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Transcriptional networks of lung airway epithelial ciliogenesis

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TRANSCRIPTIONAL NETWORKS OF LUNG AIRWAY EPITHELIAL CILIOGENESIS

BY

XIAOBO SUN

B.S., BIOTECHNOLOGY, WUHAN UNIVERSITY, 2005

THESIS

Submitted in Partial Fulfillment of the
Requirements for the Degree of

Master of Science
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DEDICATION

This thesis is dedicated to my parents and wife Qian who have supported me all the way since the beginning of my studies.

Also, this thesis is dedicated to those who believe in the richness of learning.

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ABSTRACT OF THESIS

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ABSTRACT

Motile cilia of the mammalian airway play an essential role in innate defense. The coordinated transcriptional regulation of cilial axoneme genes remains to be elucidated. Transcription factor FOXJ1 has been shown to be important in ciliogenesis; however, direct transactivation of cilia genes by FOXJ1 has not been reported. Using a combined bioinformatics and experimental approach, here, we show a transcriptional network for cilia gene expression. FOXJ1 can directly transactivate endogenous cilia genes such as *ENKURIN, EFHC2, IFT57, RIBC2 and ROPN1L* in human bronchial epithelial cells (HBEC). FOXJ1 transactivation is localized to the proximal end of the 5' flanking region of *ENKURIN* and *EFHC2* promoters. However, FOXJ1 failed to transactivate *HSPA1A* and *MNS1*, two other cilia genes, indicating there are other transcription

factor(s) involved in the ciliogenesis. Motif discovery analysis indicates ETS and RFX binding sites located in promoters of several cilia genes (*IFT57*, *HSPA1A*, *MNS1*, *RIBC2* and *ROPN1L*). QRT-PCR indicates 5 of 27 human ETS transcription factors members (ETV1, ETV5, SPDEF, SPIC and ESE1) and 4 of 7 RFX transcription factors (RFX1, RFX2, RFX5 and RFXANK) are regulated coincidently with the differentiation of human bronchial epithelial cells and the appearance of cilia, suggesting these transcription factors are involved in gene regulations during lung airway epithelial ciliogenesis. ETS family members ESE1 and ETV1 both can transactivate endogenous EFHC2 and ENKURIN while SPIC represses EFHC2. An RFX family member RFX1 transactivates endogenous ENKURIN, HSPA1A, ROPN1L, and RFX5 transactivates EFHC2, HSPA1A and RIBC2. Furthermore, another transcription factor, GATA2, transactivates endogenous ENKURIN and EFHC2, and its transactivation also locates in the proximal end of the 5' flanking region of *ENKURIN* and *EFHC2* promoters. Our work defines a transcriptional network that regulates expression of cilia genes during airway epithelial differentiation and highlights the importance of multiple transcription factors in ciliogenesis.

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Chapter 1: Introduction

Human Cilia and Cilia-Related Diseases

Cilium is an organelle found in eukaryotic cells, which projects from the cell surface and is membrane bounded. It contains a microtubule cytoskeleton, the ciliary axoneme, surrounded by the ciliary membrane. The ciliary axoneme derives from and keeps the nine-fold symmetry of centriole, which is nearly identical to, and often becomes a ciliary basal body (1).

There are two major patterns of mammalian cilial axoneme. Those in multiciliated epithelial cells are motile 9+2 type, possessing dynein arms. The other pattern is single, nonmotile 9+0 primary cilia which are found on virtually every cell in the body (2). These nonmotile 9+0 cilia do not assemble the central microtubule which appears in the motile 9+2 cilia and lack of the molecular motors, termed axonemal dyneins (both ODA and IDA). Primary cilia function as various specialized sensory structures with chemosensory, mechanosensory, or positional sensory activity. They incorporate specific receptors and channels into their ciliary membrane and thus can respond to either mechanical stimuli or defined ligands. The presence of primary cilium is important for cell cycle, cell differentiation and controlling tissue homeostasis (1). Loss of primary cilia leads to abnormal cell function, cell division and can cause diseases such as polycystic kidney disease (3, 4, 5, 6, 7). In addition, unique motile 9+0 cilia possessing dynein arms with left-right dynein (8, 9) are found at the mammalian embryonic node during development, determining left-right asymmetry of the body (10, 11, 12, 13).

The ciliary axoneme of motile cilia grows from and continues the nine-fold symmetry of the centriole, which is polymerized from αβ tubulin heterodimers and the precursor of a ciliary basal body. The protein complex of axonemes is attached to the microtubules, including outer and inner dynein arms (ODAs and IDAs), the radial spokes, and the central pair projections. The ODAs and IDAs are molecular motors with ATPase activity, and can change their conformation alternatively by utilizing the energy of ATP hydrolysis to move along their neighboring outer microtubule doublets. This activity generates the force to make the microtubule doublets slide with respect to one another, producing a sliding motion (14, 15). The radial spokes resist this sliding motion which is converted into a local bending. The highly extensible interdoublets protein, nexin, keeps adjacent doublets together during this process (Figure 1).

The wave-like or beating motion of motile cilia is used either for locomotion such as in spermatozoa, or in the case of cilia along the respiratory tract and nodal cilia, to generate mucosal or fluid flow (16-18). Non-motile (or primary) cilia are usually associated with various sensory functions and present singly on cells (16, 19, 20). Their functions are cell type-dependent, for example, specifically responsible for vision, olfaction and mechanosensation in various tissue settings.

Ciliary defects are classified into primary and secondary ciliary defects. Primary ciliary dyskinesia is a genetic disorder causing dysfunctional motility of cilia and impaired mucociliary clearance. Secondary ciliary defects are caused by a variety of pathogens, toxins and trauma. These ciliary defects can lead to a number of human

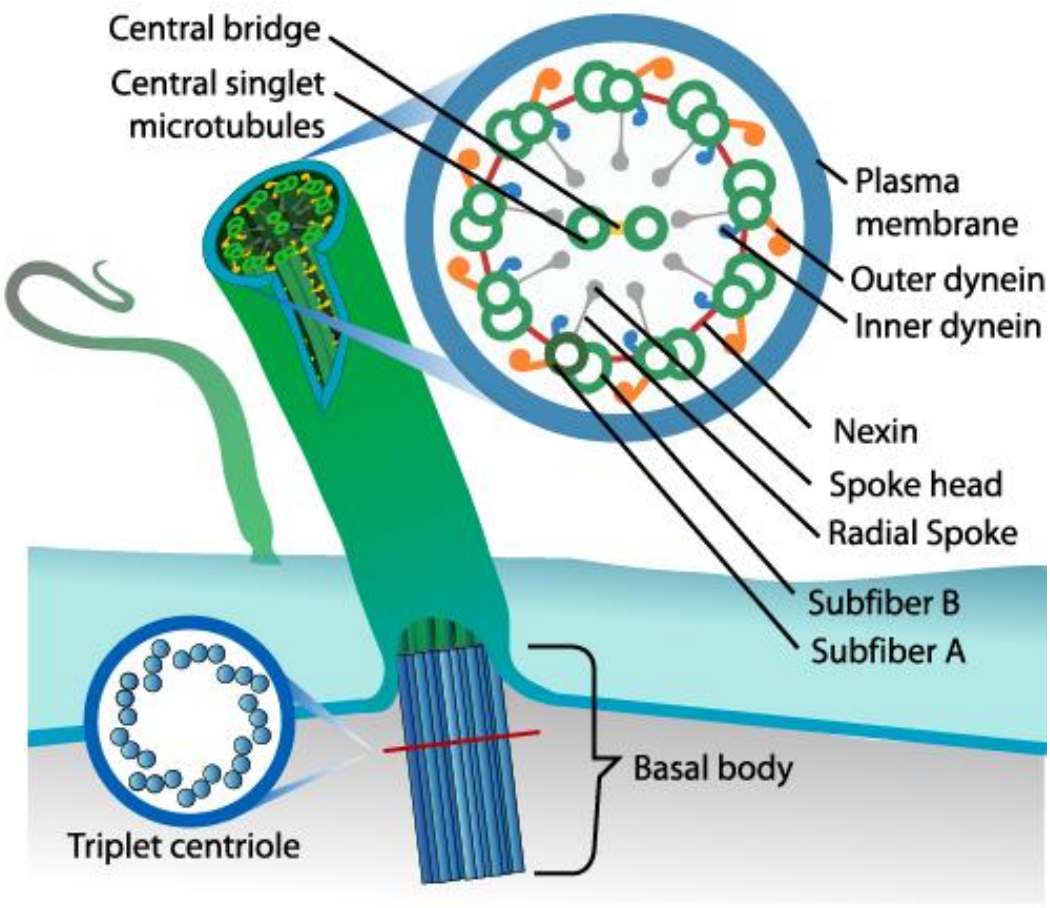


Figure 1. Axonemal Structure of Motile 9+2 Cilia: Cilia grow from basal body composed of nine microtubule triplets. The microtubule fibers in an axoneme are in a characteristic 9+2 array, with a peripheral group of nine pairs of microtubule doublets (subfiber A and B) surrounding two singlet microtubules. The microtubule doublets are held together by nexin links and joined to a central sheath by radial spokes. Two dynein arms emerge from each subfiber A and slide along neighboring subfiber B to yield a local bending.

(*Source*: http://en.wikipedia.org/wiki/File:Eukaryotic_cilium_diagram_en.svg)

diseases including polycystic kidney disease (PKD), retinal degeneration, anosmia, laterality defects, chronic respiratory problems, *situs inversus*, hydrocephalus, or infertility (17, 21-23) and other ailments such as Bardet–Biedl, Alstrom and Meckel syndromes. These aliments extend the ciliary disorder to obesity, diabetes, liver fibrosis, hypertension, heart malformations, skeletal anomalies (e.g. polydactyly), cognitive impairment and developmental defects such as exencephaly (17, 21, 24-29).

Functions of Lung Epithelial Cilia and Related Diseases

The lung and respiratory passage are constantly exposed to respiratory pathogens, toxins and foreign particles, and have evolved a very effective defense system. Lung epithelium exerts protective effects through several ways: by providing a physical barrier; through the secretion of factors that mediate immunity, inflammation, and antioxidant defense; and through mucociliary clearance. Mucociliary clearance plays an important role among these defense mechanisms for clearance of foreign particles and prevention of bacterial colonization or viral spreading. The pseudostratified columnar respiratory epithelium consists of ciliated, mucus-producing and basal cells (30). Cilia line the nasal cavity, paranasal sinuses, Eustachian tubes and middle ear of the upper respiratory tract. The lower respiratory tract is lined by ciliated cells from the trachea to the terminal bronchioles. These epithelial cells lining the airway are multi-ciliated which posses approximately 100-200 9+2 motile cilia on each cell (Figure 2).

Because the basal foot processes of individual cilia in each cell point in a similar

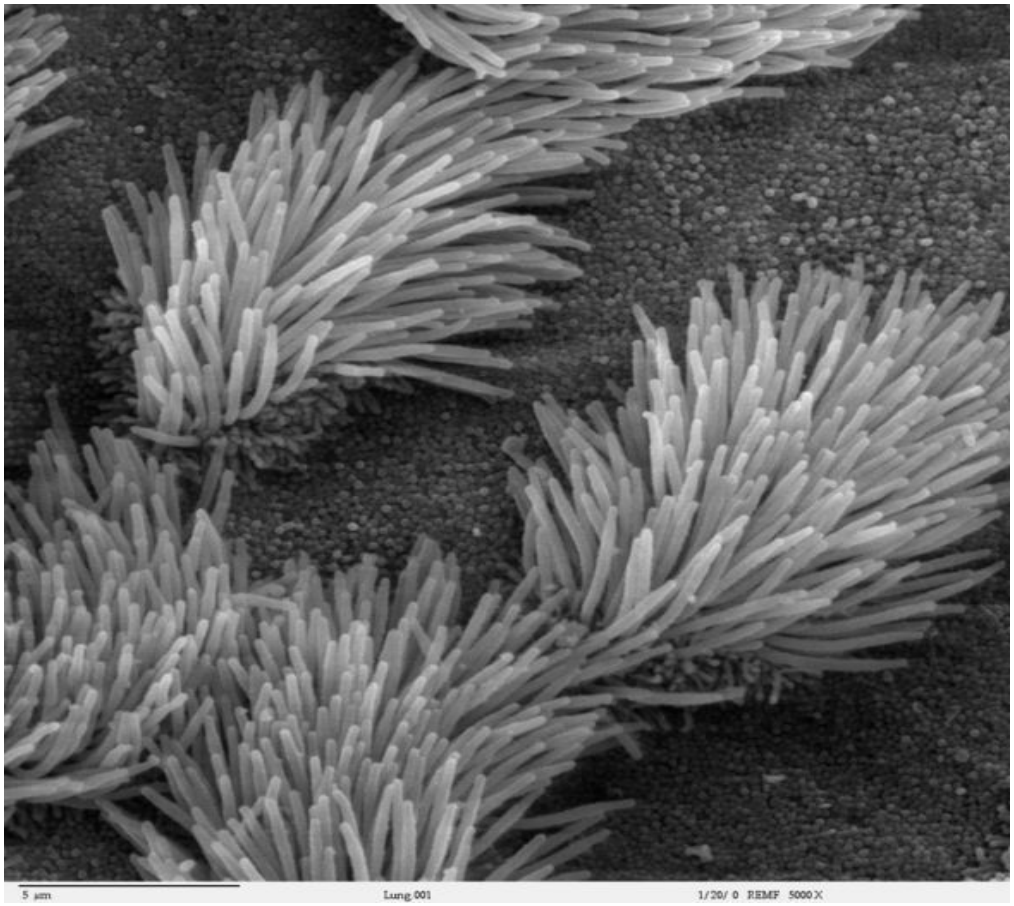


Figure 2. Scanning electron microscope image of lung trachea epithelium: These lung epithelial cells are both ciliated and non-ciliated. The rod-like cilia on multicilated cells is different in size from the sand-like microvilli on non-ciliated cell surface. (*Source*: http://remf.dartmouth.edu/images/mammalianLungSEM/source/9.html)

direction (31), the effective ciliary stroke for each cell beats in a common direction. Mucus secreted by respiratory epithelium acts as a physical barrier for capture of inhaled pathogens and particles, while the tips of cilia interact with the mucus layer to propel it along the airway surface from the lung and nasal cavities (32, 33).

