Published in final edited form as:
Dev Dyn. 2012 March ; 241(3): 563–573. doi:10.1002/dvdy.23731.

Identification of putative retinoic acid target genes downstream of mesenchymal *Tbx1* during inner ear development

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Abstract

The T-box transcription factor Tbx1 is expressed in the otic vesicle and surrounding mesoderm of the periotic mesenchyme (POM) during inner ear development. Mesenchymal Tbx1 is essential for inner ear development, with conditional mutants displaying defects in both the auditory and vestibular systems. We have previously reported that mesodermal Tbx1 loss of function mutants (Mest-KO) have reduced expression of retinoic acid (RA) metabolic genes, Cyp26a1 and Cyp26c1, in the POM, consistent with other studies showing an increase in mesodermal RA reporter expression in Tbx1-/- embryos. However, putative RA effector genes whose expression is altered downstream of increased otic mesenchymal-epithelial RA signaling have remained elusive. Here we report the identification of eighteen retinoic acid responsive genes altered in Mest-KO conditional mutants by microarray gene profiling. Nine were chosen for biological validation including quantitative RT-PCR and in situ hybridization (Otor, Mia, Col2a1, Clu, Adm, Myt1, Dlx3, Itgb3 and Itga2b). This study provides a series of newly identified RA effector genes for inner ear development downstream of mesenchymal Tbx1 that may contribute to the inner ear phenotype observed in Tbx1 loss of function mouse models.

INTRODUCTION

Haploinsufficiency of *TBX1*, a transcription factor of the T-box family, has been implicated in the etiology of velo-cardio-facial syndrome/DiGeorge syndrome/22q11.2 deletion syndrome (VCFS/DGS/22q11DS). VCFS/DGS patients display a wide range of phenotypes including hearing loss, with 30% of patients suffering from conductive hearing loss subsequent to recurrent middle ear infections and 10% suffering from sensorineural hearing loss (Digilio et al., 1999). Mouse models for *Tbx1* have similar phenotypes, with heterozygous animals displaying chronic otitis media and homozygous null mice having sensorineural hearing loss due to arrested development of inner ear structures (Vitelli et al., 2003; Liao et al., 2004).

The mammalian inner ear is composed of the vestibular system, with three semicircular canals for sensing angular acceleration as well as the utricle and saccule for sensing linear acceleration, and the cochlea of the auditory system for processing of sound. Murine *Tbx1* is expressed in the otic vesicle (OV) epithelium from which these inner ear structures derive as well as the surrounding mesoderm of the periotic mesenchyme (POM) which gives rise to the cartilaginous otic capsule during development and later the bony labyrinth surrounding the mature inner ear. Homozygous null *Tbx1* mutants have a severe inner ear phenotype with

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an absence of mature inner ear structures. There are no auditory or vestibular system components, and the otic capsule is malformed (Vitelli et al, 2003; Raft et al., 2004).

Due to this severe early phenotype in homozygous null mutants, conditional ablation studies of *Tbx1* in the OV and, separately, the POM have been used to study tissue-specific roles of this transcription factor during inner ear development. Conditional inactivation of *Tbx1* in the OV epithelium using *Pax2-Cre* mice phenocopies *Tbx1*$^{-/-}$ embryos in the inner ear, suggesting a major early role for *Tbx1* in the epithelium of the OV (Arnold et al., 2006). Conversely, ablation of *Tbx1* in the mesoderm and POM, using mesodermal Cre mouse lines such as *Mesp1-Cre* and *T-Cre*, lead to a later, less severe inner ear defect consisting of failed cochlear outgrowth, hypoplastic semicircular canals (SCs), and an enlarged endolymphatic duct (Xu et al., 2007; Braunstein and Monks et al., 2009). This suggests that, as a transcription factor, *Tbx1* in the surrounding mesoderm plays a key role in regulating signals from the POM to the OV, referred to as mesenchymal-epithelial signaling, during development of the inner ear.

Retinoic acid (RA) is a major signaling pathway implicated in both epithelial-mesenchymal signaling during otic capsule chondrogensis (Frenz et al., 1997) as well as mesenchymal-epithelial signaling to the OV during cochlear outgrowth (Braunstein and Monks et al., 2009). Earlier studies investigating the role of *Tbx1* and RA in heart development demonstrated increased RA reporter activity in *Tbx1*$^{-/-}$ embryos, including an anterior shift encompassing the developing ear (Guris et al., 2006), concurrent with decreased expression of the RA metabolic genes, *Cyp26a1* and *Cy26c1*, in the POM at E9.5 (Roberts et al., 2006). We have previously demonstrated such dysregulation of RA signaling downstream of *Tbx1* is attributable to the POM domain of *Tbx1*, as loss of *Tbx1* in the mesoderm leads to reduction in the expression of *Cyp26a1* and *Cyp26c1* in the POM, while expression of *Cyp26c1* in the ventral OV remains intact (Braunstein and Monks et al., 2009). This suggests that loss of the transcription factor *Tbx1* in the mesoderm regulates RA signaling, either directly or indirectly, in the POM during inner ear development (Braunstein and Monks et al., 2009). Additional evidence for mesenchymal-epithelial RA signaling comes from genetic studies using *Brn4*$^{+/-}$ mice. *Brn4* (*Pou3f4*) is an X-linked gene encoding a POU domain transcription factor required for brain, ear and pancreatic development (Mathis et al., 1992; Phippard et al., 1999; Hussain et al., 2002). Hemizygosity for *Brn4* leads to defects in cochlear outgrowth similar to those observed in mesenchymal *Tbx1* ablation (Braunstein et al., 2008). Furthermore, *Brn4* was shown to genetically interact with *Tbx1* for proper cochlear outgrowth, and both are required for *Cyp26c1* expression in the POM (Braunstein and Monks et al., 2009).

While RA has been implicated in mesenchymal-epithelial signaling during inner ear development, downstream effector genes for RA signaling in the OV and/or POM have not been identified. Using a gene profiling microarray approach, we have identified genes which may serve as RA targets, based upon published data in other cells or tissues, at the stage of mid-gestational auditory and vestibular system morphogenesis (E12.5).

