic stem cells, naïve CD4⁺ cells differentiate into either T helper type 1 (Th1) or T helper type 2 (Th2) cells. This switch is tightly regulated by *GATA3* (Szabo et al., 2003; Mowen and Glimcher, 2004) and involves the direct transcriptional regulation of IL5 and IL13 by *GATA3* to specify a Th2 fate (Siegel et al., 1995; Kishikawa et al., 2001; Lavenu-Bombléd et al., 2002). *GATA3* also plays a significant role in skin development and particularly in specifying inner root sheath cell vs. hair shaft cell differentiation and organization (Kaufman et al., 2003). Recently, a direct binding target of *GATA3* has been described in the first intron of the lipid acyltransferase gene *AGPAT5*, suggesting a critical role for *GATA3* in lipid biosynthesis during skin epidermal barrier acquisition (de Guzman Strong et al., 2006). Although some *GATA3* transcriptional targets of this type have been described in T-lymphocyte specification, skin differentiation and brain development (Hikke van Doorninck et al., 1999), little is known about its direct targets of action in inner ear development/differentiation.

Most previous studies of *GATA3* in the inner ear have focused on its role in embryonic development. However, expression of *GATA3* is also maintained in the mature inner ear. In a previous study (Hawkins et al., 2003) we demonstrated that *GATA3* is expressed throughout the sensory epithelium of the mature avian cochlea, but its expression in the vestibular organs is limited to a 6–10 cell wide region of supporting cells in the striola of the utricle and lagena in the utricle. This narrow region of *GATA3* expression corresponds to the location where hair cell stereocilia undergo a 180° shift in orientation (Flock, 1964) and where hair cell phenotype changes from so-called type I to type II (Fig. 1). Type I hair cells are enclosed by large calyx nerve terminals (Lysakowski and Goldberg, 1997) and are morphologically distinct from type II hair cells, which are contacted by bouton nerve terminals from afferent and efferent neurons (Jørgensen and Andersen, 1973; Jørgensen, 1989). Type I hair cells are conserved within amniotes but are lacking in anamniotes. Although type I and type II hair cells have been studied for over 30 years, specific roles for type I and type II hair cells have not yet been defined. However, the distinct morphologies between type I and type II sensory hair cells suggest specialized functions. The relationship between *GATA3* expression and these two morphological changes is not clear. Recent experiments have examined the orientation of regenerated hair cells in explants of the avian utricle following surgical ablation of the *GATA3*-expressing region. Such hair cells are normally oriented, suggesting that *GATA3* probably does not specify hair cell reversal (Warchol and Montcouquiol, in press). Instead, given its function in other developing systems, it is more likely that *GATA3* plays a role in specification of hair cell phenotype (as type I vs. type II) and/or axon guidance near the reversal zone. *GATA3* is the only marker for the striola described to date. Since the specific functions of the striola and type I hair cells have not yet been defined, identifying the genes and biological pathways specific to *GATA3* in the striola may provide functional insights into this enigmatic inner ear structure.

The present study is aimed at the identification of transcription factors in the inner ear the expression of which is regulated by *GATA3*. We specifically focused on the striola of the chick utricle, which is comprised of ~10,000 cells. We used four approaches to characterize *GATA3*-regulated gene expression. First, we used microdissection, micro cDNA amplification methods and custom gene microarrays to identify transcription factor (TF) genes specifically co-expressed with *GATA3* in the highly localized striolar region. We next utilized siRNA knockdown of *GATA3* and ectopic over-expression of *GATA3* to identify genes that are coordinately altered in their expression levels when *GATA3* expression is

disrupted. This resulted in the identification of four genes (*BMP2*, *FKHL18*, *LMO4* and *MBNL2*) that paralleled *GATA3* expression in all three comparisons. We next used chromatin immunoprecipitation (ChIP) to confirm that two of these genes (the LIM domain only 4 [*LMO4*] and muscleblind like-2 [*MBNL2*] transcription factors) are direct targets for *GATA3* binding in the chick utricular sensory epithelium. Finally, we confirmed a subset of our microarray observations by RNA *in situ* hybridizations, including *LMO4* and *MBNL2*. Taken together, our gene expression data demonstrate that regulation of specific components of *Wnt*, *FGF*, *Notch* and *BMP* signaling, as well as regulators of neurogenesis and neural survival, are differentially expressed in the striolar vs. extra-striolar regions and possibly play roles in regulating neuronal differentiation and axon guidance to specific hair cell types within the sensory maculae.