Respiratory ciliary defects contribute to a wide range of human diseases such as asthma, cystic fibrosis and chronic obstructive pulmonary disease. Primary respiratory ciliary defects include primary ciliary dyskinesia (PCD) which results in chronic sinusitis, bronchiectasis, rhinitis, otitis and “glue ear” with associated hearing loss (36, 37).

A variety of pathogens, toxins and trauma could cause the secondary ciliary defects and abnormal functions. During acute viral infections such as coronavirus, mucociliary clearance has been shown to be impaired (38), which might be due to the cytopathic loss of epithelial cilia (38-42). When infected by various common cold viruses or, RSV, a paramyxovirus, ciliated lung epithelial cells are lost. Cilia may also become withdrawn into the cell body as was seen in the nasal epithelium during an episode of coronavirus infection (43-46, 55, 56). Interestingly, infection of several respiratory viruses is unique in ciliated and nonciliated airway epithelial cells (43). In addition to viral infections, bacterial infections also compromise ciliated respiratory epithelial cells. For example, when infecting the respiratory epithelium, *Pseudomonas aeruginosa* will secrete a toxic substance named pyocyanin that will immobilize the tracheal cilia (54). Infection with *Mycoplasma pneumoniae, Bordetella pertussis* or *B. bronchiseptica* will cause derangement of ciliary attachment (50-52).

Mucociliary Differentiation of Human Airway Epithelial Cells

The commitment of an undifferentiated airway epithelial cell to a ciliated cell and subsequent maturation to the ciliated phenotype occurs through undefined molecular pathways. In the developing human lung, ciliated epithelial cells arise from undifferentiated cells in the trachea during the canalicular stage, and, as lung development proceeds, cilia progressively appear in large and then small airways (61). Ciliogenesis in airway epithelial cells is a multistep process, in which precursor structures called “amorphous bodies” give rise to centrioles that subsequently transform to basal bodies. The basal bodies then migrate and dock at the apical membrane where they give rise to axonemes on the ciliated cells (62, 63). Although some regulators have been identified for the primary ciliogenesis (64), few molecular regulators of ciliogenesis in motile ciliated cells have been identified.

Primary human bronchial epithelial cells (HBEC) can be harvested from lung tissues and cultured in an air-liquid interface (ALI), which will promote their mucociliary differentiation. In the first week of ALI culture, the cells form a stratified squamous epithelial layer. By the end of the second week, those squamous cells are no longer flattened in appearance but without other obvious morphological changes. Although cilia could not be discerned on cells of this stage,α-tubulin are observed to concentrate on the surface of individual cells, indicating that ciliary protein constituents are being synthesized and transported, and that ciliogenesis has initiated. In other cells in this culture mode, there is high concentration of MUC5B, which is the main secreted mucin by secretory cells (57), suggesting the initiation of secretory

cell differentiation of non-ciliated cells. By the end of the third week, many of the cells have arranged in the pseudostratified layer with a columnar morphology. At the end of both third and fourth week, cilia are observed on the apical membrane surface of the cells, and there is a large increase of acidic mucin on the surface of epithelium, giving rise to the characteristic features of the predominant epithelial subpopulations of ciliated and secretory cells in the airways (58, Figure 3).

Cilia Genes in Mucociliary Differentiation of HBEC Cells

Axonemes purified from primary ALI cultures of HBEC cells have been studied by proteomic analysis, through which 200 proteins have been found (59). In another study, changes in gene expression during differentiation of airway epithelial cells have been examined by Affymetrix cDNA microarray analysis, and 1488 genes were found to display two or more-fold increase at early stage of ciliogenesis. By comparing the proteomics data with the microarray data, there are 37 overlapping genes between these two data sets (Figure 4, 58). Thus these genes are very likely to have potential roles in cilia formation or functions, and it is notable that most of them have very similar expression profiles, with a dramatic increase between days 2 and 8 of ALI culture.

Among these 37 genes, a number of genes encode proteins that are components of the ciliary axoneme, such as those encoding light, intermediate or heavy chains of axonemal dyneins (*DNAH9, DNAI1*, *DNAI2*, *DNALI1*), and tubulins (*TUBA3*, *TUBA4, TUBB2*).

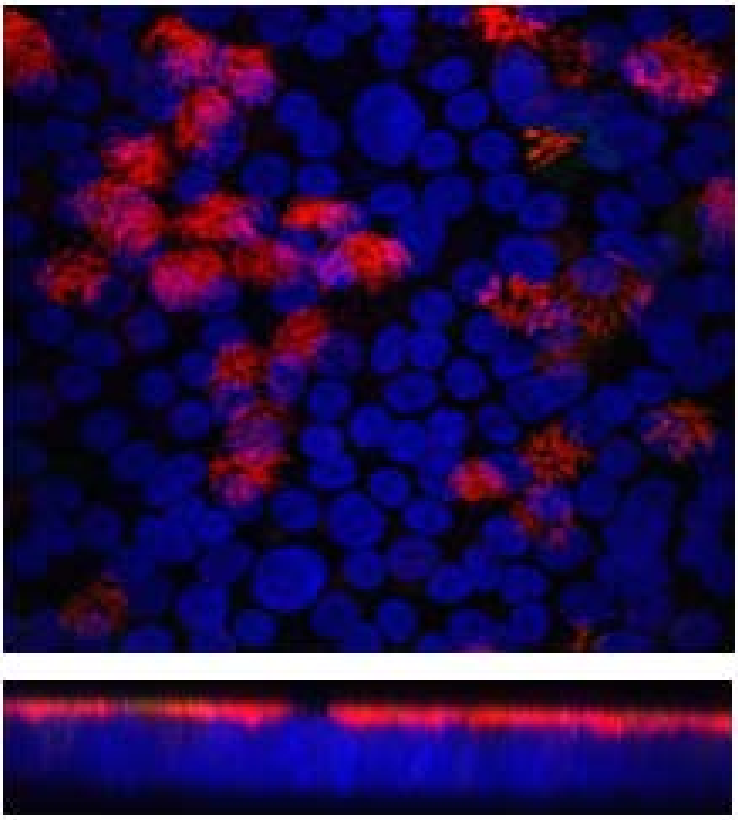
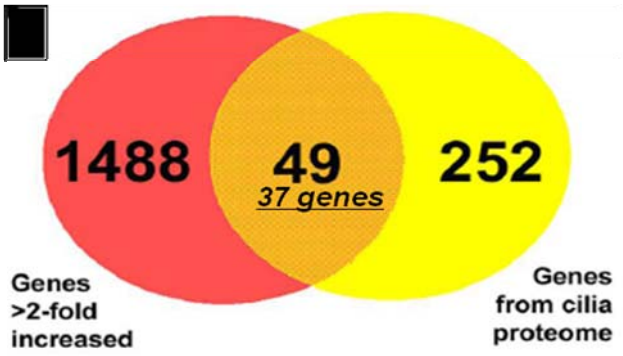


Figure 3. Mucociliary differentiation of HBEC cells during ALI culture on day 21. Acetylated α-tubulin is immunostained to mark ciliary axonemes (red) and nuclei are stained with DAPI (blue) respectively. Ciliated cells are denoted with red apical staining to show axonemal structures.

A.



B.

Affymetrix ID	Gene	Description	Maximum Fold Increase	Protein	Protein Accession
237314_at	*C10orf63*	Chromosome 10 open reading frame 63	5.36	Mus musculus male testis cDNA	AK017056
220581_at	*C6orf97*	Chromosome 6 open reading frame 97	22.04	cDNA FLJ23305 FIS	Q9H5M3
231729_s_at 226424_at 231728_at	*CAPS*	Calcyphosine	8.17	CAYP Human calcyphosine	Q13938
209194_at	*CETN2*	Centrin, EF-hand protein, 2	2.52	Human caltractin	P41208
218876_at	*CGI-38*	Brain-specific protein	73.18	Brain-specific protein	Q9Y6H0
204576_s_at 204577_s_at	*CLUAP1*	Clusterin-associated protein 1	3.37	KIAA0643 protein	O75138
240857_at	*DNAH9*	Dynein, axonemal, heavy polypeptide 9	6.83	Dynein heavy chain	Multiple
220125_at	*DNAI1*	Dynein, axonemal, intermediate polypeptide 1	4.64	Dynein intermediate chain	Multiple
220636_at 221668_s_at	*DNAI2*	Dynein, axonemal, intermediate polypeptide 2	9.75	Dynein intermediate chain	Multiple
205186_at 227081_at	*DNALI1*	Dynein, axonemal, light intermediate polypeptide 1	12.24	Dynein light chain	Multiple
239733_at	*DYDC2*	DPY30 domain containing 2	5.49	Similar to Riken 4933428D01	BC007374
219833_s_at	*EFHC1*	EF-hand domain (C-terminal) containing 1	7.52	FLJ10466 FIS	Q9NVW6
220591_s_at	*EFHC2*	EF-hand domain (C-terminal) containing 2	18.79	FLJ22601 FIS	Q9H653
213679_at	*FLJ13946*	Hypothetical protein FLJ13946	2.71	FLJ13946	Q9H849
1552716_at 232745_x_at	*FLJ23577*	KPL2 protein	3.10	KIAA1770	BAB21861
205498_at	*GHR*	Growth hormone receptor	3.39	Growth hormone receptor	AAF67170
200800_s_at 200799_at	*HSPA1A*	Heat shock 70-kD protein 1A	2.36	70-kD Heat shock proteins	Multiple
218100_s_at 222520_s_at 222519_s_at	*IFT57*	Intraflagellar transport 57 homolog (Chlamydomonas)	4.04	MHS4R2	AAK13588
219372_at	*IFT81*	Intraflagellar transport 81 homolog (Chlamydomonas)	2.15	CDV-1 protein	Q9UNY8
204703_at	*IFT88*	Intraflagellar transport 88 homolog (Chlamydomonas)	3.49	TG737	Q13099
204202_at	*IQCE*	Homo sapiens IQ motif containing E (IQCE), mRNA.	3.45	KIAA1023	Q9UPX7
206076_at	*LRRC23*	Leucine-rich repeat containing 23	10.61	B7 protein	Q92977
204783_at, 204784_s_at	*MLF1*	Myeloid leukemia factor 1	6.09	Fusion gene	AAA99997
219703_at	*MNS1*	Meiosis-specific nuclear structural protein 1	22.83	Unnamed protein product	BAA92077
206197_at	*NME5*	Non-metastatic cells 5, protein expressed in (nucleoside-diphosphate kinase)	7.98	Human nucleoside diphosphate kinase homolog (testis specific)	P56597
205037_at	*RABL4*	RAB, member of RAS oncogene family-like 4	3.12	Putative GTP-binding protein	AAH00566
222742_s_at	*RABL5*	RAB, member of RAS oncogene family-like 5	2.59	Unknown protein	AAH04522
206526_at	*RIBC2*	RIB43A domain with coiled-coils 2	6.22	Unknown protein	AAH03024
223609_at	*ROPN1L*	Ropporin 1-like	21.82	AKAP-associated sperm protein	AAG59587
220105_at	*RTDR1*	Rhabdoid tumor deletion region gene 1	3.28	Rhabdoid tumor deletion region protein	Q9UHP6
205406_s_at, 239515_at	*SPA17*	Sperm autoantigenic protein 17	4.45	HU sperm surface protein Sp17	Q15506
210033_s_at, 210032_s_at	*SPAG6*	Sperm-associated antigen 6	33.05	Sperm flagellar protein, SPAG6	O75602
210323_at	*TEKT2*	Tektin 2 (testicular)	4.30	H-Tektin-T	Multiple
209118_s_at	*TUBA3*	Tubulin, alpha 3	14.37	alpha-Tubulin	Multiple
207496_at	*TUBA4*	Tubulin, alpha 4	2.84	alpha-Tubulin	Multiple
208977_x_at	*TUBB2*	Tubulin, beta, 2	2.81	beta-Tubulin	Multiple
223636_at	*ZMYND12*	Zinc finger, MYND domain containing 12	4.74	cDNA DKFZp434N2435	AL136858

Figure 4. A). This diagram shows the overlapping genes found to be regulated in ciliogenesis by microarray analysis with proteins known to be found in the cilia axoneme by proteomic analyses. The microarray analyses (Left) identified genes displaying two or more fold increases during mucociliary differentiation of HBEC cells. The proteomic analyses (Right) identified genes encoding proteins found in purified ciliary axoneme. **B)**. The list of the 37 overlapping genes and their encoding proteins (Andrea J. Ross. etc. 2007. *Am J Respir Cell Mol Biol.*)

Another number of genes (*TEK2, CENT2, SPAG6, NME5, SPA17, IFT57, IFT81, and IFT88*) encode proteins associated with cilia, flagella or microtubules. For example, IFT57, IFT81, IFT88 are component proteins of the intraflagellar transport machinery.