RESULTS

Gene profiling to identify *Tbx1* regulated POM genes

To identify possible downstream genes of mesodermal *Tbx1*, gene profiling microarray analysis was performed on microdissected periotic regions (POM + OV) of *T-Cre* mediated conditional *Tbx1* mutants (*Mes*^{t}*-KO*) at E12.5. A total of 161 differentially expressed genes were identified, including 80 up-regulated and 81 down-regulated genes (Supplemental Table 1). Data obtained from this microarray screen were analyzed for changes in common gene networks or signaling pathways so as to explain molecularly how mesenchymal-

epithelial signaling might be perturbed in *Mes^t-KO* embryos during inner ear morphogenesis. This screen identified eighteen genes that have been shown in other systems to be downstream of RA signaling (Figure 1). All genes were altered in the direction of known response to RA, with *Adm* (Minamino et al., 1995), *Cp* (Tice et al., 2002), *Myt1* (Franco et al., 1999), *Ncam1* (De Laurenzi et al., 2000), *Pak1* (Csomos et al., 2010), and *Vwf* (Hatzopoulos et al., 1998) up-regulated and *Aplnr* (Zhong et al., 2005), *Bhmt* (Xu et al., 2010), *Col2a1* (Jimenez et al., 2001), *Clu* (Orlandi et al., 2005), *Dlx3* (Acquafreda et al., 2010), *Fgf4* (Maerz et al., 1998), *Hoxc9* (Mao et al., 2011), *Itga2b* (Liu et al., 2000), *Itgb3* (Csomos et al., 2010), *Mia1* (Dietz et al., 1996), *Msc* (Hishikawa et al., 2005), *Otor* (Roberts et al., 2000), and *S100a8* (Liu et al., 2000) down-regulated, consistent with previously published data *in vitro* or in other model systems (Table 1).

Nine genes from the RA pathway were selected for validation by quantitative RT-PCR (qRT-PCR), based on known ear expression or strongest fold-change values by microarray. The nine selected genes were *Adm, Clu, Col2a1, Dlx3, Itga2b, Itb3, Mia1, Myt1,* and *Otor*. Validation by qRT-PCR confirmed significantly altered expression of these RA responsive genes with the exception of *Col2a1* and *Mia1*, which showed a trend of down-regulation but were not significant (Figure 2).

Localization of RA regulated genes to developing inner ear

While RA regulation of these downstream genes has been implicated or established, most of these studies were conducted in either cell culture systems or in the development of organs other than the ear (Table 1). For this reason, specific temporo-spatial expression data for ear development is largely unavailable. In order to localize expression patterns of these genes to the otic epithelium and/or POM during inner ear development, *in situ* hybridization on microdissected E12.5 periotic regions, containing the OV, POM, cochleovestibular ganglion (CVG), and surrounding head mesenchyme, was performed.

We have previously reported that the E11.5 *Mes^t-KO* inner ear begins to show signs of hypoplasia, specifically in the ventral region that gives rise to the cochlea, and that E15.5 Mest-KO ears display a shortened cochlea, semicircular canal hypoplasia, and an enlarged endolymphatic duct. (Braunstein and Monks et al., 2009). In order to determine the presence and morphology of these structures at the stage of microarray analysis, E12.5 Mest-KO and control embryos were analyzed by paint filling. At E12.5, the *Mes^t-KO* cochlea is present, but shortened, as compared to the cochlea in control embryos, conditional *Tbx1* heterozygotes, at this stage (green arrows, Figure 3A,D). Dorsal structures, including the endolymphatic duct (red arrow) and canal outpouches, which will give rise to the semicircular canals of the vestibular system (yellow arrows), are present and appear morphologically normal at E12.5.

The mesenchymal gene *Melanoma inhibitory activity 1* (*Mia1*), also known as cartilage-derived retinoic acid sensitive protein (*CD-RAP*), was identified by microarray analysis as down-regulated in *Mes^t-KO* embryos (Table 1). Validation by qRT-PCR supported a trend of down-regulation, but was not statistically significant (Figure 2). Supporting the microarray findings, *in situ* hybridization revealed expression of *Mia1* in the dorsal POM of control embryos, with nearly complete loss in mutant embryos (arrows, Figure 3B,E). Such differences in validation by qRT-PCR and *in situ* hybridization might be due to statistical noise caused by slight differences in the region of microdissected tissue included in qRT-PCR analysis. This seems likely for *Mia1*, as it is expressed in POM lying at the dorsal endpoint of microdissection for qRT-PCR. Microdissected tissue for *in situ* analysis contains more tissue including head mesenchyme and surface ectoderm surrounding the developing ear, making expression changes easier to visualize. *Mia1* was identified in cell culture of

bovine chondrocytes, where its expression was significantly reduced upon treatment with RA, consistent with our microarray and *in situ* hybridization data (Dietz et al., 1996).

Otoraplin (*Otor*) was initially discovered as a homologue to CD-RAP/MIA expressed in a cochlear cDNA library, and was accordingly classified as negatively regulated by treatment with all-trans RA (atRA). Its expression was localized to the chicken cochlea by northern blot analysis, but previous attempts at *in situ* hybridization were unsuccessful (Robertson et al, 2000). Through tissue specific *in situ* hybridization, *Otor* expression was detectable in the developing mouse cochlea at E12.5. *Otor* was expressed in the base of the cochlea of control embryos, but strikingly absent from the cochlea of *Mes^{t}-KO* embryos, consistent with qRT-PCR results (green arrow, Figure 3C,F). No *Otor* expression was detected in other regions of the ear, consistent with previous data (Robertson et al., 2000).

Myelin transcription factor 1, Myt1, encodes a zinc finger transcription factor implicated in neurogenesis and pancreatic islet cell development (Romm et al., 2005). In Xenopus, *XMyT1* expression is expanded in the neural ectoderm upon treatment with RA, while inhibition of RA signaling leads to decreased expression (Franco et al., 1999). Consistent with a role in neurogenesis, *Myt1* was localized to the developing cochleovestibular ganglion (CVG), as well as the inferior vagal ganglion (CN IX) by *in situ* hybridization. Microarray and qRT-PCR analysis confirms up-regulation in *Mes^{t}-KO* embryos. However, due to the complex morphology of the CVG at E12.5, such up-regulation was not detected by *in situ* hybridization (Figure 3G,J).

