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Identification of FOXJ1 effectors during ciliogenesis in the foetal respiratory epithelium and embryonic left-right organiser of the mouse

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ABSTRACT

Formation of motile cilia in vertebrate embryos is essential for proper development and tissue function. Key regulators of motile ciliogenesis are the transcription factors FOXJ1 and NOTO, which are conserved throughout vertebrates. Downstream target genes of FOXJ1 have been identified in a variety of species, organs and cultured cell lines; in murine embryonic and foetal tissues, however, FOXJ1 and NOTO effectors have not been comprehensively analysed and our knowledge of the downstream genetic programme driving motile ciliogenesis in the mammalian lung and ventral node is fragmentary. We compared genome-wide expression profiles of undifferentiated E14.5 vs. abundantly ciliated E18.5 micro-dissected airway epithelia as well as *Foxj1*$^{+}$ vs. *Foxj1*-deficient foetal (E16.5) lungs of the mouse using microarray hybridisation. 326 genes deregulated in both screens are candidates for FOXJ1-dependent, ciliogenesis-associated factors at the endogenous onset of motile ciliogenesis in the lung, including 123 genes that have not been linked to ciliogenesis before; 46% of these novel factors lack known homologues outside mammals. Microarray screening of *Noto*$^{+}$ vs. *Noto* null early headfold embryos (E7.75) identified 59 of the lung candidates as NOTO/FOXJ1-dependent factors in the embryonic left-right organiser that carries a different subtype of motile cilia. For several uncharacterised factors from this small overlap – including *1700012B09Rik*, *1700026L06Rik* and *Fam183b* – we provide extended experimental evidence for a ciliary function.

1. Introduction

Cilia are microtubule-based organelles that project from the surface of most vertebrate and some invertebrate cell types into the extracellular space. They can be classified by their motility (motile or non-motile), number per cell (solitary or multiple) or axoneme configuration (mostly 9+0 or 9+2; i.e. 9 outer microtubule doublets without or with a central microtubule pair) (reviewed in Takeda and Narita, 2012). Cilia fulfil a diverse range of functions essential for development and health: motile cilia move extracellular fluid or the ciliated cell itself while non-motile cilia (and some motile cilia) play critical roles in signal transduction and sensory perception (Shah et al., 2009; reviewed in e.g. Eggenschwiler and Anderson, 2007; Hildebrandt and Otto, 2005; Huangfu and Anderson, 2006). Absence of functional cilia can lead to multiple defects, which are termed ciliopathies (reviewed in Fliegauf et al., 2007; Gerdes et al., 2009; Nigg and Raff, 2009; Yuan and Sun, 2013).

Motile cilia play crucial roles at various stages of mouse development. At embryonic day (E) ~7.75, rotation of motile solitary cilia in the ventral node, the transient left-right organiser, generate a directed flow in the extracellular space, which is required for the establishment of left-right asymmetry in the developing organism (Nonaka et al., 1998; Takeda et al., 1999). Around E15.5, cells in the respiratory epithelia of the lung start to differentiate into multi-ciliated cells whose coordinated whip-like beating is responsible for muco-ciliary airway clearance throughout adult life (Jain et al., 2010; reviewed in Stannard and O'Callaghan, 2006). In the first week after birth, motile multi-ciliated cells in the ependymal epithelia of the mouse brain ventricles are essential for homeostasis of the cerebrospinal fluid movement (Banizs et al., 2005; Jacquet et al., 2009; Spassky et al., 2005). Correspondingly, ciliopathies caused by the dysfunction or lack of motile cilia, termed Primary Ciliary Dyskinesia (PCD), are associated with situs randomisation, respiratory problems and hydrocephalus (reviewed in Praveen et al., 2015).