Calcium homeostasis is important for many aspects of ciliary and flagellar functions, and proteins encoded by some genes (*ENKURIN, CAPS, EFHC1, EFHC2*) bind calcium ions or are involved in calcium transport. Enkurin is a calmodulin protein co-localizing with calcium channels, which is suggested to be an adaptor to localize calcium signal transduction machinery to calcium channels (60). *EFHC2* encodes a protein very similar to EFHC1 protein, and is proposed to be a calcium protein associated with the axoneme and plays a role in cilial axoneme assembly and stability.

In addition, several genes encode proteins with different functions. For example, HSPA1A is a member of heat shock protein 70 family, and in conjunction with other heat shock proteins, stabilizes existing proteins against aggregation to mediate the folding of newly translated proteins in the cytosol and in organelles. It is also involved in the ubiquitin –proteasome pathway (91). *ROPN1L* encodes a sperm protein, which interacts with A-kinase anchoring protein AKAP3 and allows ROPN1L to be targeted to specific subcellular compartments (92). MNS1 is a meiosis-specific structural protein expressed at the pachytene stage during spermatogenesis, and thought to be involved in the control of meiotic division and germ cell differentiation through regulation of pairing and recombination during

meiosis (89, 90). Other genes (*RABL4, RABL5, RIBC2)* encode proteins with unknown functions in cilia.

FOXJ1 Transcription Factor in Ciliogenesis

FOXJ1 (also called HFH-4) is a forkhead box (f-box) transcription factor expressed in ciliated cells located in the upper and lower airway, choroid plexus, ependyma, oviducts, testis and embryonic node (65-68). Colocalization of FOXJ1 and β-tubulin IV but not Clara cell secretory protein confirmed that FOXJ1 is expressed in ciliated epithelial cells rather than nonciliated columnar epithelial cells in the respiratory tract (73). Much evidence indicates a direct relationship between Foxj1 expression and ciliogenesis: During the murine lung development, the expression of Foxj1 immediately precedes the appearance of cilia in airway epithelial cells, which is in the late pseudogladular stage at embryonic day 15.5 (65). In the infection of some respiratory viruses such as the paramyxovirus respiratory syncytial virus (RSV), loss of cilia is associated with loss of Foxj1 expression, and during airway repair, the appearance of ultrastructural component of ciliogenesis is associated with re-expression of Foxj1 (69, 74). It is noteworthy that interruption of Foxj1 gene in genetically engineered mice leads to the loss of left-right dynein (*Dnahc11*) expression and absence of cilia (66, 70). In airway cells that have been already committed to ciliated cell phenotype, Foxj1 promotes the expression of capastatin, an inhibitor of the protease calpain, leading to the stabilization of cytoskeletal anchoring proteins ezrin and EBP-50. Ezrin and EBP50 both are associated with basal bodies of

cilia in the pulmonary epithelium. Thus, Foxj1 helps the docking of basal bodies to the apical membrane and the induction of mechanisms of axoneme assembly (71, 72). Although mutations in the FOXJ1 gene have not yet been identified in human diseases with abnormalities of cilia formation (75), FOXJ1 is still be considered to be a potential master regulator of ciliogenesis in airway epithelial cells.

ETS Transcription Factor Family

ETS is one of the largest families of transcription factors, and all members are identified to have a highly conserved DNA binding domain, the ETS domain, a winged helix-turn-helix structure that binds to DNA sites with a central GGA motif sequence. As well as DNA binding functions, the ETS domain is also involved in protein-protein interactions suggesting transcriptional co-regulation with other transcription factors. ETS factors act as a transcriptional activator, repressor, or both (78). There are 28 ETS genes in human and they are divided into 11 subfamilies (Figure 5, [80]). The extensive conservation in DNA-binding ETS domain among these genes leads to a substantial of redundancy in their DNA binding capacity. It is thought that interactions with other proteins are one way in which specific binding to DNA is achieved (79). There is limited similarity outside the ETS DNA binding domain. This sequence divergence contributes to the individual properties of ETS-domain transcription factors, altering their DNA binding specificity and interactions with other transcription factors (76, 77). These unique properties, coupled with distinct expression pattern, culminate in their distinct biological roles. The ETS family is present through out the body and is involved in wide variety of functions

Subfamily	Members and their homologs
ELF	ELF1, NERF/ELF2, MEF/ELF4
ELG	GABPα, ELG
ERG	ERG, FLI1, FEV
ERF	ERF/PE2, ETV3/PE1
ESE	ESE1/ESX/ELF3, ESE2/ELF5, ESE3/EHF
ETS	ETS1, ETS2, POINTED
PDEF	PDEF/SPDEF/PSE
PEA3	PEA3/E1AF/ETV4, ERM/ETV5, ER81/ETV1, ER71/ETV2
SPI	PU.1/SPI, SPIB, SPIC
TCF	ELK1, SAP1/ELK4, NET/SAP2/ELK3, LIN
TEL	TEL/ETV6, TEL2/ETV7, YAN

Figure 5. The members and their homologs of ETS family and they are divided into 11 sub-families. (Gutierrez-Hartman A.et.al. 2007. *Trends Endocrinol Metab*).

including the regulation of cellular differentiation, cell cycle control, cell migration,cell proliferation, apoptosis and angiogenesis. Multiple Ets factors have been reported to be associated with cancer, such as through gene fusion (81, 82). It is worthy to mention that ETS family members have been proven to expressed in stratified epithelial cells, including the lung epithelium (85, 86)

Motif discovery analysis of the promoters of mouse lung gene subsets as well as their human homologues, which are found to transactivate several genetic programs during lung embryogenesis and lung cell differentiation (88, 89), identified ETS family members as candidate regulators in both sets of promoters. This analysis suggests that ETS family potentially regulates crucial lung functions and development (88).

RFX Transcription Factor Family

RFX proteins belong to the winged-helix family of transcription factors. They share a characteristic 76-amino acid DNA binding domain that binds to an X-box motif, and conserved in many eukaryotes (99, 100). Five RFX genes (RFX1,-2,-3,-4 and -5) have been identified in mice and human genome (101). RFX factors are involved in the regulation of cell cycle (93, 94, 95), brain development and neuronal functions (96, 97), and ciliogenesis (98).

In mice and humans, RFX-1 is the first gene found as a candidate major histocompatibility complex (MHC) class II promoter binding protein (103). It also has been proved to a transactivator of hepatitis B virus enhancer (104). It is primarily found in the human brain with high expression in cerebral cortex and Purkinje cells

(105). RFX-2 and -4 both have highest expression in human testis (106, 107). In mice, RFX-4 has been implicated in dorsal patterning of brain development and may regulate circadian rhythm in human (112, 113). RFX-3 is expressed in ciliated cells including pancreatic endocrine cells, ependymal cells and neuronal cells (108-110). RFX-5 is a transcriptional activator of MHCII genes. It also mediates the formation of an enhanceosome complex containing RFXANK, RFXAP, CREB, and CIITA (111).

The roles of RFX family in ciliogenesis of human airway epithelial cells remain largely unknown. Similar to the discovery of ETS family members by motif discovery, our lab has recently identified binding sites of RFX family members over-represented in lung gene networks in mouse and in their human homologue promoters. These findings predicate that RFX family members are candidate transcriptional regulators in mammal lung development and functions (88).

General Features of Transcription Factor GATA2 and other GATA factors

The GATA family of transcription factors controls multiple developmental processes by regulating tissue-specific gene expression. The zinc finger DNA binding domains bind to W(A/T)GATAR(A/G) motifs of gene promoter regulatory elements (118, 119).

GATA1, GATA2 and GATA3 regulate the development of different hematopoietic lineages---erythroid, hematopoietic progenitor and T-lymphoid, respectively (120-122). Similarly, GATA4, GATA5 and GATA6 have been shown to be involved in cardiac, genitourinary, and multiple endodermal developmental events

(123-125).

GATA2 was first cloned from chicken reticulocyte and shown to be present all along the development of erythoid cells (126). It is also expressed in other hematopoietic cells, neurons and cells of developing heart, liver, pituitary and in trophoblasts (127-132).

The roles of GATA transcription factors in human lung epithelial cells differentiation and ciliogenesis remain elucidated. Our lab has shown that binding motifs of GATA3 and GATA4 are over-represented in the gene promoters of mouse and human lung genes, respectively (88). These findings predicate that there might be multiple *cis*-regulatory elements of entire GATA family functioning in both organisms, and GATA family may have roles in lung epithelial cell differentiation and ciliogenesis.

Hypothesis and Rationale

The lung epithelial cells are highly specialized, differentiated cells originating from common progenitors in the lung during development. They have unique functions involved in airway host defense, gas exchange and ion transport (88, 136). The lung epithelium is a pseudostratified layer consisting of basal cells, secretory cells and columnar ciliated cells. Mucociliary clearance is an important host defense mechanism, and defects in mucociliary clearance are associated with various respiratory disorders and diseases.

Transcription of cilia genes is significantly increased during lung epithelial differentiation and ciliogenesis. Transcription factor FOXJ1 has been identified as the

only one transcription factor during *in vitro* differentiation of air way epithelial cells. However, direct transactivation of cilia genes by FOXJ1 was not elucidated. In addition, through motif discovery and bioinformatics analyses, multiple binding sites for other families of transcription factors, including RFX, ETS and GATA, were located in many of the cilia gene proximal promoter regions.

In this thesis, we hypothesize that FOXJ1, ETS, RFX and GATA transcription factors regulate the expressions of cilia genes in human bronchial epithelial cells, alone or together.

To verify this, we address the following aims:

Aim 1: Analyze the expression patterns of ETS and RFX family transcription factors during human bronchial epithelial cells differentiation and ciliogenesis, and locate binding motifs of these transcription factors in the 5' proximal promoters of cilia genes.

Aim 2: Experimentally assess transactivation of distinct cilia genes by the transcription factors FOXJ1, ETS, RFX and GATA2 through reporter assay, followed by qRT-PCR detection of the changes of endogenous cilia genes.

Aim 3: Show the direct binding of FOXJ1 onto the promoters of selected cilia genes.

Chapter 2: Materials and Methods

Cell Cultures

Cells were kept at 37°C with 5% CO_2. Normal human bronchial epithelial (NHBE) cells were purchased from Lonza and plated onto collage-coated 100mm dishes (Becton-Dickinson, Biocoat) and grown in BEGM media (Lonza) to approximately 80% confluency (~4-5days). Then the cells were plated onto collagen coated transwell inserts (Corning) with BEGM plus DMEM (Sigma-Aldrich) media in the basolateral and apical chambers. NHBE cells are allowed to grow to 100% confluency (~3-7 days) at which time the media in the chamber was removed and the cells were grown under air-liquid interface (ALI) for differentiation (14-28 days). HBEC3KT, kindly provided by Dr. John. D. Minna (137), were maintained in L-Glutamine containing Keratinocyte-SFM medium (GIBCO, Invitrogen) in flask coated with FNC mix (AthenaES™'s).

Bioinformatic Analysis of Cilia Gene Promoters

Proximal promoters of 37 cilia genes were acquired from human genome build 36 (v.1, March 2006) through UCSC genome browser. For each gene, the proximal promoter was defined as 1000 base pairs upstream of transcriptional start site and the downstream 5'UTR exon sequences were included to accommodate for the possible existence of additional *cis*-regulatory elements.

The ExPlain tool of TRANSFAC Suite (BIOBASE Knowledge Library) was used to predict transcription factor binding motifs on the cilia gene promoters. The profile

containing a matrix for the binding motifs of a set of transcription factors (ETS family for example) was created by choosing them from existing matrices (Figure 6), and the cut-offs value was set to be custom CSS (core similarity score) 0.75 and MSS (matrix similarity score) 0.85. Potential binding motifs of specific transcription factors on cilia gene promoters were analyzed and searched by uploading promoter sequences from FASTA files, and match them with a specific matrix using the Match Workflow Wizard.

Construction of Protein Expression Vectors

Total RNAs were isolated with TRIzol (Invitrogen) according to the protocol provided by manufacturer. RNAs were extracted from NHBE cells under ALI culture for 0, 2, 4 days and 3 weeks. Five hundred nanograms of total RNA were used to synthesize the first-strand cDNA with Quanti-Tech Reverse Transcription Kit (Qiagen).

Full length coding sequences of transcription factors FOXJ1, ETV1, ESE1, SPIC, SPDEF, ETV5, RFX1, RFX5 and GATA2 were amplified and flag-tagged through PCR with FastStart *Taq* DNA polymerase (Roche). The primers used are outlined in Table 1 (restriction enzyme sites are shown in italics, and the antisense of FLAG-tag coding sequences are underlined).

PCR products were cloned into pTarget T Vector (Promega). Competent NEB-5α cells (NEB) were transformed with ligation products and selected through white/blue colonies in Agar plates containing 100ug/ml Ampicillin. White colonies were cultured

a)

b)

Figure 6. Matrices of the binding sequences of a) ETS family (identifier: V$ETS_Q6) and b) RFX family (identifier: V$RFX_Q6), for predicting their potential binding sites on cilia gene promoters.