Results

Striola vs. extra-striola microarray comparisons

As an initial screen for genes that are potentially regulated by *GATA3*, we compared gene expression in cells of the striola (which includes the narrow strip of *GATA3*-expressing reversal zone cells) to expression in the medial extra-striolar region. Our rationale for investigating just transcription factors and components of known signaling pathways was that changes in these molecules frequently act as important switches in genetic programming. Sensory epithelia from mature chick utricles were isolated and micro-dissected into striolar and extra-striolar portions (see Fig. 1). Separation of these distinct sensory regions is more easily accomplished in the chick utricle, compared to the utricles of mice. RNAs from these pooled samples were then compared on a custom oligonucleotide microarray that primarily interrogates transcription factor gene expression (Messina et al., 2004), but also includes oligonucleotides specific to major signaling pathways. All comparative microarray hybridizations consisted of 2 biological samples and 4 technical replicates for each biological sample, including dye switch experiments. To our knowledge this is the first such comparison ever conducted and identified 38 genes that are up-regulated and 45 down-regulated at the striola (Fold change > 2.0 and p-value < 0.05) (Table 1). Notably, the four genes that showed the highest relative levels of expression in the striola (*KCNIP4*, *DKK2*, *NGN2*, and *HEY2*) have been shown to affect neuronal differentiation (Falk et al., 2002; Sakamoto et al., 2003; Xiong et al., 2004; Guder et al., 2006). For example, the bHLH transcription factor *NGN2* can induce neuronal cell fate in mouse neural stem cells (Hu et al., 2005). Expression of *NGN2* within the striola was up-regulated by 7.85 fold, compared to the extra-striolar region. In contrast, we observed reduced striolar expression of *WNT3A* and *WNT5* (−7.55 and −5.37 fold changes respectively) and two hairy and enhancer of split (*HES*) paralogs, *HEYL* and *HRY* (−5.02 and −6.99 fold changes respectively). *HES* genes are components of Notch/Delta signaling, and both *HEYL* and *HRY* are known to physically interact with *GATA* proteins and inhibit transcriptional activity (Kathiriyi et al., 2004; Fischer et al., 2005). From this dataset we also identified known components of *WNT*/beta-catenin signaling (*DKK2*, *FZD5*, *FZD7*, *WNT3*, *WNT3A*, *WNT5A*), *FGF* signaling (*FGF16* and *FGF20*), *Notch* signaling (*HEY2*, *HEYL* and *HRY*) and *BMP* signaling (*BMP2*, *BMP4* and *BMP15*) (Table 1). Overall, this comparison revealed a complex pattern of gene expression changes that strongly implicate differential expression of *WNT*, *FGF*, *Notch* and *BMP* signaling pathways within these distinct regions of the sensory maculae.

GATA3 RNAi knockdown comparisons

To better discriminate between gene expression changes within the striola that are associated with *GATA3* expression and those that might be coincidental, we utilized RNAi knockdowns in cultured chick utricles (the entire utricle including striola plus extra-striola regions) to

identify genes that potentially act downstream of *GATA3*. Since *GATA3* expression is maintained in the adult utricle, it very likely plays a critical and active role regulating direct targets in the adult striola. We compared gene expression profiles of pure sensory epithelia from whole, explanted utricles transfected *in vitro* via electroporation with siRNAs for either *GATA3* or a GFP control. In order to identify both direct and indirect consequences of *GATA3* knockdown, epithelial cells were harvested 48 hours after RNAi treatment. Immunohistochemical labeling indicated that knockdown of *GATA3* is maintained at the striola 48 hrs. post siRNA treatment (Fig 2A,B). We identified 63 genes that were up-regulated and 10 genes down-regulated (including *GATA3* itself) in response to *GATA3* siRNA knockdowns (Fold change > 2.0 and p-value < 0.05) (Table 2). The *BAR* homeobox transcription factor 1 (*BARX1*) and *BARH*-like homeobox 1 (*BARHL1*) genes exhibited the largest down-regulation in expression (−9.31 and −6.68 fold changes respectively). *BARHL1* encodes a homeodomain transcription factor involved in sensorineural development. It is expressed in migrating neurons of the CNS as well as in sensory hair cells, where it is required for long-term survival and maintenance (Bulfone et al., 2000; Li et al., 2002). *BARX1* regulates transcription of two *WNT* antagonists, *sFRP1* and *sFRP2* (Kim et al., 2005). Consistent with our earlier observation that *WNT* signaling is differentially regulated in the striola compared to the extra-striola regions, we identified three components of *WNT* signaling that were up-regulated in *GATA3* knockdowns (*WNT3*, *LRP5* and *FZD5*) and one Wnt gene (*WNT5B*) that was down-regulated. Expression of the Fibroblast Growth Factor *FGF16*, which was specifically down-regulated at the striola, was up-regulated in *GATA3* knockdowns.