Transcription factors of the distaless homeobox family are known to play important roles in the developing vestibular system of the inner ear (Robledo et al., 2006). One member of this family, *Dlx3*, was down-regulated by microarray and qRT-PCR analysis. *Dlx3* is expressed in the dorsal region of the OV at E12.5 of control embryos, including the developing endolymphatic duct and canal outpouches (yellow arrows, Figure 3H), consistent with previously reported dorsal expression in chick (Brown et al., 2005). Expression was lost in the developing semicircular canals (SC) of *Mes^{t}-KO* embryos, while expression in the endolymphatic duct remained intact (red arrow, Figure 3K). A recent report using an oral squamous cell carcinoma line treated with atRA suggests that *Dlx3* is negatively regulated by RA (Acquafreda et al., 2010).

Clusterin (*Clu*) is a widely expressed protein serving both pro- and anti-apoptotic functions during development and in mature tissues, with major functions in prostate development and Alzheimer's disease (Ahuja et al., 1994; Calero et al., 2005). Expression in the developing inner ear has been described, with early widespread OV expression (Ahuja et al., 1994) and later localization to the ventral cochlear epithelium (Ficker et al., 2004). Evidence for RA regulation of *Clu* comes from studies in vascular smooth muscle cells, where treatment with atRA led to decreased *Clu* expression (Orlandi et al., 2005). Consistent with this data, E12.5 expression is localized to the ventral cochlear portion of the OV and is completely absent in *Mes^{t}-KO* embryos at this stage (green arrows, Figure 3I,L).

Localization by *in situ* hybridization for three RA-responsive genes encoding Adrenomedullin (*Adm*), Integrin alpha 2b (*Itga2b*), and Integrin beta 3 (*Itgb3*), though attempted, failed due to lack of ability of probes to detect specific expression. *Adm* is a vasodilator peptide expressed in smooth muscle cells, where its expression is positively regulated by RA exposure (Minamino et al., 1995). Consistent with these reports, *Adm* expression was increased 1.44-fold in *Mes^{t}-KO* embryos by qRT-PCR (Figure 2). *Adrenomedullin* expression was previously identified in the mouse cochlea by RT-PCR and further localized to the stria vascularis by immunohistochemistry (Tono et al., 2002). *Itga2b* and *Itgb3* are known to be down-regulated by RA in a leukemia cell line (Liu et al., 2000;

Csomos et al., 2010), though expression during inner ear development has not been characterized. However, integrins are known to play important roles in mesenchymal condensation and chondrogenesis, suggesting possible expression of both *Itga2b* and *Itgb3* in the POM (Schubert et al., 2010). In summary, all gene expression changes detected by microarray analysis were in the direction predicted downstream of increased RA levels, some of which may be effectors of cochlear or vestibular morphogenesis based on tissue expression patterns.

Clu$^{-/-}$ embryos display normal cochlear morphology

Due to the strong expression of *Clu* in control cochleae and a striking loss of expression in *Mes*^{t}*-KO* embryos at E12.5 (Figure 3I,L) and E11.5 (Figure 4A-B), loss of ventral *Clu* is an attractive candidate for the cochlear phenotype of mesodermal *Tbx1* conditional knockout embryos (Xu et al., 2007; Braunstein and Monks et al., 2009). To determine if loss of *Clu* leads to defects in gross cochlear morphology, E15.5 *Clu* homozygous null embryos were analyzed via paint filling. No defects in inner ear morphology were detectible in these mutants as compared to *Tbx1* heterozygous control embryos at the same stage (Figure 4C-D), suggesting that loss of *Clu* is either compensated for by another, to date unidentified, gene, or that *Clu* plays a minor role, if any, in the cochlear outgrowth phenotype observed in *Mes*^{t}*-KO* embryos.

DISCUSSION

RA has previously been implicated in earlier stages of inner ear development, such as otic induction, where increased signaling expands competence to form otic placode and is necessary for proper Fgf signaling from the hindbrain in zebrafish (Hans and Westerfield, 2007). A role for RA during early murine inner ear development has also been described, with RA treatment prior to E9.0 leading to widespread morphological defects and embryonic resorption and later exposure at E10 *in vivo* (Frenz et al., 1996) and at E12.5-E14 leading to specific cochlear defects in an explant culture system (Frenz et al., 1997). More recently, a specific temporo-spatial role for RA in establishing anterior-posterior axis identity in the avian OV has been established, with early exposure (E7.75-8.25) leading to posteriorization, marked by expanded *Tbx1* expression, and exposure at E8.5 resulting in fewer gene expression and morphology changes (Bok et al., 2011). RA is also known to play a role in condensation of the POM to form the cartilaginous otic capsule, with treatment of cultured POM+OV unable to form cartilage in the presence of exogenous atRA (Frenz et al., 2000).

However, elucidating the *in vivo* roles of RA in later stage murine ear development, during mid-gestation, has been difficult due to widespread teratogenicity of RA treatment. This is particularly important in studying events such as cochlear coiling, as avian cochleae do not form a coiled structure as mice and higher vertebrates do. Mesodermal knockout of *Tbx1* provides an excellent system to bypass early RA teratogenicity and study the slightly later, tissue-specific roles of RA during inner ear morphogenesis in the mouse system.

Previous studies have revealed increased RA signaling in the absence of *Tbx1* expression, including the region of the developing E9.5 inner ear (Roberts et al., 2006; Guris et al., 2006). This early increase in RA signaling appears to be mediated in part by reduction in the genes encoding cytochrome P450 enzymes responsible for RA degradation, *Cyp26a1* and Cyp26*b1*. We have previously reported a similar reduction in *Cyp26a1* and *Cyp26c1* gene expression in the periotic mesenchyme when *Tbx1* is ablated in the same cell population using the mesodermal *T-Cre* line (Braunstein and Monks et al., 2009).