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Cilia are complex organelles build from several hundreds of factors only a fraction of which are characterised (Arnaiz et al., 2009; Gherman et al., 2006; Inglis et al., 2006). These factors need to be provided in a coordinate way in order to produce functional cilia (reviewed in Choksi et al., 2014b). A key regulator of motile ciliogenesis in the mouse is the forkhead family transcription factor FOXJ1 (Blatt et al., 1999; Brody et al., 2000; Chen et al., 1998; Tichelaar et al., 1999). FOXJ1 is expressed in all murine tissues forming motile cilia including ventral node (9+0 solitary cilia), respiratory and ependymal epithelia (9+2 multiple cilia; (reviewed in Costa et al., 2001) where it is essential to instruct the formation of motile cilia (Alten et al., 2012; Brody et al., 2000; Chen et al., 1998), a function conserved in other species including *Xenopus* and zebrafish (Stubbs et al., 2008; Vij et al., 2012; Yu et al., 2008). *Foxj1* deficient cells that started to differentiate into ciliated cells fail to transport basal bodies to the cell membrane or to anchor them to the cortical actin skeleton; subsequent axonemal outgrowth is impaired (Alten et al., 2012; Brody et al., 2000; Gomperts et al., 2004; Huang et al., 2003; Pan et al., 2007; You et al., 2004). Although mainly associated with motile cilia, *Foxj1* is also expressed in a few murine tissues that may not carry motile cilia, including the floor plate of the neural tube (Cruz et al., 2010), the forebrain and olfactory bulbs (Jacquet et al., 2011), epithelia of developing teeth (Venugopalan et al., 2011) or cells of the immune system (Lin et al., 2005, 2004). *Foxj1* induces the growth of cilia on floor plate cells and on cultured, primary ciliated NIH3T3 fibroblasts (Cruz et al., 2010).

NOTO, a homeodomain transcription factor, is expressed distinctly in the ventral node of the mouse embryo and the left-right organiser in other vertebrate species (Ben Abdelkhalek et al., 2004; Didon et al., 2013; Gont et al., 1996; Stein and Kessel, 1995; Talbot et al., 1995). It transcriptionally activates *Foxj1* and acts as a regulator of ciliogenesis. Lack of NOTO causes defects in number, length, ultra-structure and docking of cilia as well as randomisation of laterality of the organism; this phenotype closely resembles *Foxj1* null (Alten et al., 2012; Beckers et al., 2007). Only a few NOTO targets have been identified yet, including *Foxj1* and *Dynlrb2* (Alten et al., 2012; Beckers et al., 2007). Expression of *Foxj1* and of its target *Dynlrb2* is strongly reduced in *Noto*-deficient nodes and expression of FOXJ1 in *Noto*-deficient embryos rescues the expression of NOTO targets, ciliogenesis and ciliary motility indicating that the function of NOTO in ciliogenesis is predominantly exerted through FOXJ1 (Alten et al., 2012).

Systematic screens for FOXJ1 target genes in various species and tissues identified candidates involved in a range of functions required for ciliogenesis indicating a primary role of FOXJ1 in the expression of a subset of the hundreds of components required for the generation of functional motile cilia (Choksi et al., 2014a; Didon et al., 2013; Gomperts et al., 2004; Jacquet et al., 2009; Newton et al., 2012; Stubbs et al., 2008; Yu et al., 2008). Additional screens were conducted to identify factors required for the generation of functional cilia based on transcriptome and proteome analyses, gene expression patterns and analysis of gene coexpression networks in PCD and gene knockdown (e.g. Arnaiz et al., 2009; Austin-Tse et al., 2013; Geremek et al., 2014; Gherman et al., 2006; Hoh et al., 2012; Inglis et al., 2006; Ivliev et al., 2012; Ross et al., 2007). However, it has never been comprehensively analysed, which FOXJ1 targets and factors associated with motile ciliogenesis are expressed during endogenous ciliogenesis in the mammalian foetal lung and embryonic ventral node. The genetic programme of motile ciliogenesis activated by FOXJ1 is still incompletely known.