***GATA3* over-expression microarray comparisons**

As a reciprocal experiment to our siRNA knockdowns, we next identified genes differentially expressed in response to *GATA3* over-expression. Using a pMES vector expressing *GATA3* and eGFP under the control of a chick beta-actin promoter we over-expressed *GATA3* in dissociated epithelial cells from the chick utricle (see Fig. S1 in supplementary material). Transfection efficiency was determined to be 24% by comparing eGFP expression to total DAPI stained nuclei (n = 136). We quantified changes in gene expression between the *GATA3* over-expressing samples and those transfected with an eGFP/pMES (empty) vector. We identified 12 genes that are up-regulated and 11 down-regulated in response to *GATA3* over-expression (Fold change > 2.0 and p-value < 0.05) (Table 3). The genes showing the most dramatic up-regulation in response to *GATA3* over-expression are the *WNT*/beta-catenin signaling modulators *WNT9A* and *SFRP2*.

Downstream effectors of *GATA3* expression

To identify genes that are potentially directly regulated by *GATA3* we compared expression changes across all three conditions. This is a particularly conservative approach given that *GATA3* can in various circumstances act as either an activator or a repressor (Siegel et al., 1995; Lavenu-Bombled et al., 2002; Mantel et al., 2007). It is quite possible that dramatic down-regulation or up-regulation of *GATA3* may not have immediately reciprocal effects on actual *in vivo* target genes in a simplistic model of target selection. Nevertheless, we adopted this filtering approach to identify a set of genes that would be strong candidates for direct regulation. We identified 4 genes with similar or reciprocal expression patterns to *GATA3*: *BMP2*, *FKHL18*, *LMO4* and *MBNL2* (Table 4). For this comparison, we expanded our datasets to include more modest fold changes (>1.3 fold, p-value < 0.05) across all 3 conditions (see Tables S1, S2 and S3 in supplementary material). As described below, it is clear that at least two of these genes are indeed *in vivo* targets of *GATA3*.

We next employed chromatin immunoprecipitation (ChIP) to confirm the direct interaction of *GATA3* with two of the four predicted target genes. This experiment was conducted using

dissociated epithelial cells from the chick utricle that had been transfected with the pMES vector, to over-express *GATA3*. Putative GATA binding sites were computationally identified, using TF Search (Heinemeyer et al., 1998), upstream of the transcription start sites of *LMO4* and *MBNL2*. Searches within the regions surrounding the other two genes did not reveal convincing putative *GATA3* targets. However, as previously noted, biologically functional *GATA3* sites are not strictly confined to promoter-proximal sites and broader search parameters revealed numerous potential *GATA3* sites. Primers were designed surrounding the putative GATA binding sites adjacent to the *LMO4* and *MBNL2* genes. These were used to PCR amplify those regions after ChIP pull-down with a *GATA3* polyclonal antibody (see Table S4 in supplementary material). PCR products for each candidate region were compared to products from a mock antibody pull-down, in order to identify enrichment by *GATA3* ChIP (Fig. 3). We identified enrichment of 1 region upstream of the *LMO4* transcription start site, -627 to -818, containing two putative GATA binding sites and another region upstream of the *MBNL2* transcription start site, -1574 to -1950, containing 8 putative GATA binding sites. These data strongly support the classification of these two genes as being directly regulated by *GATA3*.

To independently verify the expression patterns predicted by our microarray comparisons and confirm that those expression patterns were consistent with direct regulation by *GATA3*, we conducted RNA in situ hybridizations on whole mount chicken utricles (Fig. 4). In agreement with our microarray data we found that the area of *LMO4* expression surrounds and encloses the striolar region. Our microarray data indicate that *MBNL2* exhibits a reciprocal pattern of expression to that of *GATA3*. In agreement with this, we found that *MBNL2* is not expressed at the striola, but is confined to the medial region of the utricle bordering the striola. This is consistent with a model in which *GATA3* acts to repress *MBNL2* expression, but positively regulates *LMO4* expression at the striola.