Consistent with decreased expression of *Cyp26a1* and *Cyp26c1* enzymes in *Mes*^{t}*-KO* embryos, two putative T-sites were identified in the region of mouse chromosome 19

containing these contiguous genes (see Materials and Methods). The first lies approximately 50 bp upstream of the published *Cyp26a1* promoter (Loudig et al., 2000) while the second is approximately 500 bp upstream of the *Cyp26c1* coding sequence, with the distance between these two putative T-sites spanning a total interval of less than 20kb. Given this small genomic interval, TBX1 protein binding to either of these T-sites could regulate transcription of one or both *Cyp26* genes. In order to determine if TBX1 can bind these putative T-sites, electrophoretic mobility shift assays were performed using a GST-tagged TBX1 protein and oligonucleotides encompassing the putative T-sites. Neither putative T-site was found to bind TBX1 in this assay, suggesting that regulation of *Cyp26a1* and *Cyp26c1* occurs either indirectly or via unidentified T-sites in the region (Data not shown).

In the E12.5 *Mes^{t}-KO* conditional *Tbx1* mutant, we have identified changes in over 160 genes. After pathways analysis, we identified eighteen genes previously described as regulated by retinoic acid using either *in vitro* or *in vivo* approaches in other model organisms. All eighteen genes show changes in transcriptional up- or down-regulation in the direction predicted by the presence of increased RA signaling in *Mes^{t}-KO* embryos by microarray, while seven out of nine genes assayed remained significant after qRT-PCR validation. The expression changes, while significant and obvious upon *in situ* hybridization, do not display large fold change values by these quantitative methods. This is likely due to the large amounts of tissue present in the E12.5 periotic region used for these studies as well as the specific expression patterns in very small portions of that tissue, thus diluting gene expression levels. Taking this into account, it is likely that changes in genes such as *Mia1* might actually have physiologic significance, as suggested by clearly reduced expression in the small region of dorsal POM by *in situ* hybridization, despite the small fold-change on the microarray and/or statistical insignificance upon qRT-PCR validation. It is also important to note that, due to these dilution effects, there are likely many more genes changed in the periotic region of *Mes^{t}-KO* mutants than have been identified by this approach. The list presented, however, represents those genes identified to have significant expression changes in the periotic region in addition to previous data suggesting regulation by RA signaling.

The significant gene changes can be divided into two broad expression domains: the otic epithelium, giving rise to the inner ear proper, and the periotic mesenchyme, giving rise to the bony labyrinth. Genes expressed specifically in the otic epithelium, such as *Adm, Clu, Dlx3*, and *Otor*, may play a role in the inner ear phenotype observed in *Mes^{t}-KO* mutants, with the exception of *Clu*, which showed no cochlear phenotype in our analysis. Similarly, genes expressed in the POM, such as *Mia*, may contribute more directly downstream of mesenchymal Tbx1 to defects in the otic capsule or to inner ear morphogenesis. *Itga2b* and *Itgb3* were unable to be localized by *in situ* hybridization due to unavailability of specific probes and absence of previous expression data during inner ear development. However, integrins are known to play a key role in mesenchymal condensation and chondrogenesis via an interaction with *Mia1* (Schubert et al., 2010), suggesting the possibility that these two genes interact and contribute to inner ear and/or otic capsule defect observed in *Mes^{t}-KO* embryos (Braunstein and Monks et al., 2009).

Interestingly, exogenous RA signaling has been shown to act upstream of the otic epithelial expression domain of *tbx1* in zebrafish (Radosevic et al., 2011). Thus, RA might act both upstream and downstream of *tbx1*. In our previous studies, alteration of *Tbx1* expression was not detected in the OV domain of *Mes^{t}-KO* embryos, concurrent with increased RA (Braunstein and Monks et al., 2009), suggesting that there may be species-specific or temporal differences in regulation of *Tbx1* downstream of RA signaling.

Failed cochlear outgrowth is perhaps the most striking phenotype observed in *Mes^{t}-KO* embryos. In order to investigate the mechanism of this defect, we sought knockout mouse

models for *Otor* and *Clu* to provide further genetic data for this phenotype. The only available mouse model, *Clusterin*, though expressed specifically in the developing cochlea and strikingly lost in *Mes^{t}-KO* embryos, failed to produce a cochlear phenotype when inactivated. Development of future mouse models for genes such as *Otor* might provide the missing link between altered RA signaling and cochlear morphology defects observed in *Mes^{t}-KO* embryos. Alternatively, the cochlear phenotype might arise due to expression changes undetected in our microarray analysis or a combination of changes in multiple genes and signaling pathways.

Together, this data aids in the construction of a putative model for RA mesenchymal-epithelial signaling downstream of *Tbx1* (Figure 5). In this model, *Tbx1* in the POM regulates the *Cyp26a1* and *Cyp26c1* RA metabolic genes in the same tissue via an indirect mechanism or via unidentified T-sites, as evidenced by previous data demonstrating altered *Cyp* gene expression in the POM, but not OV, of *Mes^{t}-KO* mutants (Braunstein and Monks et al., 2009) and the inability of *Tbx1* to bind to the two putative T-sites identified to date). Reduced RA breakdown, through diminished *Cyp26* expression in the POM, leads to increased RA in the periotic region at E12.5, as supported by increased *RARE-LacZ* reporter activity in *Tbx1$^{-/-}$ embryos where Cyp26a1* and *Cyp26c1* expression is similarly reduced in the POM (Guris et al., 2006). Expression of RA-responsive genes in the OV (*Dlx3, Adm, Clu, Otor*), and POM itself (*Mia*) is affected in response to increase RA signaling in *Mes^{t}-KO* embryos at E12.5, further supporting this model.

As an important environmental and dietary teratogen, RA signaling plays a role in countless organs during vertebrate development. Early perturbation of RA signaling surrounding the developing ear leads to defects in otic placode specification and subsequently major defects in otic development. Timed exposure to RA has not been well studied in mice due to the complexity of such experiments, and can also lead to widespread teratogenic effects. However, loss of mesenchymal *Tbx1* provides a useful model by which the later actions of physiologic RA can be studied in ear development. More importantly, the identification of RA target gene changes in the developing ear and periotic mesenchyme, taken with previous data reporting altered *Cyp26* gene expression in mesenchymal *Tbx1* mutants, adds specific RA target genes to the only mesenchymal-epithelial signaling pathway thus far identified during inner ear development.