Table 1. PCR primers for amplifying cDNAs of transcription factors

Name	Sequences
FOXJ1 upstream	5'-aatggcggagagctggctgcg-3'
Downstream	5'-a*aagctt*ttacttatcgtcgtcatccttgtaatccaagaaggcccccacgctggc-3' (*Hind*III)
GATA3 upstream	5'-a*agatct*atggaggtgacggcggacc-3' (BglII)
Downstream	5'-a*tctaga*ttacttatcgtcgtcatccttgtaatcacccatggcggtgaccatgc-3' (XbaI)
ETV1 upstream	5'-at*accggt*ccatggatggattttatgaccagcaag-3'(*Age*I)
Downstream	5'-at*ctcgag*ttacttatcgtcgtcatccttgtaatcatacacgtagccttcgttgtagg-3' (*Xho*I)
ETV5 upstream	5'-at*accggt*ccatggacgggttttatgatcagcaag-3' (*Age*I)
Downstream	5'-at*ctcgag*ttacttatcgtcgtcatccttgtaatcgtaagcaaagccttcggcatagg-3' (*Xho*I)
ESE1 upstream	5'-at*accggt*ccatggctgcaacctgtgagattag-3' (*Age*I)
Downstream	5'-at*atcgat*ttacttatcgtcgtcatccttgtaatcgttccgactctggagaacctcttc-3' (*Cla*I)
SPIC upstream	5'-at*accggt*ccatgacgtgtgttgaacaagacaag-3'(*Age*I)
Downstream	5'-at*ctcgag*ttacttatcgtcgtcatccttgtaatcgcaatcatggtgatttagctc-3' (*Xho*I)
SPDEF upstream	5'-at*accggt*ccatgggcagcgccagcccgggtctg-3' (*Age*I)
Downstream	5'-at*atcgat*ttacttatcgtcgtcatccttgtaatcgatggggtgcacgaactggtag-3' (*Cla*I)
RFX1 upstream	5'-a*aagctt*atggcaacacaggcgtatac-3 ' (*HindIII*)
Downstream	5'-a*tctaga*ttacttatcgtcgtcatccttgtaatcgctggagggcagcgcctgcac-3' (*Xba*I)
RFX5 upstream	5'-a*aagctt*atggcagaagatgagcctg-3' (*HindIII*)
Downstream	5'-a*tctaga*ttacttatcgtcgtcatccttgtaatctgggggtgttgcttttgggtc-3' (*Xba*I)

in 2ml 2xYT medium overnight at 37°C with shaking at 250rpm. Plasmids were extracted with GenElute Plasmid Miniprep Kit (Sigma-Aldrich) according to the manufacture's protocol, and verified through restriction enzymatic cleavage analysis and sequencing.

Construction of Luciferase-Reporter Plasmids

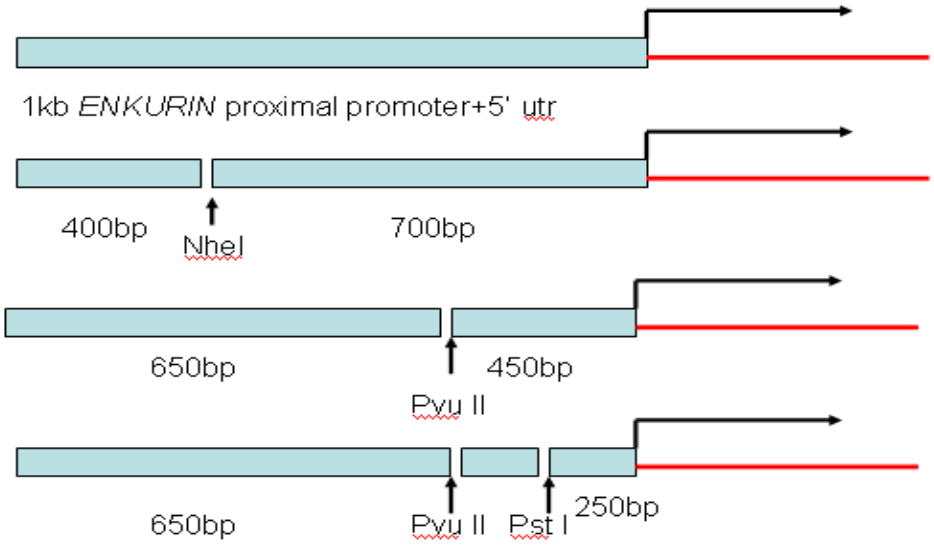
Genomic DNAs were extracted from A549 cells with TRIzol (Invitrogen) according to the protocol provided by the manufacture and used as templates in the PCR with primers to amplify proximal promoters (1000 bases upstream) and 5' UTR exon of cilia genes *ENKURIN*, *EFHC2* and *IFT57*. The primers used are in Table 2 (restriction enzyme sites were shown in italics).

The PCR products were purified with QIAquick PCR Purification Kit (Qiagen) and digested, then ligated into pGL3-Basic vectors (Promega) treated with corresponding restriction enzymes. All the reporter plasmids were verified through sequencing. Reporter plasmids containing truncated proximal promoters and 5' UTR of *ENKURIN* and *EFHC2* were also constructed and were digested with NheI/HindIII and BstEII/HindIII respectively. Two truncated fragments (distal 400bp and proximal 700bp) of *ENKURIN* promoters as well as proximal 600bp of *EFHC2* promoters were acquired (Figure 7). The cohesive end (BstEII) of proximal 600bp fragment of *EFHC2* promoter was converted to blunt end through T4 polymerase treatment before HindIII digestion. Distal 400bp *ENKURIN* promoter, proximal 700bp *ENKURIN* promoter, proximal 600bp *EFHC2* promoters were inserted into pGL3-Basic vector treated with NheI, NheI/HindIII, SmaI/HindIII respectively. Proximal 450bp and

Table 2. PCR primers for amplifying proximal promoter and 5'UTR exon of cilia genes.

Name	Sequence
ENKURIN upstream	5'-at*ctcgag*gagtcccgacccggggaag-3' (*Xho*I)
Downstream	5'-at*agatct*ggccaccaaatgactcct-3'(*Bgl*II)
EFHC2 upstream	5'-a*ctcgag*aattataaaaacgacggctc-3' (XhoI)
Downstream	5'-a*aagctt*acgttgcctggagacgggaag-3' (HindIII)
IFT57 upstream	5'-a*cccggg*catccaccacttacctcatt-3' (XmaI)
Downstream	5'-a*agatct*cgccgccagtacagccacgac-3' (HindIII)

a)



b)

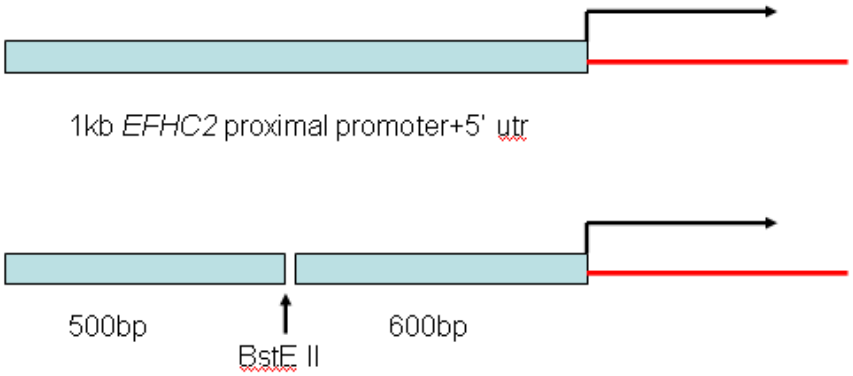


Figure 7. a) Promoter structures of *ENKURIN* in luciferase reporter vectors. b) Promoter structures of *EFHC2* in luciferase reporter vectors.

250bp *ENKURIN* promoter were further acquired by treating the reporter plasmid containing proximal 700bp *ENKURIN* promoter with PvuII/HindIII, PstI/HindIII, then ligated to pGL3-Basic vector treated with SmaI/HindIII. All reporter plasmids were propagated in NEB-5α cells (NEB) and verified through restriction enzymatic analysis and sequencing.

Maxiprep of Plasmids

Large amounts (~1-2μg/μl) of plasmids were acquired using Spin Doctor BAC Prep Kit& Spin Doctor Super Clean BAC Prep Kit (Gerard Biotech). The procedure was according to the protocol provided by the manufacturer with modifications as follows: after the first centrifugation, 0.8ml endotoxin removal resin (Promega) was added to supernatants and shaken for 10mins at room temperature. The resin was removed by centrifuging at 4000rpm shortly. The DNA pellets in the last step was re-suspended in 500μl Tris-HCl (pH8.0) and kept in 57 water bath. After re-suspension, each tube was centrifuged at maximum speed for 5mins and transferred to a new tube for subsequent transfections.

Transfection and Reporter Gene Assay

Immortalized hBEC3KT cells were seeded onto 6-well plate (2.5×10^5 cells per well, for endogenous gene transcription assay) or 48-well plate (2×10^4 cells per well, for reporter assay) 24 h before the transfection. FuGENE HD (Roche) was used for transfection according to the protocol provided by manufacturer. Typically, for each well of 6-well plate, 250ng of expression vectors, and empty expression vectors

(CMV3) were added to a total 500ng in 100µl serum-free GIBCO Opti-MEM medium (Invitrogen) with 2µl FuGENE HD. For each well of 48-well plate, 100 ng of reporter plasmid, 25 ng of transcription factor expression vector, and empty expression vector were added to a total 200ng in 17ul Opti-MEM medium with 1µl FuGENE HD. Each transfection of a 48-well plate was carried out in duplicates. Twenty-four hours after transfection, for 6-well plate, cells were washed cold PBS, and RNA was isolated for RT and qRT-PCR analysis with TRIzol (Invitrogen) according to the manufacturer's protocol. For 48-well plate, 24 h after transfection, cells were washed once with cold PBS followed by adding 100 µl of 1xlysis buffer (Promega) per well, and kept at -70°C for 1 h. After 30 min of rotation at room temperature, 5 µl of lysates was mixed with 25 µl of substrate (Promega) to detect luciferase activity with Turner.

Quantitative Real-time PCR

Total RNAs isolated from transfected hBEC3KT and differentiated NHBE cells on day 0, 2, 4 and three weeks were extracted and reverse transcribed as aforementioned. In parallel, GAPDH CDS was amplified as an internal control with primers GS (atcactgccacccagaagac) and primer GR (ttactccttggaggccatgtg). To detect mRNA level of ETS family and RFX family, and their changes during NHBE cell differentiation, qRT-PCR forward and reverse primers were designed. Besides, to identify the effects of transcription factors on endogenous cilia gene expression after transfection, qRT-PCR primers for cilia genes (*ENKURIN, EFHC2, IFT57, HSPA1A, MNS1, RIBC2 and ROPN1L*) were also designed (Table 3). QuantiTect SYBR Green

PCR Kit (Qiagen) was used with forward and reverse primers (final concentration 300nM each) according to the manufacturer's protocol in an ABI 7300 real-time PCR system (Applied Biosystem). The quantitative PCR results were analyzed using ABI's 7300 System SDS software, RQ Study.

Chromatin Immunoprecipitation Assay

To verify whether FOXJ1 directly binds to the proximal 250bp *ENKURIN* promoter and regulates its expression, chromatin immunoprecipitation assay was performed with ChIP-IT Express Enzymatic Kit (Active Motif) according to the manufacturer's protocol. In brief, twelve wells of NHBE cells cultured for 3 weeks were digested with 0.25% trypsin-EDTA solution and washed once with cold PBS, then fixed with 1% formaldehyde for 10 min at room temperature. After centrifugation and washing with cold PBS once, the residual formaldehyde was neutralized with glycine (0.125 M). Released nuclei from collected cells were enzymatically treated at 37 ^{o}C for 10 min in 400µl volume. 50µl of sheared chromatins were used for immunoprecipitation in 500µl volume with normal goat serum as a control, or polyclonal antibody against FOXJ1 (Santa Cruz). *ENKURIN* proximal 250bp promoter was amplified with EPF (gcacagtcaacgacgcttagcaat) and EPR (tctccgtccctttcttcactgctt). Surfactant protein D was used as a negative control to verify the specific enrichment of *ENKRUIN* proximal promoter by FOXJ1 antibody, and amplified with primers SPDF (tggaatgacagggcttgtggagaa) and primer SPDR (aggatattggcagcatgagggtct). Specific enrichment of *ENKURIN* proximal promoter was

quantified with software described in qRT-PCR section, and normalized to nonspecific precipitation of last exon of surfactant protein D.

Table 3. qRT-PCR primer sites for ETS, RFX family and cilia genes.

Name	Sequence
ELF1 upstream primer	agctagaaccagtaccatgcagga
downstream primer	agacacaaccactggaacttgggt
ELF2 upstream primer	cctgaaggtgaaacagattgagcc
downstream primer	acatcgagaccatcctggctaaca
ELF4 upstream primer	actacctccaccatgctcgtctct
downstream primer	tggatgttgctgggcactgaaga
GABP-α upstream primer	gcctacgatgaactatgagaaactcagtcg
downstream primer	tgccttgaactttacaaatcatgtccc
ELG upstream primer	cgatcttcgtccagctaacaaacttgc
downstream primer	cagctccttggagaaatggctgat
FLI1 upstream primer	aggagtggatcaatcagccagtga
downstream primer	caccagcttgctgcatttgctaac
FEV upstream primer	tctcttcaaggacgggaagaaccc
downstream primer	agaaactgccacagctggatct
ERF upstream primer	gatgaattacgacaagctgagccg
downstream primer	gaaattgaacttgtaggtgaaccgt
ETV3 upstream primer	ccgcaggaaatgcaaaccaca
downstream primer	tgaaggatcctcttgttgtaatagtatctg
ESE1 upstream primer	acagcaacatgacctacgagaagc
downstream primer	ttgccaaacttgtagacgagtcgc
ESE2 upstream primer	agagtactaccctgcctttgagca
downstream primer	cacacatggcgcttagtccagtat
ESE3 upstream primer	tgccaagtgccacaccaaa
downstream primer	tcctgggttcttgtctgggttcaa
ETS1 upstream primer	ttatcagctggacaggagatggct
downstream primer	aggccacggctcagtttctcataa
ETS2 upstream primer	tgccagtcattcatcagctggact
downstream primer	gcttctcgtagttcatcttgggct

Table 3 (Cont.)