Discussion

In this study, we report the first large-scale study of regionalized differences in gene expression in the vestibular sensory organs. We focused on genes that are differentially expressed in the striolar vs. extra-striolar regions of the chick utricle, and which are associated with the expression of the zinc finger transcription factor *GATA3*. Most notable among the detected differences were those involving genes implicated in *WNT* signaling and neurogenesis. Our data indicate that the neurogenesis regulators, *KCNIP4*, *NGN2*, and *HEY2*, may be correlated with the presence of type I vestibular hair cells. *KCNIP4* is particularly interesting, as it encodes a potassium channel-interacting protein that regulates membrane excitability (Holmqvist et al., 2002; Rhodes et al., 2004) and has also been shown to inhibit *WNT* signaling by promoting presenilin (PS1) mediated degradation of β -catenin (Kitagawa et al., 2007). Consistent with this observation we also identified 6 modulators of *WNT* signaling that are differentially regulated at the striola. Three secreted *WNT* ligands, *WNT3*, *WNT3a* and *WNT5a*, and two *WNT* receptors, *FZD5* and *FZD7*, are specifically down-regulated in the striolar region. In contrast, the striola expresses high levels of the *Wnt* modulator Dickkopf2 (*DKK2*). *DKK2* acts as a context-dependant agonist or antagonist of *WNT*/beta-catenin signaling depending on the presence of its co-factor Kremen-2 (Mao and Niehrs, 2003). Although there is no known chick ortholog to Kremen-2, we did note that *GATA3* over-expression resulted in strong upregulation of the *WNT* antagonist *SFRP2* (5.84 fold change). Together, our data suggests a model in which *GATA3* expression leads to down-regulation of *WNT* signaling at the striola. In support of this, a previous study identified overrepresentation of *WNT* signaling, *Notch* signaling, *FGF* signaling and *BMP* signaling gene expression in *GATA3* conditional knockouts of mouse skin epidermis and hair follicles (Kurek et al., 2007). *GATA3* is also required for differentiation and organization of hair follicles during skin development and regeneration (Kaufman et al.,

2003). Our data suggest that *GATA3* may regulate these same signaling pathways in the utricle striola. In addition, *GATA3*-regulated differences in *Wnt* signaling may play an important role in type I vs. type II hair cell differentiation and/or function. Further studies, based upon the candidate genes identified here, should help to resolve these issues.

In addition to implicating candidate effectors within the striola, we also used chromatin immunoprecipitation to identify two direct gene targets of *GATA3* and an additional two genes whose expression consistently varies with *GATA3* levels and localization. *BMP2* has previously been shown to act downstream of *Wnt* signaling during osteoblast differentiation (Rawadi et al., 2003; Morvan et al., 2006) and our gene expression data suggest that *GATA3* may inhibit *BMP2* at the striola, although it is not clear whether this is direct or indirect regulation. Of the confirmed direct targets, the LIM domain only 4 gene (*LMO4*) encodes a cystein-rich transcription regulator containing two LIM domains. The zinc finger binding domains of *LMO4* are structurally similar to *GATA* zinc fingers (Perez-Alvarado et al., 1994). However, no specific LIM-DNA interaction has been reported. Rather, LIM family members are thought to act as part of a transcriptional complex that is mediated by protein-protein interactions: LIM family members have been shown to interact with *GATA* transcription factors during hematopoiesis (Osada et al., 1995; Wadman et al., 1997) and in the spinal cord, *LMO4* interacts with *GATA* transcription factors to regulate the balanced generation of inhibitory and excitatory neurons (Joshi et al., 2009). In the inner ear, *LMO4* is detected in the mouse otic placode at E8.5 and by E10.5 it is expressed primarily in the dorsolateral regions of the otic vesicle that will eventually form the vestibular organs (Fekete and Wu, 2002; Burton et al., 2004; Deng et al., 2006). This pattern appears to be coincident with *GATA3* expression, which also occurs throughout the otic placode at E8 and is also restricted to the dorsolateral otic vesicle by E10.5 (Grace Lawoko-Kerali, 2002). Our gene expression and *in situ* hybridization results confirm this pattern of spatial and temporal co-expression in the mature utricle as well. Taken together, these data suggest that *GATA3* and *LMO4* may partner during ear development. In addition, our ChIP data indicate that *LMO4* is itself a direct target of *GATA3* activity within the mature utricle and very likely throughout inner ear development.

We also identified the muscleblind-like 2 (*MBNL2*) gene as a direct binding target of *GATA3* in the utricle. In this case, given the fact that *MBNL2* gene expression shows a reciprocal pattern to *GATA3*, we would postulate that *GATA3* represses *MBNL2* in the striolar region. The Muscleblind family of proteins regulate alternative exon splicing during differentiation in many cell types, including muscle, neurons and photoreceptors. *MBNL2* can also function as an RNA binding protein essential for integrin $\alpha 3$ subcellular localization (Adereth et al., 2005; Maya Pascual, 2006). Interestingly, a role for integrins in hair cell differentiation and stereocilia maturation has previously been described (Littlewood Evans and Muller, 2000). Taken together these observations suggest that *GATA3* may play a role in subcellular localization of integrins and in the regulation of alternative splicing between type I and type II sensory hair cells in the utricle.