EXPERIMENTAL PROCEDURES

Mouse Matings

Conditional *Tbx1* mutants were obtained by crossing *T-Cre;Tbx1$^{+/-}$* males with *Tbx1$^{flox/flox}$* females, resulting in *T-Cre;Tbx1$^{flox/+}$* (Control) and *T-Cre:Tbx1$^{flox/-}$* (*Mes^{t}-KO*) embryos as previously described (Braunstein and Monks et al., 2009). *Clu* (*Clusterin*) heterozygotes (Jackson Laboratories, Maine, USA: Stock#00562) were intercrossed to obtain *Clu$^{-/-}$* embryos, and crossed into the *Tbx1$^{+/-}$* (Arnold et al., 2006) background to obtain *Clu$^{-/-}$;Tbx1$^{+/-}$* embryos for paint filling analysis.

Paint Filling Analysis

Embryos of appropriate genotype were dissected at E12.5 or E15.5 in cold PBS, cut below the forelimb, fixed overnight in Bodian Fixative, washed in 100% Ethanol, cleared in methyl salicylate and injected with 0.1% white correction fluid in methyl salicylate as previously described (Braunstein and Monks et al., 2009).

Microdissection and RNA isolation

Freshly dissected embryos were immediately transferred to ice cold PBS and cut below the forelimb and above the mandible to retain the periotic tissue. This tissue was then hemisected along the neural tube and neural tissue was removed. Forceps were used to remove the remaining jaw and tissue surrounding the periotic region, leaveing only otic epithelium and surrounding, condensed, periotic mesenchyme. Left and right periotic tissue was combined and homogenized by vortexing in Qiagen Buffer RLT with ß-mercaptoethanol and stored at -80°C until all samples were dissected. RNA was then isolated according to the manufacturer's protocol (Qiagen RNeasy Micro Kit).

Microarray Analysis

cDNA was synthesized using the NuGen Ovation kit from pooled left and right ears of the same embryo. Affymetrix Mouse Gene ST 1.0 microarrays were performed on 5 *Mes^{t}-KO* embryos and 5 Control (conditional heterozygous) embryos. Data was normalized via the GC-RMA method and a t-test was performed. Samples with p values <0.05 were considered significant and filtered for fold change greater than +/-1.25, resulting in 68 upregulated and 64 downregulated genes in *Mes^{t}-KO* vs. conditional *Tbx1* heterozygous controls. Raw data and analysis details have been submitted to the NCBI Gene Expression Omnibus database to comply with MIAME guidelines.

qRT-PCR Validation

RNA from 3 separate *Mes^{t}-KO* and 3 Control embryos was isolated with samples for microarray analysis but used for qRT-PCR validation. Validation was carried out in triplicate for each sample using TaqMan based assays (Applied Biosystems) for *Col2a1* (Mm01309652_g1), *Clu* (Mm00442773_m1), *Adm* (Mm00437438_g1), *Mia1* (Mm00444563_m1), *Otor* (Mm00498571_m1), *Itga2b* (Mm00439741_m1), *Itgb3* (Mm00443980_m1), and *Myt1* (Mm00456190_m1) as well as *Gapdh* and *Tbx1* as controls (Mm00448948_m1) on an Applied Biosystems 7900HT. Data analysis was performed by SDS 2.2 and RQ Manager (Version 1.2) software (Applied Biosystems) with a confidence interval of 95%.

Microdissected *in situ* hybridization

Microdissected tissue-specific *in situ* hybridization was performed as previously described, with minor modifications (Nichols et al., 2008). Briefly, embryos were fixed in 4% PFA for 24hrs, dehydrated in a graded methanol series, and stored at -20°C until use. Embryos were then rehydrated in a methanol series to PBS, placed in 0.4% PFA, and microdissected in a manner similar to that used for microarray analyses, except that head mesoderm surrounding the periotic region could not be removed due to changes in tissue architecture caused by fixation. Primer sequences and conditions for PCR-generated riboprobes are provided in Supplemental Table 2. All *in situ* hybridizations were carried out on periotic regions from at least 5 separate embryos of each genotype.

Electrophoretic Mobility Shift Assay

Two conserved putative T-sites were identified in the genomic interval of mouse chromosome 19 containing *Cyp26a1* and *Cyp26c1* loci by manual inspection; one 50bp upstream of the *Cyp26a1* promoter (UCSC Genome Browser; NCBI37/mm9; chr19:37,722,095) and the other 500bp upstream of the *Cyp26c1* transcriptional start site (chr19;37,760,086). Electrophoretic mobility shift assay for putative T-sites, mutated versions of those T-sites, and Pitx2, a positive control previously identified as a direct target of TBX1 in heart development, was performed as previously described (Nowotschin et al., 2006), except that purified GST-tagged TBX1 was used.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Dr. Tingwei Guo (Albert Einstein College of Medicine) for his assistance generating the microarray heatmap, as well as Dr. Bernd Fritzsch (University of Iowa) for his training and assistance in microdissected *in situ* hybridization techniques. We acknowledge support of this research by NIH grant DC05186 to B.E.M.