SPDEF upstream primer	tcctcaaggagttgctactcaagc
downstream primer	acctgggctgagtcctcaattt
ETV4 upstream primer	atgaattacgacaagctgagccgc
downstream primer	aacttgtacacgtaacgctcaccagc
ETV5 upstream primer	atgaactatgacaagctgagccgc
downstream primer	agggcatctgggtcacagacaaat
ETV1 upstream primer	gataaacttagccgttcactccgc
downstream primer	gggcttctggatcacacacaaact
ETV2 upstream primer	tagcagctgcatccgttggact
downstream primer	aattcatgcccggctttctcttgc
SPI upstream primer	actggaggtgtctgacggcga
downstream primer	actggtacaggcggatcttcttct
SPIB upstream primer	tctcggacagcgagtcggatgag
downstream primer	aactggtacaggcgcagcttctt
SPIC upstream primer	gtacaacccactcttctccagcaa
downstream primer	atgccatctccggattatacaggg
ELK1 upstream primer	ttggaggcctgtctggaggctgaa
downstream primer	agctcttccgatttcaggtttggg
ELK4 upstream primer	ttatgacaaactcagccgagccct
downstream primer	tgtacacaaacttctgaccattcac
ELK3 upstream primer	tggagcagccttagtccagttgct
downstream primer	tggcatgtggccattaagcagtgt
ETV6 upstream primer	ccctgcgccactactacaaactaa
downstream primer	tcggccactcatgatttcatctgg
ETV7 upstream primer	gccaagatcttccgagttgtggat
downstream primer	ttataatagtggcgcagggcacga
ERG upstream primer	ctctccacggttaatgcatgctaga
downstream primer	ggcctagttgtaattctttgcgtagc
RFX1 upstream primer	tgccagccagtcttactctcaca
downstream primer	cctcagccgtctcatagttgtcca
RFX2 upstream primer	gatcgctgtgatgggagagttcaa
downstream primer	gctcatcgcccatgtcatctttgt
RFX3 upstream primer	agtggtcaacagacaggcacatct
downstream primer	ttctccaaactctggaagtgctcg
RFX4 upstream primer	actgtcagagaatactggacactg
downstream primer	gccgacaatgttcaccaccgt
RFX5 upstream primer	tcctgctacagcagcatctcatct
downstream primer	ttggtttagatgaccgttcccgag
RFXANK upstream primer	tcaccaccgaagccgactct
downstream primer	tggttctcgatcacctgttgcact

Table 3 (Cont.)

RFXAP upstream primer	ggatcgtcctgcaagacctactcttt
downstream primer	ctgctgttgtctttgctcca
ENKURIN upstream primer	tccgatgaagaaagggaggcagtt
downstream primer	agcctctgcttgcggatcttcttt
EFHC2 upstream primer	agctacccaagaattgcccaccta
downstream primer	tatcgaacaggtagccatccgctt
IFT57 upstream primer	acagatgctgcagaatggagccta
downstream primer	cacttctgtgctggtgcatttggt
HSPA1A upstream primer	aatctcagagccgagccgacaga
downstream primer	atctccaccttgccgtgttggaa
RIBC2 upstream primer	agctctggacagcagcaacctca
downstream primer	tgtgtgaaatagtctcccgtgggt
ROPN1L upstream primer	agactcagatgtgtctcccttgga
downstream primer	agacctatcatgccgttcttcctg
MNS1 upstream primer	gagcatgctacaaacttactaggc
downstream primer	cctgaactcttcaccaagcaga

Chapter 3: Results

FOXJ1 in human airway epithelial differentiation and ciliogenesis

FOXJ1 is a master regulator during the ciliogenesis; however, few target genes of FOXJ1 have been identified thus far. Mutation of the FOXJ1 gene is not correlated with abnormality of ciliogenesis (75). The expression of thirty-seven cilia genes, which encode axonemal components of cilia, increases significantly during human lung epithelial ciliogenesis (58), corresponding to the increased expression of FOXJ1. These genes are likely transcriptional targets of FOXJ1.

By quantitative RT-PCR, we assessed the relative mRNA levels of FOXJ1 as well as several cilia genes (*ENKURIN, EFHC2, IFT57*) during differentiation of NHBE cells under ALI cultures (0 day and the 21 day). We found that the expression of FOXJ1 and these cilia genes increased significantly in NHBE cells at day 21 of differentiation, when compared to undifferentiated NHBE cells lacking a ciliated cell population on day 0 (Figure 8). It is noteworthy that FOXJ1 expression was highly up-regulated.

To test whether ENKURIN, EFHC2 and IFT57 are transcriptional regulation targets of FOXJ1, we constructed an expression vector of FOXJ1 through cloning of coding sequence into pTarget expression plasmid; and reporter plasmids containing the proximal promoters (1000 bases upstream of transcriptional start site and 5′ UTR sequences) of *ENKURIN, EFHC2* and *IFT57* were constructed with pGL3 to direct expression of the reporter gene luciferase. FOXJ1 expression vectors and the reporter

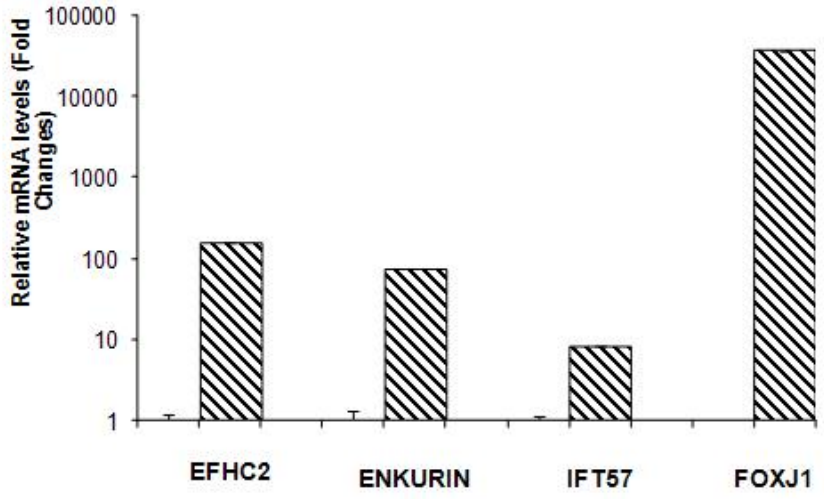


Figure 8. Expression of *FOXJ1, EFHC2, ENKURIN* and *IFT57* were up-regulated during lung epithelial differentiation and ciliogenesis. Fully differentiation of NHBE cells cultured under ALI situation will achieved after 21 days. Total RNAs were extracted from D0 and D21 ALI cells for quantitative RT-PCR detection of the relative mRNA levels of FOXJ1 and three cilia genes, mRNA levels of D0 were set as 1.

plasmids were cotransfected into HBEC3KT cells in 48-well plate and 24 hours later, cellular lysates were mixed with luciferase substrates for reporter assay. Results showed FOXJ1 can markedly transactivate the *ENKURIN*, *EFHC2* and *IFT57* promoters (Figure 9).

Overexpression of FOXJ1 in HBEC3KT cells is able to induce the expression of cilial axoneme genes *EFHC2*, *ENKURIN*, *RIBC2* and *ROPN1L*, while *IFT57*, *HSPA1A* and *MNS1* were not induced dramatically (Figure 10), indicating FOXJ1 can only selectively transactivate the cilial axoneme genes *EFHC2*, *ENKURIN*, *RIBC2* and *ROPN1L*. The inability of FOXJ1 to induce expression of endogenous *IFT57* while capable of transactivating the *IFT57* promoter will be discussed later.

To identify the FOXJ1 responsive *cis*-element(s) located in *EFHC2* and *ENKURIN* proximal promoters, we truncated their proximal promoters and cloned them to pGL3 plasmid as described, and then cotransfected with FOXJ1 expression plasmid in HBEC3KT cells. *Cis*-elements responsive to FOXJ1 transactivation in the *ENKURIN* promoter were located in the proximal 250bp sequences, while the distal 400bp of the promoter didn't contain *cis*-elements responsive to FOXJ1 (Figure 11). Similarly, the proximal 600bp of *EFHC2* promoter could be transactivated by FOXJ1 (Figure 12). These results indicate that the *cis*-elements responsive to FOXJ1 transactivation are located in the proximal region of *ENKURIN* and *EFHC2* promoters.

Next, chromatin immunoprecipitation (ChIP) was employed to analyze if FOXJ1 could directly bind to the 250bp proximal region of *ENKURIN* promoter. Twelve

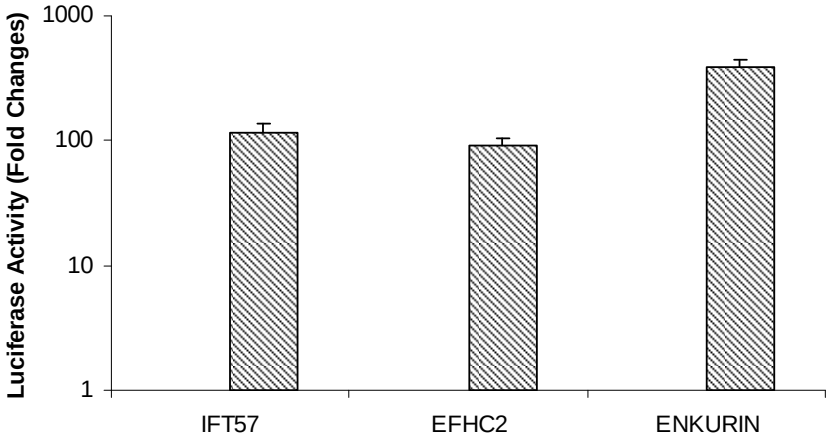


Figure 9. FOXJ1 transactivates *IFT57, EFHC2* and *ENKURIN* promoters. Reporter plasmids containing 1000bp proximal promoter and 5′ UTR of *IFT57*, *EFHC2* and *ENKURIN* were co-transfected with FOXJ1 expression plasmid respectively. Luciferase activities were measured 24h later. Luciferase activity of reporter plasmids co-transfected with control expression plasmids (empty CMV3) was set as 1.

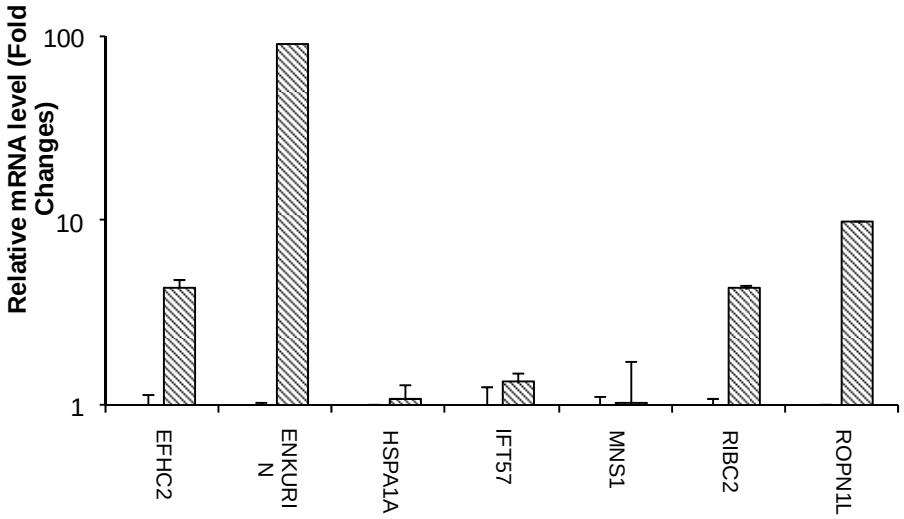
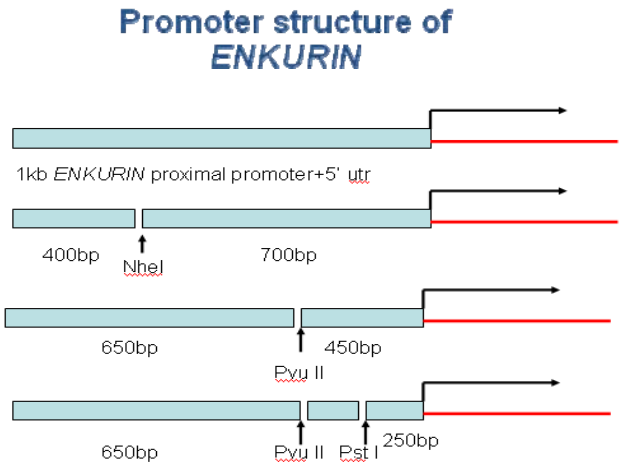


Figure 10. Effects of FOXJ1 on endogenous cilia gene expression. Total RNAs were extracted from HBEC3KT cells transfected with FOXJ1 expression vectors. The mRNA levels of cilia genes (*EFHC2,ENKURIN, HSPA1A, IFT57, MNS1, RIBC2* and *ROPN1L*) were assayed by quantitative RT-PCR. Control groups were transfected with empty CMV3 expression plasmid, whose mRNA levels of cilia genes were set as 1.