Overall, the current study represents the first large-scale investigation of *GATA3* and its function in the vestibular organs. The identification of two direct DNA binding targets lays the groundwork for eventually building the entire pathway of *GATA3* interactions in the utricle. Our gene expression data strongly suggest that at least one of these interactions is with *WNT* signaling and that *WNT* signaling, or suppression of *WNT* signaling, may play a pivotal role in the striola reversal zone. Our dataset also provides many differentially expressed genes that have not yet been parsed into known pathways. These will be good candidates for future exploration of the exact function of the striola and its conservation within amniote species.

Experimental Procedures

Tissue dissection

10–21 day post hatch White Leghorn chicks were CO₂ asphyxiated and decapitated, heads were immersed in chilled 70% ethanol for 10 min. Utricles were removed and immediately placed in chilled Medium 199 with Hanks salts.

Separation of striolar and extrastriolar regions of the utricle

Explanted utricles were placed in Medium-199 with Hanks salts and iridectomy scissors were used to cut away the edges of the sensory organs (which are comprised of nonsensory transitional epithelium). The remaining sensory tissue was then cut along the anterior-posterior axis, in order to separate the lateral portion of the epithelium (which contains the striola, the GATA3-expressing region and all type I hair cells) from the medial portion (which does not express GATA3 and is populated exclusively by type II hair cells – Fig. 1). Striolar and extrastriolar regions from 10 utricles were separated into two groups and were incubated for 60 min. in 500 µg/ml thermolysin (at 37°C). A fine needle was then used to remove the sensory epithelium from each of the utricular fragments, and mRNA from the striolar and extra-striolar groups was extracted with 100 µl Trizol.

siRNA transfection

Whole utricle specimens were treated 1 mM streptomycin in Medium 199 with Earles salts supplemented with 10% FBS for 24 hours. At this point specimens were rinsed 3× with fresh Medium199/10% FBS and cultured for an additional 24 hours to recover. Whole utricle siRNA transfections were performed by electroporation. Utricles were transfected with 21mer synthetic siRNAs (Ambion) at a final concentration of 100 nM siRNA in 30 µl H₂O under the following conditions: 50 Volts, 30 ms pulse for 10 pulses. Specimens were returned to Medium 199/10% FBS for an additional 48 hours and pure sensory epithelia were isolated using previously published methods (Warchol, 2002).

Whole utricles were transfected with either *GFP* synthetic siRNA target sequence (AGGAGAAGAAGCTTTTCACT) or an equal concentration of *GATA3* synthetic siRNA target sequence (AAGGCAGGGAGTGTGTAAATT).

GATA3 overexpression

Dissociated sensory epithelia were plated in 96 well cultures, 5 wells per sample. 4 days post plating, ~ 30% confluency, cells were transfected with a pMES vector containing an internal ribosome entry site regulating expression of *GATA3* and eGFP under control of a chick beta-actin promoter. Controls were transfected with a vector containing eGFP only. Transfections were performed using recommended concentrations for Lipofectamine 2000 (Invitrogen). 24 hours post transfection cells were harvested in 100 µl Trizol per well, 5 wells were combined for each biological sample.

cDNA amplification and microarray hybridization

RNA isolation and cDNA synthesis was carried out as previously described (Hawkins et al., 2003), with the following modifications. Following cDNA amplification from the streptavidin coated beads cDNA was used for *in-vitro* transcription (T7 MegaScript Kit, Ambion). Following LiCl precipitation and 75% ethanol wash, 1 µg poly (A)⁺ RNA was used for indirect labeling using Genisphere 3DNA Array 50 Kit following manufacturers protocols and hybridized to a custom oligonucleotide microarray at 42C

Microarray design and analysis

All microarray hybridizations were conducted on a custom microarray consisting of 50–70 mer oligonucleotides designed to the majority of human transcription factor genes (Messina et al., 2004). Probes were designed to coding regions and have been shown to accurately report expression in chicken under appropriate conditions (Hawkins et al., 2003). Microarray comparisons were Lowess normalized, genes with intensity below background as determined by control spots were removed. All comparative microarray hybridizations consisted of 2 biological samples and 4 technical replicates for each biological sample, including dye switch experiments. A one-sample t-test was used to identify genes that showed statistically consistent changes in gene expression with a P-value < 0.05 across all replicates for each condition. All microarray data has been deposited with NCBI GEO with accession number GSE14792.

RNA in situs

Primers were designed to generate 200–400 bp PCR amplicons from chicken sequences. A second round of PCR was used to add T7 promoters to either the 5' or 3' end. PCR products were gel purified and verified by DNA sequencing. PCR templates were used to separately generate DIG labeled *in-vitro* transcripts (Ambion T7 MegaScript Kit) for both sense and anti-sense strands. Utricles were obtained from 10–21 day old White Leghorn chicks and processed for whole mount in situs following published protocols (Henrique et al., 1995) Utricles were labeled and mounted in glycerol/PBS (9:1) and imaged.