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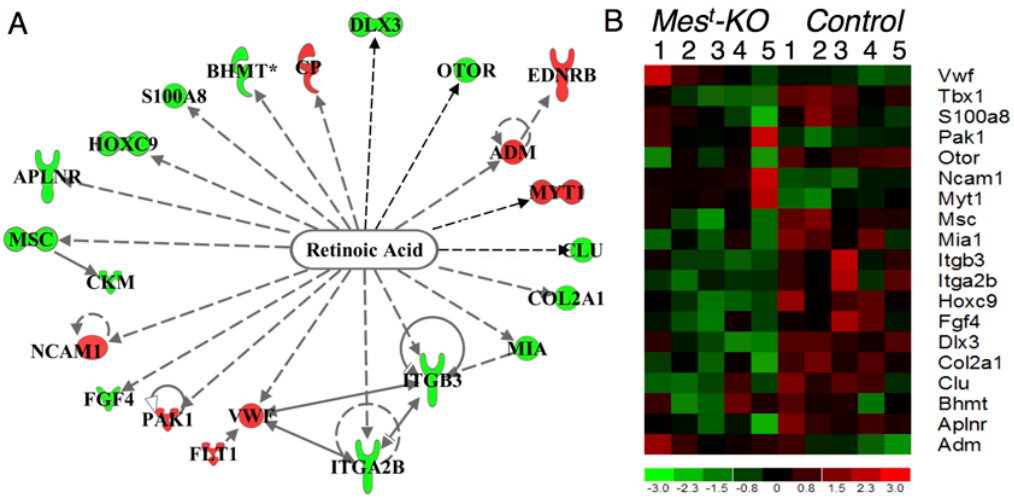

Figure 1. Altered expression of retinoic acid responsive genes in *Mes^t-KO* embryos consistent with up-regulated RA signaling

(A) Ingenuity Pathways Analysis identifies expression changes in 19 genes responsive to RA levels, overlayed with expression values from E12.5 microarray analysis. Up-regulated (red) and down-regulated (green) genes are altered in a manner consistent with increased RA levels in *Mes^t-KO* embryos. (B) Heat map of E12.5 microarrays representing five *Mes^t-KO* (left) and five Control (right) chips with RA genes represented on the right along with the control probe *Tbx1*, decreased in expression after conditional *Tbx1* albation. Genes with negative response to treatment with RA are down-regualted (green) on mutant arrays, while those described to be positively regulated by RA are up-regulated (red) on mutant arrays.

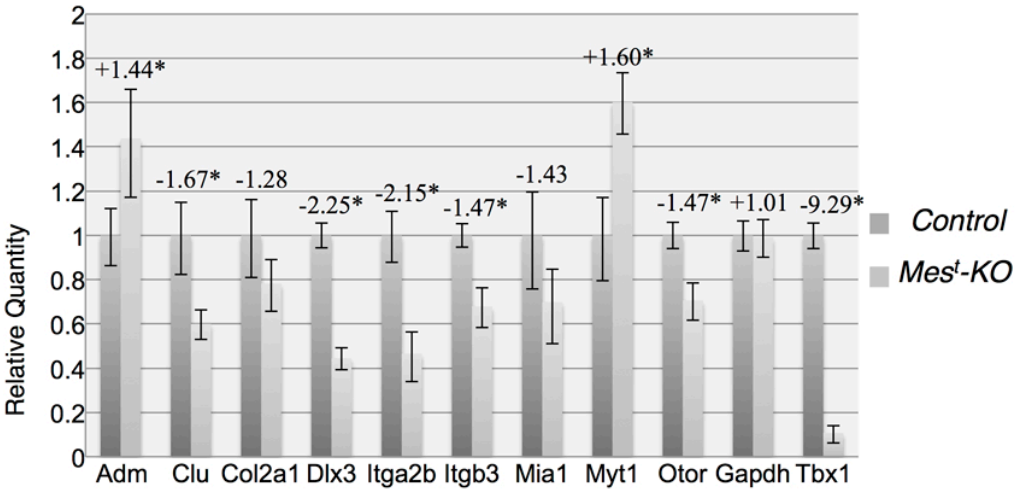

Figure 2. qPCR validation of RA target genes

Genes shown to be regulated by RA in other systems (Table 1) were validated by qPCR on microdissected *Mes^{t}-KO* periotic regions (OV+POM) using TaqMan probes. As expected, *Adm*, *Clu*, *Dlx3*, *Itga2b*, *Itgb3*, *Myt1*, *Otor*, and control probe *Tbx1* were significantly changed in T-Cre KO versus control ears. *Col2a1*, *Mia1*, and control probe *Gapdh* were not significantly changed, though both *Col2a1* and *Mia1* show a trend towards down-regulation that is not statistically significant. Fold change values are shown above (*Mes^{t}-KO* vs. Control) and asterisk indicates significance with $p<0.05$.