A)



B)

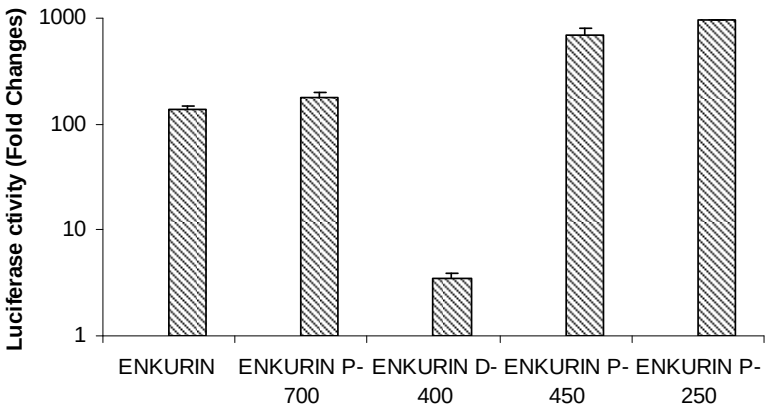
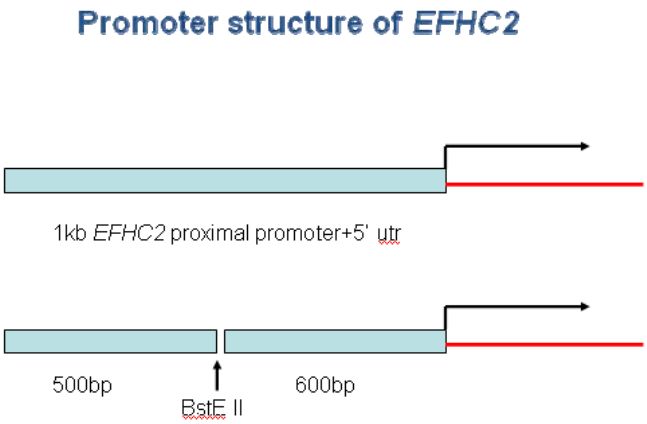


Figure 11. *Cis*-element(s) responsive for FOXJ1 transactivation were located in the proximal promoter regions of *ENKURIN*. A) *ENKURIN* promoter (proximal 1000bp+5′UTR) were truncated at 700 bp, 450 bp, 250 bp proximal to the transcriptional start site, and cloned into the luciferase reporter gene construct. In addition the distal 400 bp of the proximal promoter was also cloned into the luciferase expression construct. B) Subsequent cotransfection with FOXJ1 expression plasmid was used to determine promoter activity as measured by luciferase activity assay.

A)



B)

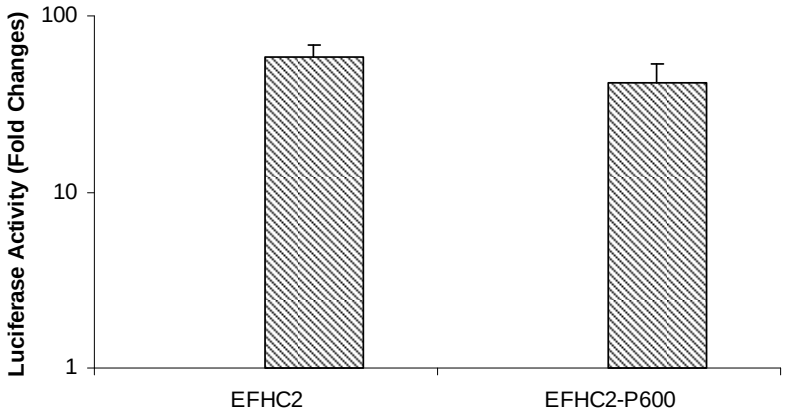


Figure 12. *Cis*-element(s) responsive for FOXJ1 transactivation were located in the proximal promoter regions of *EFHC2*. A) *EFHC2* truncated proximal 600bp fragment was cloned to pGL3 luciferase- reporter plasmid. B) Subsequent co-transfection with FOXJ1 expression plasmid.

wells of NHBE cells under ALI culture for three weeks were treated as described for ChIP analysis. Chromatin precipitates were enzymatically sheared into 200bp~400bp pieces. Goat polyclonal antibody against FOXJ1 (Santa Cruz) was used for specific enrichment of *ENKURIN* proximal promoter and non-immunized goat serum was used as negative control. Quantitative PCR showed that the FOXJ1 polyclonal antibody, but not the normal goat sera, specifically enriched the proximal *ENKURIN* promoter (Figure 13), indicating *ENKURIN* is an authentic transcriptional target of FOXJ1, and the *cis*-elements for FOXJ1 transactivation are located in the proximal *ENKURIN* promoter.

ETS transcription factor family in human airway epithelial differentiation and ciliogenesis

Although FOXJ1 was the only transcription factor identified by microarray to be increased during the differentiation of NHBE cells (58), it failed to transactivate several endogenous cilia genes. We analyzed the transcript levels of other transcription family members such as ETS proteins, which are expressed in stratified epithelium such as the pulmonary airways (85, 86). In lung epithelial cells, ETS family members are involved in transcriptional regulation and differentiation of several differentiated components of the airways (83, 84, 87). Previously, this laboratory has discovered ETS binding motifs overrepresented in the promoters of cilia genes. ETS binding motifs were found on promoters of mouse lung gene subsets as well as their human homologues, regulating the lung cell differentiation (88, 89).

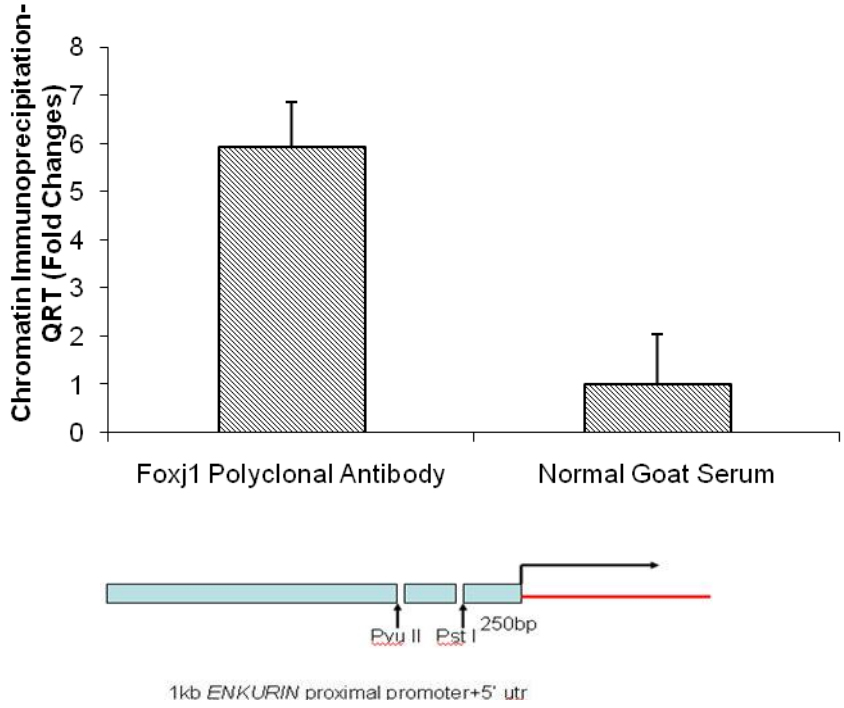


Figure 13. Direct binding of FOXJ1 to the proximal promoter of *ENKURIN*. Sheared chromatins from NHBE cells of three-week ALI culture were co-immunoprecipitated with goat polyclonal antibody against FOXJ1 or normal goat serum. The specific enrichment of *ENKURIN* proximal promoter sequence was quantified by quantitative PCR as detailed in *Materials and Methods*.

Thus ETS family members are potential transcriptional regulators involved in lung epithelial ciliogenesis in addition to FOXJ1. Quantitatitive RT-PCR was employed to detect the changes of expression patterns of all twenty-eight ETS family members in differentiated, ciliated NHBE cells under ALI culture. Results showed that the expression of ETV1, ETV2, ETV6, ETV7, ESE1, ESE2, ESE3, SPDEF and SPIC were significantly increased during the differentiation, while the expression of ETV4 and ETV5 were decreased. The level of other ETS factors did not show significant changes during ciliogenesis, and some, such as SPI, SPIB and ELG, were expressed in very low level in both unciliated and ciliated NHBE cells, indicating they are not likely involved in the lung epithelial ciliogenesis (Figure. 14).

The initial stage of ciliogenesis occurs between 2d and 4d apical upon exposure to the air. To further define more acutely the temporal induction of ETS family members, we assessed the expression level changes of ETS factors, during this stage to further identify those that are most likely involved in regulation of ciliogenesis. As Figure 15 shows, by quantitative RT-PCR of identification of the eleven dramatically changed ETS factors in Day 21 of ALI cells, the mRNA level of eight (ESE1, ETV1, ESE2, ESE3, SPIC, ETV2, ETV6, SPDEF) were significantly up-regulated during the initial stage of epithelial differentiation, while ETV5 significantly down-regulated. These results suggest that these nine ETS factors, are most likely involved in regulation the initiation of NHBE differentiation.

To identify transcription factors in regulation of cilial axoneme genes, motif discovery analysis as detailed in *Methods and Materials* was utilized to examine the

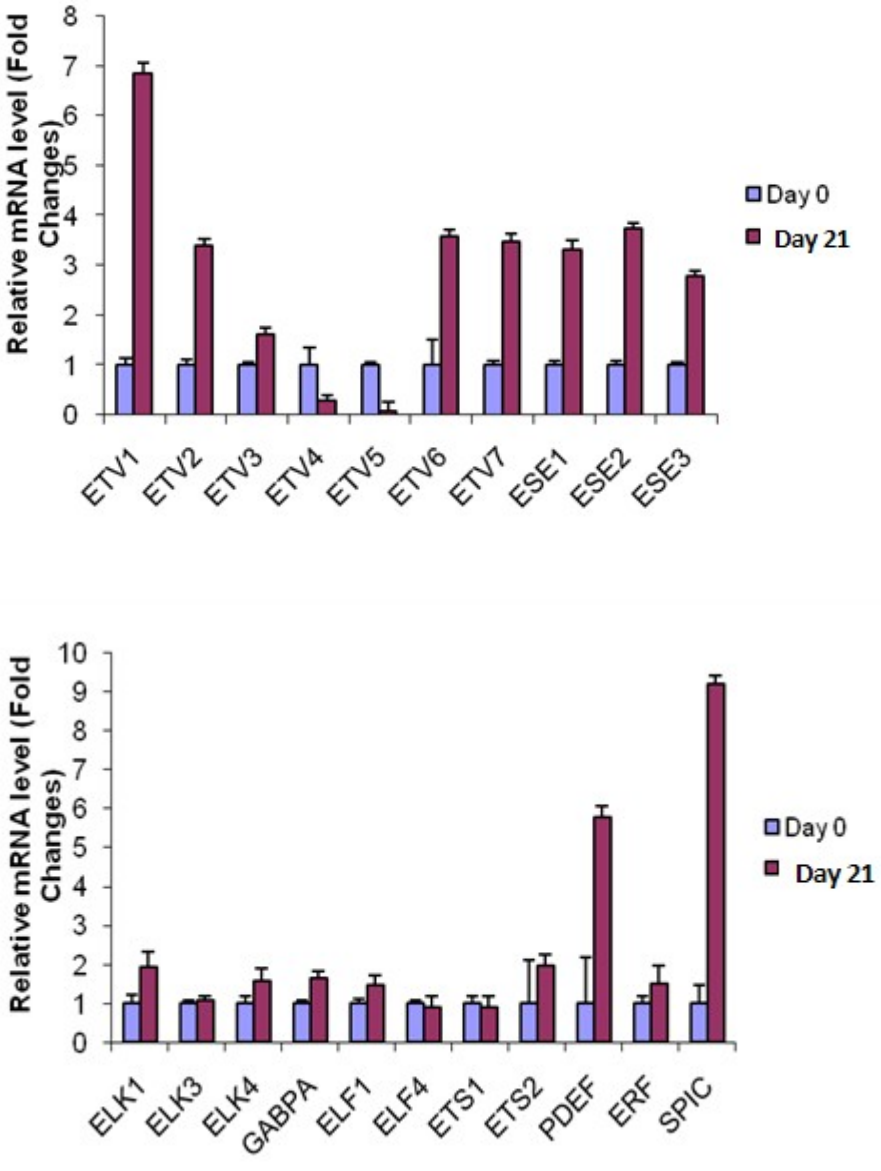


Figure 14. Expression level changes of ETS transcription factors during lung epithelial ciliogenesis. Total RNAs were extracted from unciliated (ALI Day 0) and ciliated (ALI Day 21) NHBE cells. The mRNA levels of ETS transcription factors were detected by quantitative RT-PCR. For comparison, the mRNA levels in unciliated cells (D0) were set as 1.

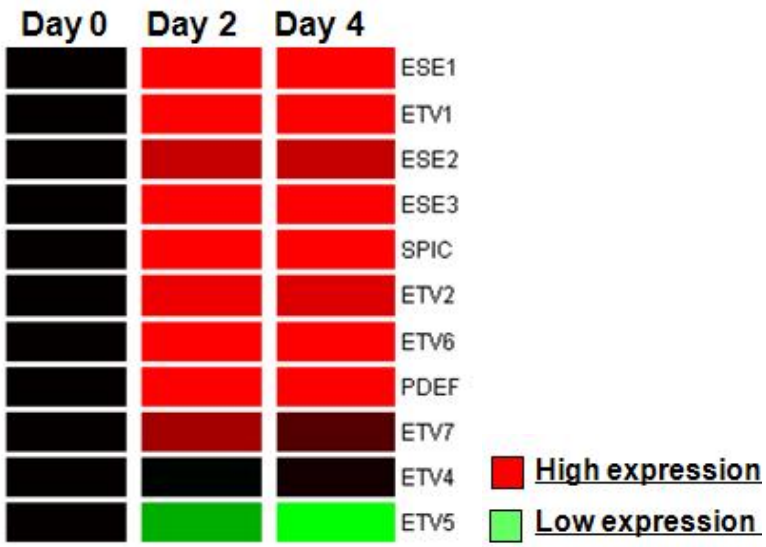


Figure 15. Expression level changes of ETS factors during the initiation of NHBE cell differentiation and ciliogenesis. Total RNAs were extracted from NHBE cells under ALI culture at Day 0, Day 2 and Day 4, followed by quantitative RT-PCR to detect the mRNA levels of chosen ETS genes (heatmap, deep red: mRNA levels increased over 2 fold; deep green, mRNA levels decreased over 2 fold). Day 0 was set as calibrator (black).

proximal promoter (proximal 1000bp plus 5′ UTR) of cilia genes to predict ETS binding sites. As shown in Figure 16, eight and seven ETS binding motifs were identified in *ROPN1L* and *HSPA1A* promoters among all cilia genes, respectively. Four ETS sites were identified in the proximal promoter region of *ENKURIN* as well. So we picked up *ROPN1L, HSPA1A* as well as four other cilia genes of interests (*ENKURIN, EFHC2, IFT57* and *MNS1*) for further analyses to test if their expressions are regulated by the five ETS factors. HBEC3KT cells were transfected with expression plasmids of ETV1, ESE1, SPDEF, SPIC and ETV5 in 6-well plates. Twenty four hours after transfection, mRNA level of these cilia genes were quantified by quantitative RT-PCR. We found that ETV1 and ESE1 were able to transactivate endogenous *EFHC2* and *ENKURIN,* and SPIC repressed the expression of EFHC2 (Figure 17). These five ETS factors had no effects on expression of endogenous *ROPN1L, HSPA1A, MNS1* and *IFT57* (data not shown).