ChIP

Chromatin immunoprecipitation was performed in dissociated sensory epithelia over-expressing GATA. Sensory epithelia from 10 utricles were physically dissociated and plated in 6 well cultures and transfected with a *GATA3* expressing pMES vector as previously described. ChIP was conducted following recommended protocols (Active Motif). Specifically, enzymatic shearing was conducted for 10 min and IP was performed with the *GATA3* goat polyclonal IgG sc-22205 (Santa Cruz) at a concentration of 3 µg/100 µl or a mock antibody negative control. Predicted GATA binding sites were identified up to 2000 bp upstream of predicted target genes using TF Search (Heinemeyer et al., 1998). PCR primers were designed flanking putative GATA binding sites (see Table S4 in supplementary material) and ChIP pull down products were amplified for 30 cycles with a 55 °C annealing temperature followed by an additional 30 cycles and imaged by agarose gel electrophoresis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Avian utricle hair cell patterns. The striola region contains the Type I hair cells and the extrastriola region is populated by the Type II hair cells. GATA3 is expressed in a 6–10 cell wide strip of cells corresponding to the striola reversal zone. Sensory hair cells undergo a 180° shift in orientation at the striola reversal zone.

Figure 2. Immunohistochemical labeling with a GATA3 antibody (green) in siRNA treated whole avian utricles. GATA3 immunoreactivity is localized to the 6–10 cell wide strip of cells at the striola reversal zone in the A) control GFP siRNA treated sample and knocked down in the B) GATA3 treated siRNA.

Figure 3.

Direct *in-vivo* interactions with GATA3 were demonstrated by ChIP in dissociated sensory epithelia over-expressing GATA3. PCR amplification with primers flanking putative GATA binding sites identified enrichment in the anti-GATA antibody (+) ChIP compared to a mock antibody control (-) in a 192 bp product, LMO4-3, from -818 bp to -627 bp upstream of LMO4 and two overlapping 165 bp and 232 bp products from -1738 bp to -1574 bp (MBNL-8) and -1950 bp to -1719 bp (MBNL-9) upstream of MBNL2.

Figure 4.

In situ hybridizations confirm expression patterns of LMO4 and MBNL2 predicted by our microarray and ChIP data. Immunohistochemical labeling with a GATA3 antibody (green) and RNA in situ hybridizations with antisense probes to LMO4 and MBNL2 in whole mount chick utricles. GATA3 and LMO4 are expressed at the striola reversal zone and MBNL2 is expressed in the medial region of the utricle bordering the striola reversal zone.

Table 1

Genes differentially expressed in the striola vs. extra-striola.

Gene	Average Fold Change	P-value	Gene	Average Fold Change	P-value
KCNIP4	11.19	7.32×10^{-03}	KLHL9	-19.34	1.75×10^{-02}
DKK2	10.92	2.68×10^{-04}	MGC16733	-15.25	3.81×10^{-03}
NEUROG2	7.86	1.17×10^{-02}	ENO1	-10.46	4.33×10^{-04}
HEY2	6.53	5.81×10^{-03}	WNT3A	-7.55	2.62×10^{-04}
BRD9	5.66	2.35×10^{-02}	CYLD	-7.44	2.90×10^{-04}
GATA3	5.45	1.12×10^{-02}	HRY	-6.99	1.23×10^{-03}
NR0B1	5.04	1.52×10^{-02}	RORA	-6.63	1.26×10^{-03}
LOC90322	4.55	2.51×10^{-02}	FEZL	-6.06	3.44×10^{-04}
RAX2	4.09	2.49×10^{-02}	BMP4	-5.63	1.46×10^{-02}
MYT1L	4.00	1.11×10^{-02}	WNT5A	-5.37	9.07×10^{-03}
TCEA2	3.93	3.13×10^{-02}	PMX1	-5.27	9.96×10^{-03}
FGF20	3.92	2.00×10^{-02}	FHL2	-5.26	1.32×10^{-02}
IHH	3.55	5.90×10^{-03}	HOXD8	-5.14	5.17×10^{-03}
SATB1	3.50	3.41×10^{-04}	HEYL	-5.02	2.36×10^{-03}
MLL3	3.38	6.15×10^{-03}	ZNF652	-4.74	2.86×10^{-02}
KIAA0293	2.94	3.32×10^{-02}	BAPX1	-4.69	2.71×10^{-03}
PDEF	2.92	2.59×10^{-03}	NRTN	-4.61	2.68×10^{-03}
HOXA6	2.83	4.93×10^{-03}	BTBD5	-4.60	1.76×10^{-02}
TBX22	2.69	1.75×10^{-02}	MTF1	-4.34	6.23×10^{-04}
TCFL1	2.68	2.54×10^{-02}	RXR	-4.32	3.72×10^{-04}
SUV39H1	2.64	1.74×10^{-02}	GLI3	-4.18	9.42×10^{-04}
LOC416414	2.56	8.85×10^{-03}	FGF16	-3.64	4.75×10^{-02}
SCA2	2.53	3.75×10^{-05}	SP4	-3.51	1.18×10^{-03}
PAX3	2.50	4.72×10^{-02}	CDK5RAP1	-3.42	1.81×10^{-02}
BS69	2.49	1.07×10^{-02}	HNF1	-3.21	7.33×10^{-04}
NCOR2	2.42	1.12×10^{-02}	ACVR1B	-3.12	1.72×10^{-02}
FOXL2	2.38	3.65×10^{-03}	BMP2	-3.12	1.20×10^{-02}