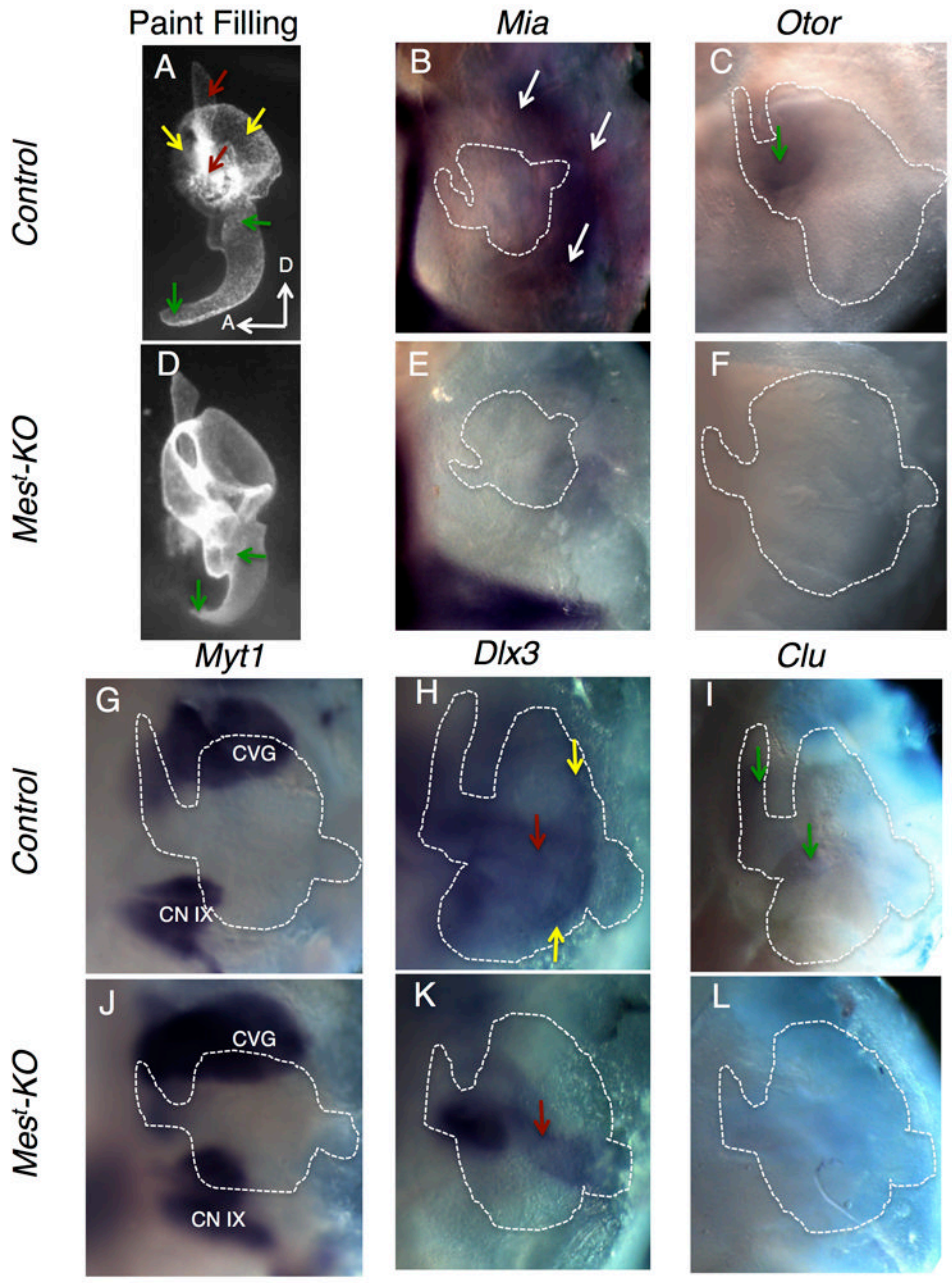

Figure 3. Tissue localization of RA target genes in the developing ear

In situ hybridization on microdissected periotic tissue was performed to localize expression changes to the OV, POM, or CVG during ear development. Structures are labeled with color-coded arrows; red representing the endolymphatic duct (ED), yellow representing the canal outpouches of the vestibular system, and green representing the cochlea. *Mes^t-KO* embryos display a shortened cochlea as early as E12.5, while the developing endolymphatic duct and canal outpouches (OP) appear unaffected at this stage in *Mes^t-KO* embryos (A,D). *Mia1* is expressed in the developing POM surrounding the dorsal OV (arrows, B), with nearly complete loss of expression in mutants (E). *Otoraplin*, closely related to *Mia1*, is expressed in the base of the developing cochlea (arrow, C), and expression is completely absent in mutants (F). *Myt1* marks the developing CVG (CN VIII), as well as the inferior

CN XI ganglion, in both mutants and controls (G,J) inner ear is outlined for reference, but out of the focal plane. *Dlx3* is expressed in the outpouches and ED of the vestibular system of control embryos (H), with loss of OP expression in mutants, though ED expression is in tact (arrow, K). *Clu* displays specific expression from the base to the apex of the cochlea (arrows, I). *Mes^{t}-KO* embryos display loss of expression throughout the cochlea (L). OV otic vesicle, POM periotic mesenchyme, CVG cochleovestibular ganglion, CN IX inferior cranial nerve IX ganglion, OP canal outpouches.

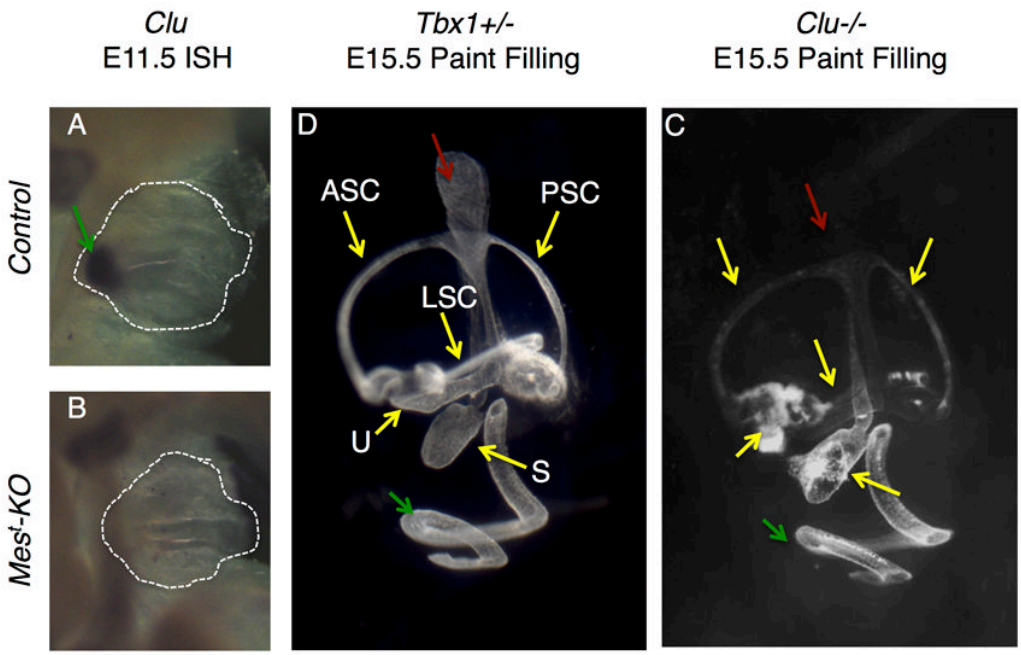

Figure 4. Loss of *Clu* is not responsible for defects in cochlear morphology

(A-B) *In situ* hybridization for *Clu* shows reduced expression in *Mes*^{t}*-KO* embryos as early as E11.5 (B) as compared to conditional *Tbx1* heterozygous controls, where expression is already apparent in the ventral OV which will form the cochlea (arrow, A). (C-D) Homozygous knockout of *Clu* leads to no morphological defects in the cochlea or other inner ear structures at E15.5, as assayed by paint filling analysis and compared to *Tbx1* heterozygous controls. ED endolymphatic ducts (red arrow), ASC/PSC/LSC anterior/posterior/lateral semicircular canals (yellow arrows), U utricle (yellow arrows), S saccule (yellow arrows), CD cochlear duct (green arrow).