RFX transcription factor family in human airway epithelial differentiation and ciliogenesis

The RFX family of transcription factors is associated with cilia formation in many eukaryotic species including human being (102, 114-117). However, their roles in ciliogenesis of lung epithelial cells remain largely unknown. Recently, our lab has identified RFX binding motifs in promoters of human and mouse gene subsets that are associated with fully differentiated lung epithelium, suggesting RFX factors are possibly involved in lung epithelial differentiation (88). No information exists regarding the expression or function of RFX family transcription factors in human

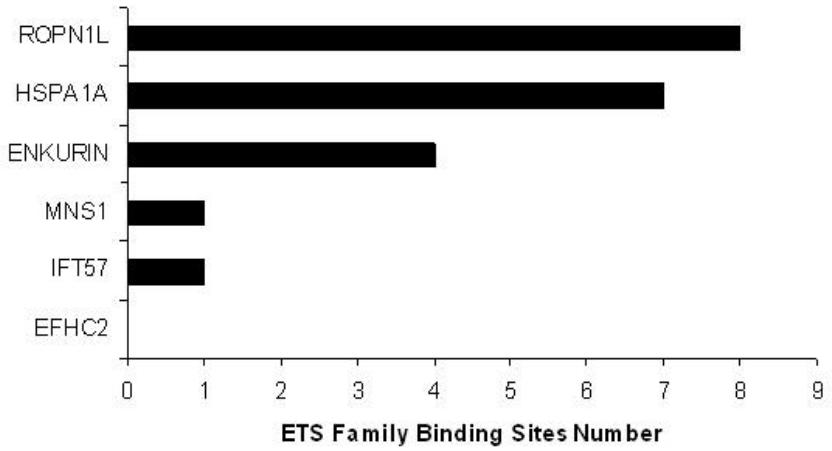


Figure 16. Numbers of ETS binding motifs in proximal promoters of cilia genes. The numbers of ETS binding motifs on proximal 1000bp plus 5′ UTR region of cilia gene promoters were predicted by motif discovery analysis as detailed in *Methods and Materials.*

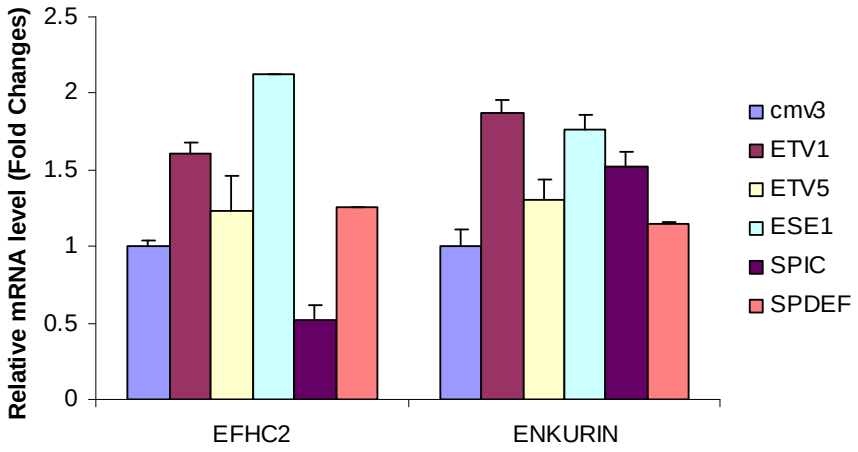


Figure 17. ETV1 and ESE1 transactivate endogenous *EFHC2* and *ENKURIN*, while SPIC represses *EFHC2*. HBEC3KT cells were transfected with expression plasmids of ETS factors (ETV1, ESE1, SPIC, SPDEF, ETV5). The effects of these transcription factors on mRNA levels of *EFHC2* and *ENKURIN* were analyzed by quantitative RT-PCR. The mRNA levels in cells transfected with empty CMV3 plasmid was set as calibrator (whose signal was set as 1).

airway epithelium. Similar to ETS family of transcription factors, we first identified RFX family members during the initial stage of differentiation and ciliogenesis of NHBE cells by quantitative RT-PCR. As Figure 18 shows, RFX1, RFX3, RFXAP and RFX5 were up-regulated significantly during differentiation and ciliogenesis, while other RFX members did not show obvious changes, indicating these RFX factors may be associated with the initiation of NHBE differentiation and ciliogenesis.

To identify the possible transcriptional regulation targets of RFX factors, we analyzed the proximal promoters of cilia gene promoters for RFX binding motifs. The numbers of binding motifs in seven cilia gene promoters (*MNS1, RIBC2, ROPN1L, HSPA1A, ENKURIN, EFHC2* and *IFT57*) are shown in Figure 19, in which *MNS1* and *RIBC2* have most among all cilia genes.

We tested effects of RFX1 and RFX5 on the endogenous expression of the seven cilia genes. As showed in Figure. 20, RFX1 transactivated endogenous *ENKURIN, HSPA1A* and *ROPN1L*, and RFX5 trans-activated endogenous *EFHC2, HSPA1A* and *RIBC2* (Figure 20). No obvious trans-activating effects were observed on other cilia genes.

GATA2 in Human Lung Airway Epithelial Differentiation and Ciliogenesis

GATA2 regulates the development of hematopoietic progenitor, and is expressed in many cell types such as cells of developing heart, liver, pituitary and trophoblasts (121, 127-132) and has been shown to be an important regulator of differentiated

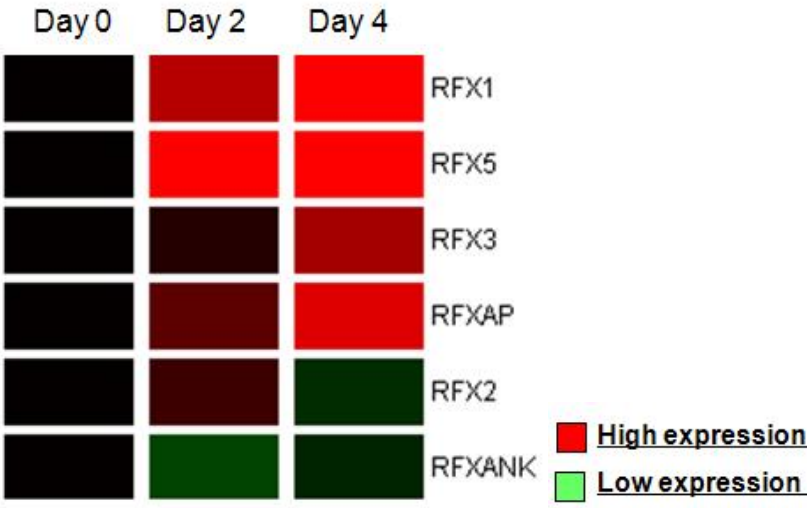


Figure 18. Expression level changes of RFX factors during the initiation of NHBE cell differentiation and ciliogenesis. Total RNAs were extracted from NHBE cells under ALI culture at Day 0, Day 2 and Day 4. The mRNA levels of RFX factors were quantified by quantitative RT-PCR (deep red: >2 fold upregulation; deep green: >2 fold down-regulation). The mRNA level on Day 0 was set as calibrator (black).

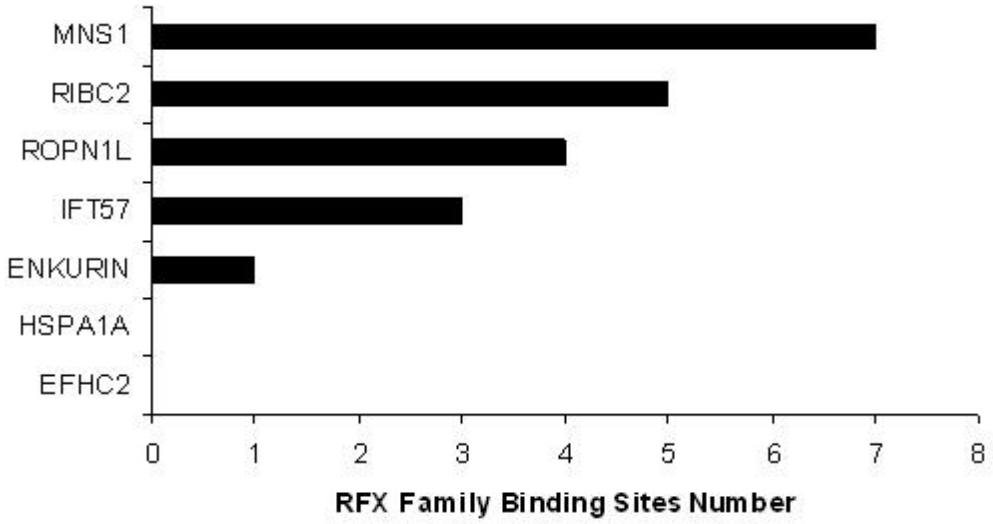


Figure 19. Numbers of RFX binding motifs in proximal promoters of cilia genes. RFX binding motifs in proximal 1000bp plus 5′ UTR region of the seven cilia gene promoters were identified through motif discovery as detailed in *Methods and Materials*.

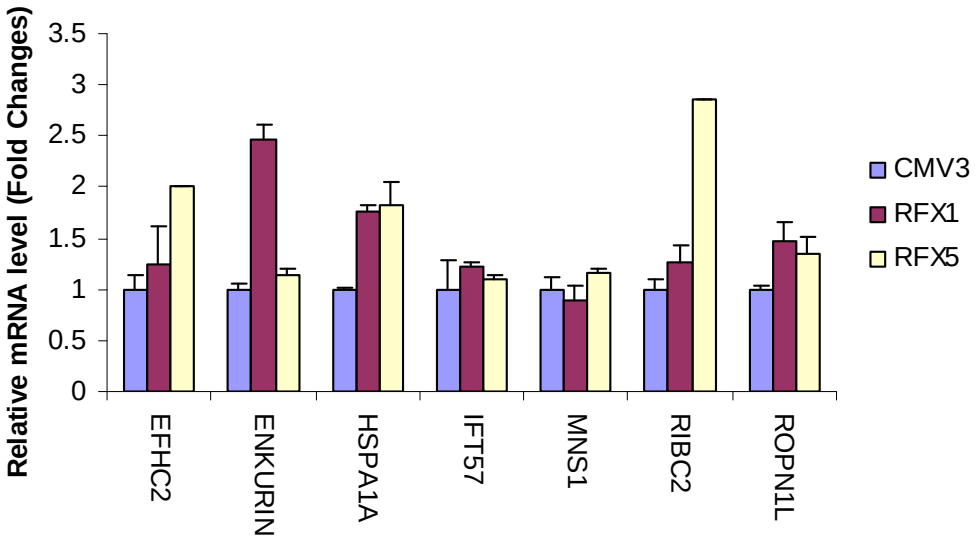


Figure 20. RFX1 transactivates endogenous *ENKURIN, HSPA1A* and *ROPN1L*; and RFX5 transactivates endogenous *EFHC2, HSPA1A* and *RIBC2* (over 1.5 fold change). HBEC3KT cells were transfected with RFX1 and RFX5 expression plasmids. Their effects on mRNA levels of the seven cilia genes (*MNS1, RIBC2, ROPN1L, HSPA1A, ENKURIN, EFHC2* and *IFT57)* were analyzed by quantitative RT-PCR. The mRNA levels of the cells transfected with empty expression plasmid (CMV3) was set as calibrator (whose signal was set as 1).

epithelium in embryogenesis. Its role in human airway epithelial cells differentiation

is still unclear. Previously, we have identified multiple GATA binding motifs in the promoters of gene subsets associated with lung epithelial differentiation. To verify this presumption, HBEC3KT cells were cotransfected with expression plasmids of GATA2 and reporter plasmids containing *ENKURIN* or *EFHC2* promoters directing the repression of reporter gene luciferase. The reporter assay showed GATA2 transactivated both *ENKURIN* and *EFHC2* promoters (Figure 21).

The GATA2 transactivation on the endogenous *ENKURIN* and *EFHC2* gene promoters was assessed in HBEC3KT cells following transfection of a GATA2 expression plasmid. The mRNA levels of *ENKURIN* and *EFHC2* were detected 24 hours later. As showed in Figure. 22, both endogenous promoters were transactivated by GATA2. To further locate the *cis*-element(s) responsive to transactivation, GATA2 expression vector and reporter plasmids containing truncated promoter fragments of *ENKURIN* and *EFHC2* were cotransfected as aforementioned. Results showed that GATA2 significantly trans-activated proximal *ENKURIN* (700bp and 400bp) and *EFHC2* (600bp) promoters, indicating the *cis*-element(s) responsive for transactivation of GATA2 were located in the proximal promoter regions of the two cilia genes (Figure 23).