This study systematically analyses the motile ciliogenic programme downstream of FOXJ1 during mouse embryogenesis focusing on the emergence of cilia in two tissues that form distinct subtypes of motile cilia, the lung and the ventral node. We perform genome-wide, microarray-based screens for FOXJ1/NOTO effectors using tissue samples directly isolated from wildtype mice at different embryonic stages and from well-characterised genetic knock-out mouse strains for *Foxj1* (Brody et al., 2000) and *Noto* (Ben Abdelkhalek et al., 2004). We present a list of 326 candidate genes activated by FOXJ1 in the foetal lung, including 123 novel candidates, and a list of 59 candidate genes active both in lung and node, including 19 novel candidates. Only a small fraction of genes identified in the lung and node are overlapping, suggesting a large diversity between the ciliogenic programme in both mouse tissues. For several candidates, we analyse expression in various murine tissues and expression and localisation in non-motile ciliated IMCD3 cells and provide further evidence for a cilium-related function of novel and uncharacterised candidates, including *1700012B09Rik*, *1700026L06Rik* and *Fam183b*.

2. Results

2.1. A bipartite screen for FOXJ1 effectors in the differentiating respiratory epithelium of foetal mice

Foxj1 is expressed in the trachea and large bronchi of the mouse from ~E15.5 on and progresses from there towards distal airway epithelia (Blatt et al., 1999; Clevidence et al., 1994; Hackett et al., 1995; Tichelaar et al., 1999), closely followed by specification of undifferentiated epithelial cells into motile multi-ciliated cells (Fig. 1A). By E18.5, ciliated cells are abundant throughout the respiratory epithelium (Jain et al., 2010; Rawlins et al., 2007; Toskala et al., 2005), amounting to ~40% of cells in large and ~20% in smaller airways (Pack et al., 1981; Tsao et al., 2009) interspersed with other cell types such as secretory cells. In the absence of *Foxj1*, i.e. in young, unspecified epithelial cells or in older *Foxj1*-deficient epithelia, no motile cilia are generated; instead, the unspecified cells fated to become motile ciliated carry a single, immotile primary cilium (Brody et al., 2000; Jain et al., 2010).

In order to identify genes involved in motile ciliogenesis of the foetal lung, we carried out two genome-wide microarray screens, one comparing the transcriptomes of undifferentiated E14.5 with motile multi-ciliated E18.5 epithelial wildtype tissues (subscreen A) and one comparing the transcriptomes of *Foxj1*$^{+}$ and *Foxj1* deficient lungs at E16.5 (subscreen B). The former screen (A) was designed to identify genes whose expression is up-regulated during specification of the respiratory epithelium, including differentiation of motile ciliated cells, and the latter screen (B) to identify FOXJ1 target genes. The overlap of the results of both subscreens was expected to be enriched in factors involved in motile ciliogenesis of the foetal lung (Fig. 1).

2.2. Tissue-level changes in the transcriptome of the developing respiratory epithelium in foetal mice

We laser micro-dissected respiratory epithelia from E14.5 and E18.5 wildtype lung sections, isolated the epithelium-specific total RNA and comparatively analysed the transcription profile at both developmental stages by microarray hybridisation (Fig. 1C, Fig. S1A). 3,872 probe sets (corresponding to 3,562 unique EntrezGene IDs) that represented different genes on the array were found to be significantly up-regulated at least twofold during maturation of the respiratory epithelium (see Section 4.3 for filter criteria).

An initial sampling confirmed that our data set indeed reflected physiological gene expression changes within the respiratory epithelium. The ten genes most strongly up-regulated (between 382- and 188-fold) include seven genes selectively expressed in motile ciliated respiratory cells (*Sec14l3*, *Sntn*) or Club/Clara cells (*Calca, Cyp2f2*, *Pon1, Scgb1a1/CCSP*, *Sftpd*) and two genes with unknown function (*Fam216b*, *Tmem212*) (Table 1). Several other genes known to be specifically activated in motile ciliated cells were identified as upregulated in our data (e.g. *Foxj1*, *Dnah9*, *Rsph4a*) (Table 1).

In order to directly test the microarray results we performed section *in situ* hybridisation (SISH), comparing the expression of several candidates in E14.5, E16.5 and E18.5 lungs. Probes for 31 genes (that