Gene	Average Fold Change	P-value	Gene	Average Fold Change	P-value
JAG1	2.33	1.70×10^{-02}	FZD7	-3.01	1.48×10^{-02}
HMGB3	2.28	1.06×10^{-02}	MADH9	-3.00	1.26×10^{-02}
STAT5A	2.28	4.09×10^{-02}	BMP15	-2.99	3.49×10^{-02}
LMO4	2.28	4.26×10^{-02}	DACH1	-2.92	3.23×10^{-02}
IRX1	2.26	5.27×10^{-03}	FZD5	-2.82	3.99×10^{-03}
CLOCK	2.24	3.63×10^{-02}	CDK9	-2.80	1.51×10^{-02}
PMX2B	2.22	9.35×10^{-04}	IKZF2	-2.78	4.56×10^{-02}
LHX3	2.17	7.11×10^{-04}	TRAF4	-2.77	1.41×10^{-02}
RBBP5	2.13	6.59×10^{-03}	WNT3	-2.72	2.24×10^{-03}
LARP1	2.11	4.20×10^{-02}	PLTP	-2.71	4.25×10^{-02}
TCF1	2.10	3.21×10^{-02}	HOXB9	-2.69	1.20×10^{-04}
			TGIF	-2.45	4.13×10^{-03}
			BUB3	-2.28	3.79×10^{-02}
			GDF8	-2.22	4.59×10^{-02}
			ARNTL2	-2.16	2.63×10^{-02}
			BANP	-2.15	2.25×10^{-02}
			RNF12	-2.07	1.66×10^{-02}
			MADH7	-2.00	1.53×10^{-02}

Average fold changes are displayed as levels in the utricle striola relative to the extra-striola. Values are > 2-fold and p-value < 0.05.

Table 2

Genes differentially expressed in GATA3 siRNA treatments.

Gene	Average Fold Change	P-value	Gene	Average Fold Change	P-value
NUP153	12.06	2.01×10^{-02}	MYOD1	2.70	6.75×10^{-03}
HRIHFB2122	7.09	2.75×10^{-03}	MYT1L	2.67	1.23×10^{-03}
VEGFC	7.06	1.84×10^{-02}	NFE2L2	2.62	3.31×10^{-02}
SIAH1	6.94	1.50×10^{-02}	GTF2E2	2.61	2.75×10^{-02}
BRD1	6.29	2.68×10^{-03}	ZFP91	2.55	3.05×10^{-02}
PMX2B	5.28	5.59×10^{-03}	ARNTL2	2.49	2.89×10^{-02}
HOXA7	5.04	9.25×10^{-03}	SHH	2.48	3.56×10^{-02}
ALX4	4.95	3.61×10^{-02}	MTF1	2.45	2.94×10^{-02}
RXRG	4.88	2.42×10^{-02}	RFX3	2.44	1.25×10^{-02}
LHX8	4.86	3.62×10^{-02}	FMR2	2.41	1.13×10^{-02}
BCL11B	4.76	9.73×10^{-03}	MDN1	2.41	4.68×10^{-03}
CHD1	4.56	2.12×10^{-02}	FZD5	2.40	2.56×10^{-02}
CITED2	4.52	3.62×10^{-02}	NEUROD6	2.40	2.26×10^{-02}
FOXP1	4.50	8.76×10^{-03}	CYLD	2.38	8.00×10^{-04}
MYBL1	4.47	3.22×10^{-02}	NHLH2	2.34	1.74×10^{-02}
SPOP	4.37	1.47×10^{-02}	HSF2BP	2.33	4.04×10^{-02}
ARTN	4.26	2.87×10^{-02}	TRIM50A	2.29	2.15×10^{-02}
PCMT1	4.24	2.15×10^{-02}	FOG2	2.27	5.94×10^{-03}
FGF16	4.14	1.45×10^{-02}	MORF4	2.23	2.08×10^{-02}
FEZL	4.08	1.63×10^{-02}	SOX21	2.13	3.91×10^{-02}
FOXH1	4.01	2.07×10^{-02}	SOX1	2.09	3.55×10^{-02}
FELZ	3.86	1.51×10^{-02}	TITF1	2.03	3.43×10^{-02}
EEF1A1	3.84	1.95×10^{-02}	MLLT6	2.03	2.64×10^{-02}
PMX1	3.73	2.82×10^{-02}	CREB3L2	2.01	4.64×10^{-02}
ID3	3.71	1.35×10^{-02}	BCL6B	2.01	1.87×10^{-02}
DAZAP1	3.63	4.14×10^{-02}			