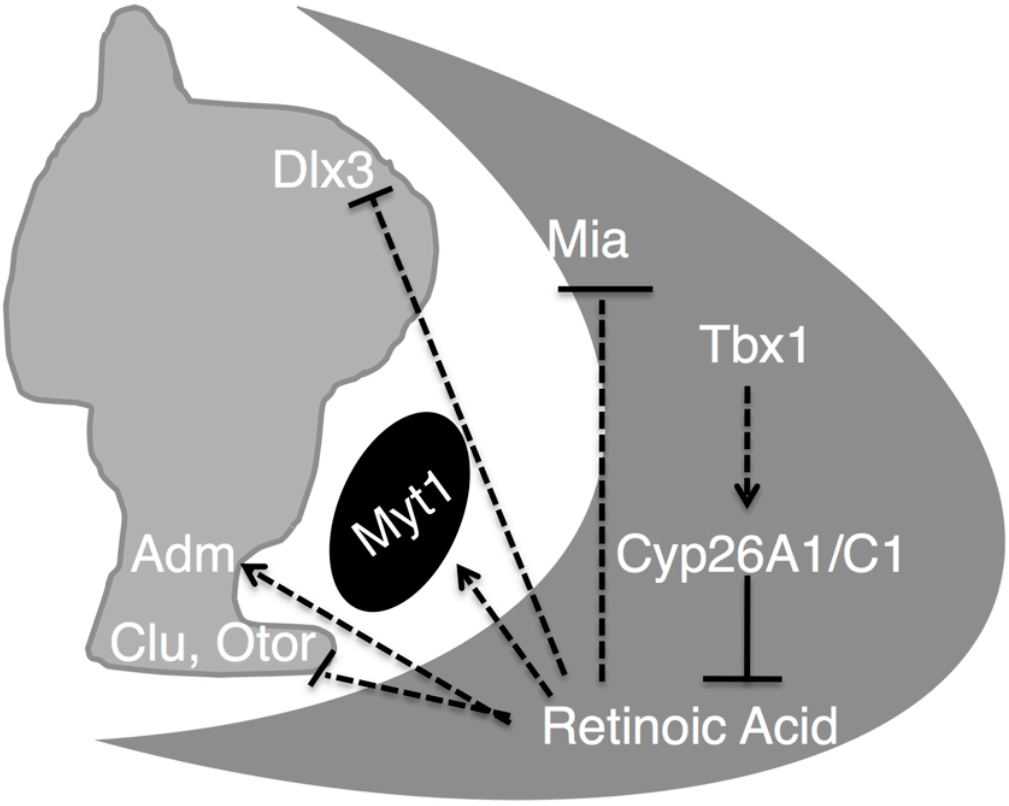

Figure 5. Model of RA target gene regulation by mesenchymal *Tbx1*

Tbx1 in the POM (dark gray) indirectly promotes expression of RA metabolic genes *Cyp26a1* and *Cyp26c1* which, in turn, breakdown/inhibit RA signaling to the OV (light gray). During inner ear development, RA activates the expression of *Adm* in the CD and *Myt1* in the CVG (black) while inhibiting expression of *Dlx3* in the developing vestibular system and *Otor* and *Clu* expression in the CD. CD cochlear duct, CVG cochleovestibular ganglion, POM periotic mesenchyme.

Table 1

Identification of RA regulated genes by microarray

Gene Symbol	Gene Name	Fold Change (KO vs Control)	p-value	Predicted response to RA	Reference for RA regulation
Adm	Adrenomedullin	1.4	0.026	INCREASE	Minamino et al., 1995
Col2a1	Collagen II, subunit a1	-1.47	0.006	DECREASE	Jimenez et al., 2001
Clu	Clusterin	-1.4	0.019	DECREASE	Orlandi et al., 2005
Dlx3	Distalless-like homeobox 3	-1.6	0.005	DECREASE	Acquafreda et al., 2010
Itga2b	Integrin alpha 2, subunit b	-1.31	0.017	DECREASE	Liu et al., 2000
Itgb3	Integrin beta 3	-1.29	0.014	DECREASE	Csomos et al., 2010
Mia1	Melanoma inhibitory activity	-1.45	0.039	DECREASE	Dietz et al., 1996
Myt1	Myelin transcription factor 1	1.58	0.044	INCREASE	Franco et al., 1999
Otor	Otoraplin	-1.28	0.009	DECREASE	Roberts et al., 2000
Cp	Ceruloplasmin	1.37	0.017	INCREASE	Tice et al., 2002
Bhmt	Betaine-homocysteine methyltransferase	-1.27	0.002	DECREASE	Xu et al., 2010
S100a8	S100 calcium binding protein A8	-1.29	0.025	DECREASE	Liu et al., 2000
Hoxc9	Homeobox C9	-1.3	0.001	DECREASE	Mao et al., 2011
Aplnr	Apelin receptor	-1.27	0.2	DECREASE	Zhong et al., 2005
Msc	Musculin	-1.34	0.037	DECREASE	Hishikawa et al., 2005
Ncam1	Neural cell adhesion molecule 1	1.26	0.016	INCREASE	De Laurenzi et al., 2000
Fgf4	Fibroblast growth factor 4	-1.26	0.048	DECREASE	Maerz et al., 1998
Pak1	p21 protein activated kinase 1	1.3	0.049	INCREASE	Csomos et al., 2010
Vwf	Von Willebrand factor	1.32	0.0235	INCREASE	Hatzopoulos et al., 1998

Microarray analysis of *Mes^{t}-KO* E12.5 periotic tissue (OV and surrounding POM) reveals changes in genes regulated by retinoic acid (RA). Gene expression changes are consistent with previously published data implicating either activation or repression by treatment with retinoic acid in cell culture or other model organisms, as indicated. Fold change versus control and p-values from microarray analysis are presented for these selected genes. For full microarray expression data, see Supplemental Table 1