Gene	Average Fold Change	P-value	Gene	Average Fold Change	P-value
IVNS1ABP	3.57	2.67×10^{-02}			
KLHL3	3.51	3.17×10^{-02}	Gene	Average Fold Change	P-value
ZBTB26	3.44	1.38×10^{-02}	BARX1	-9.31	3.28×10^{-02}
DEAF1	3.38	2.64×10^{-02}	BARHL1	-6.68	2.44×10^{-02}
ALDH3A2	3.11	1.31×10^{-02}	JUN	-3.64	1.57×10^{-02}
TNRC9	3.08	2.06×10^{-02}	POU1F1	-3.53	2.19×10^{-02}
SUPT4H1	3.03	1.91×10^{-02}	WNT5B	-2.67	3.18×10^{-02}
WNT3	3.01	4.11×10^{-02}	CSRP1	-2.25	2.71×10^{-02}
LRP5	2.97	1.12×10^{-02}	ATF7	-2.21	1.11×10^{-02}
MADH7	2.86	1.17×10^{-02}	ETV4	-2.16	1.94×10^{-02}
LAF4	2.77	2.13×10^{-02}	PPARBP	-2.05	1.77×10^{-02}
ZNF71	2.71	4.33×10^{-02}	LMO4	-2.01	3.28×10^{-02}

Average fold changes are displayed as levels in the GATA3 siRNA knockdown relative to the control GFP siRNA. Values are > 2 -fold and p-value < 0.05 .

Table 3

Genes differentially expressed in GATA3 over-expression.

Gene	Average Fold Change	P-value	Gene	Average Fold Change	P-value
GATA3	7.28	2.71×10^{-04}	WNT9A	-3.68	2.40×10^{-03}
WNT9A	6.24	1.28×10^{-02}	SFRP2	-3.04	1.18×10^{-03}
SFRP2	5.84	4.61×10^{-02}	TAF172	-2.6	2.08×10^{-03}
TAF172	3.59	1.83×10^{-02}	BMP15	-2.5	1.38×10^{-03}
BMP15	2.68	3.83×10^{-02}	SOX8	-2.48	2.00×10^{-02}
SOX8	2.67	3.90×10^{-02}	TCF7	-2.28	4.21×10^{-03}
TCF7	2.55	1.97×10^{-02}	EDAR	-2.16	5.17×10^{-03}
EDAR	2.49	4.40×10^{-02}	PXN	-2.15	1.28×10^{-02}
PXN	2.33	1.91×10^{-02}	GTF2E2	-2.04	2.07×10^{-02}
GTF2E2	2.17	4.20×10^{-03}	MADH9	-2.03	2.95×10^{-02}
MADH9	2.16	5.95×10^{-05}	HNK1	-2.03	4.08×10^{-05}
HNK1	2.01	2.05×10^{-02}			

Average fold changes are displayed as levels in the GATA3 over-expression experiment relative to the GFP vector only control. Values are > 2 -fold change p-value < 0.05 .

Table 4

Genes with similar or reciprocal expression patterns to *GATA3* across all three conditions.

Gene	Striola	P-value	GATA3 Knockdown	P-value	GATA3 Over-expression	P-value
BMP2	-3.12	1.20×10^{-2}	1.44	2.15×10^{-2}	-1.79	1.52×10^{-2}
FKHL18	1.57	1.68×10^{-2}	-1.66	4.38×10^{-2}	1.44	1.41×10^{-2}
GATA3	5.45	1.12×10^{-2}	-1.53	6.13×10^{-5}	1.84	1.42×10^{-2}
LMO4	2.28	4.26×10^{-2}	-2.01	3.28×10^{-2}	1.42	1.43×10^{-3}
MBNL2	-1.47	2.84×10^{-2}	1.85	1.35×10^{-2}	-1.58	4.44×10^{-2}

All values are derived from Supplementary Tables S1, S2 and S3. Average fold changes > 1.3 and p-value < 0.05 in all three conditions.