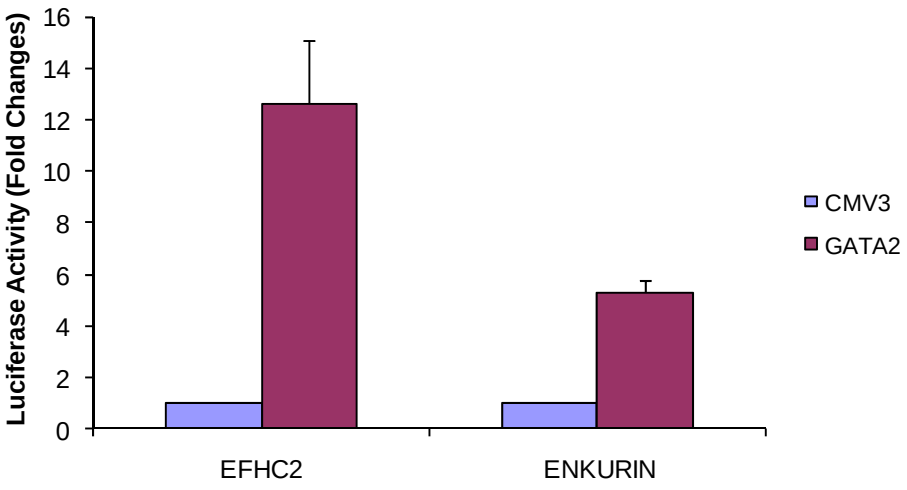


Figure 21. GATA2 transactivates *EFHC2* and *ENKURIN* promoters. Reporter plasmids containing 1000bp proximal promoter and 5′ UTR of *EFHC2* or *ENKURIN* was co-transfected with GATA2 expression plasmid. Luciferase activity was measured 24h after co-transfection. Reporter plasmid co-transfected with empty CMV3 expression plasmids was set as control, whose activity was set as 1.

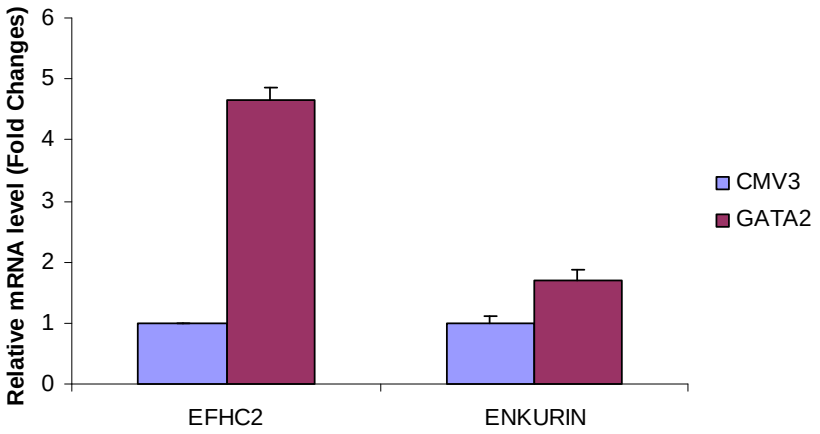


Figure 22. GATA2 transactivates endogenous *EFHC2* and *ENKURIN*. HBEC3KT cells were transfected with empty vector (CMV3) or GATA2 expression vector respectively. The mRNA levels of *EFHC2* or *ENKURIN* were quantified by qRT-PCR. The mRNA levels in cells transfected with empty expression vector (CMV3) was set as 1.

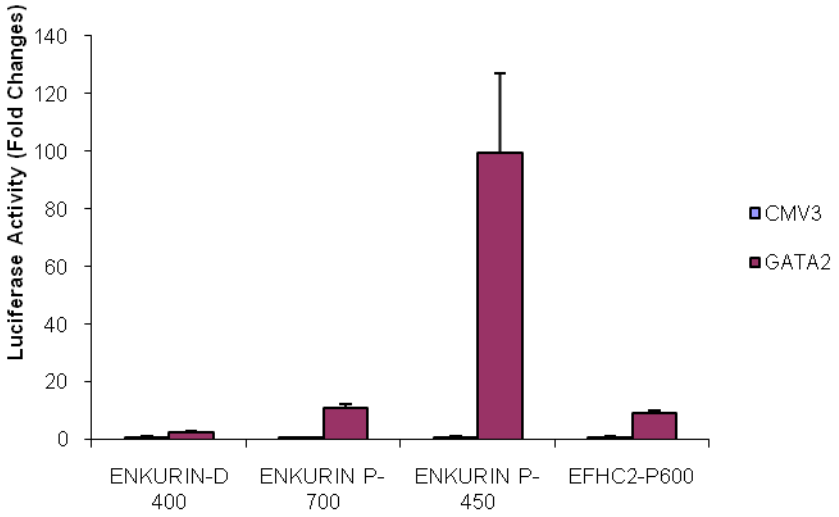


Figure 23. GATA2 transactivates proximal regions of *ENKURIN* and *EFHC2* promoters. HBEC3KT cells was transfected with GATA2 expression vector and reporter plasmids containing truncated distal 400bp, proximal 700bp, proximal 450bp of *ENKURIN* promoter, as well as proximal 600bp of *EFHC2* promoter. Luciferase activities were measured 24 hours after transfection. Cells transfected with empty CMV3 expression plasmid were set as value of one (1).

Chapter 4: Discussion

Ciliated cells in human airways are important components of host defense as part of the mucociliary clearance mechanism. Ciliary defects are caused by an ever growing recognition of genetic disorders and are often characterized by chronic or recurrent infections of the respiratory tract. In addition, viral infections lead to dysfunction of cilia which likely contributes in part to the overall presentation of lung disease. The transcriptional regulation of human airway epithelial ciliogenesis is still largely undefined. In this study, transcription factors that confer the coordinated expression of genes that encode ciliary axoneme structural proteins in ciliogenesis are identified and transcriptional targets of each transcription factor are defined.

FOXJ1 is a master regulator of motile cilia and is up-regulated dramatically during differentiation of NHBE cells *in vitro*. Quantitative RT-PCR data showed that the expression of several cilia genes (*ENKURIN*, *EFHC2*, *IFT57*, *MNS1*, *RIBC2*, *ROPN1L* and *HSPA1A*) are markedly increased during differentiation of human airway epithelia. We found that FOXJ1 transactivated the promoters of several cilia genes in reporter gene assays, as well as transactivated the endogenous cilia genes of human differentiated airway epithelial cells. Using studies of truncated promoters, the *cis*-elements responsive for transactivation of FOXJ1 were located into the proximal promoter regions of *ENKURIN* and *EFHC2*. Chromatin immunoprecipitation assays provided further support that FOXJ1 binds an unknown cis-active element in proximal promoter region of *ENKURIN*.

Although FOXJ1 endogenously transactivated the promoters of several cilia genes, it failed to transactivate other cilia genes, such as MNS1 and IFT57. Previous studies have indicated that overexpression of FOXJ1 alone does not confer ciliogenesis or differentiation of unciliated lung epithelia. These findings suggest that other transcription factors are also important in regulation of ciliogenesis of lung epithelial cells. Through motif discovery, multiple binding sites for ETS, RFX and GATA transcription factors were mapped in the promoters of a number of cilia genes, indicating cilia genes are potential targets of these transcription factors.

The twenty-eight factors of ETS transcription family are involved in a variety of mammalian developmental process at the cellular, tissue and organ level, including hematopoiesis, immune function, lymph/angiogenesis, neurogenesis/neuromuscular function, spermatogenesis, early embryonic patterning, and development of extraembryonic tissues (138-149). All members of this transcription factor family have the ETS DNA-binding domain which binds to sequences that contain a central GGA motif. However, individual ETS domain protein can select nucleotides over an 11-base-pair sequences centered on this GGA motif. ETS transcription factors are regulated, in part, by phosphorylation. Extracellular signals are transduced through a series of mitogen-activated protein kinases (MAPKs) which result in activation of downstream proteins such as ERK, JNK and p38, which in turn phosphorylate and regulate the activity of ETS transcription factors (78). Although the overall functions and roles of ETS family in lung epithelial differentiation is still unknown by now, ETS family members have been shown to be involved in the transcriptional regulation

and differentiation of mammalian lung and its epithelium differentiation. For example, aberrant expression of ELF5 disrupts lung branching and inhibits epithelial differentiation (83); In adult mouse lung, ETV5 is expressed by alveolar epithelial cells and regulates caveolin-1 transcription in mouse lung epithelial cell lines (84); In human lung epithelial cells, ESE-1, -2, -3 have regulatory effects on expression of the host defense genes lysozyme, chitinase and secretoglobins (87).

Many RFX factors are involved in cilia formations and functions in different eukaryotes. For example, in *C. elegans*, the only known RFX factor to date regulates *daf-19* which is a key regulator of ciliogenesis (102). In zebra fish, *rfx-2* is specifically expressed in multiciliated cells of the pronephros and loss of its expression results in cyst formation and loss of multicilia (114). *Rfx2*, *foxj1* and *ift88* are recently shown to be regulated by FGF signaling which regulates cilia length in diverse epithelial during embryo development (151). Most importantly, in mice, RFX-3 functions in ciliogenesis are conserved. It controls the growth of mouse embryonic node cilia, and loss of RFX-3 leads to significantly truncated but functional node cilia (115). RFX-3 loss-of-function also results in hydrocephalus with differentiation defects of ciliated ependymal cells of the choroid plexus and subcommisural organ (116). Moreover, RFX-3 mutant mice show primary cilial growth defects on islet cells and subsequent insulin secretion failure and impaired glucose tolerance (117).

Although ETS and RFX families are associated with cilia formation in many cell types and in many other simpler organisms, their roles in ciliogenesis of lung

epithelial cell subsets are still unknown and remain to be elucidated. We have performed quantitative RT-PCR to determine the expression levels of all twenty eight ETS factors and six RFX factors during the differentiation and ciliogenesis of NHBE cells under ALI culture. Among the twenty-eight ETS family members, the expression of nine (ETV1, ETV2, ETV6, ETV7, ESE1, ESE2, ESE3, SPDEF and SPIC) were significantly increased during the differentiation, while ETV4 and ETV5 were decreased. ESE1, ETV1, SPIC and SPDEF were most significantly up-regulated while ETV5 down-regulated most. RFX1 and RFX5 were the only RFX factors dramatically up-regulated, RFX3 and RFXAP were also up-regulated to some extent, while RFX2 and RFXANK slightly down-regulated. Based on these findings, we focused our studies on the five ETS factors (ESE1, ETV1, SPIC, SPDEF, ETV5) and the two RFX factors (RFX1 and RFX5) as putative transcriptional regulators in the initiation of ciliogenesis. Motif discovery analysis of cilia gene promoters identified ETS or RFX binding motifs in the proximal 1.2 kb region upstream of the first exon, suggesting putative binding sites for these transcription factors. Among the 37 cilia genes, promoters of *MNS1*, *RIBC2* and *ROPN1L* have the most RFX binding motifs, and promoters of *ROPN1L*, *HSPA1A* and *ENKURIN* had the most ETS binding motifs, suggesting these gene promoters are most likely to have potential RFX and ETS binding sites for transcription regulation. These five cilia genes as well as *EFHC2* and *IFT57* were selected to assess the transactivation ability of ectopic ETS or RFX in endogenous gene expression studies. Among the five ETS transcription factors (ETV1, ESE1, SPIC, SPDEF, ETV5), ETV1 and ESE1 were shown to transactivate both

EFHC2 and *ENKURIN* expression, and SPIC repressed *EFHC2* expression. None of the ETS factors has obvious effects on the expression of *ROPN1L*, *HSPA1A, MNS1* and *IFT57*. The transcriptional regulation of these ETS factors on the cilia genes may be directly or indirectly, cooperative or independent, which requires further study. Although *HSPA1A* and *ROPN1L* have most ETS binding motifs, these binding motifs are not functional or they require other factors that are not yet apparent.

RFX1 and RFX5 overexpression was also assessed for the ability to regulate endogenous cilia structural gene expression. *ENKURIN*, *HSPA1A* and *ROPN1L* were transactivated by RFX1, and *EFHC2*, *HSPA1A* and *RIBC2* by RFX5. *IFT57* and *MNS1* were not regulated by either RFX1 or RFX5. Our findings provide the first evidence for ETS- or RFX-mediated cilia gene induction in any metazoan system.

Our motif discovery also identified GATA elements in the promoters of ciliary axoneme genes. Although GATA family members GATA4 and GATA6 are involved in regulation of mammalian organogenesis by transactivating BMP-4 (150), so far, GATA6 is the only known GATA factor important in lung-specific gene expression and development (133-135). In this study, we investigate the effects of GATA2 on cilia gene expressions. Over-expression of GATA2 in HBEC cells led to the transactivation of *ENKURIN* and *EFHC2*, and this transactivation was located to proximal promoter region of *ENKURIN* and *EFHC2*. Transactivation of endogenous cilia genes in lung epithelial cells further supports GATA2 as a direct transactivator of those cilia genes.

The genomic program conferring differentiation, development, or other complex

cell changes operates in part through the coordinated regulation of gene expression. This program is executed by the recognitions of *cis*-regulatory DNAs by regulated transcription factors and thus the control of specific gene expression. These regulatory inputs and functional outputs of genes constitute a network-like architecture commonly referred as transcriptional regulatory networks. Transcriptional regulatory networks provide specific causal links between genomic expression patterns and biological processes such as differentiation or development (152, 153). Many transcription factors are expressed across multiple tissues and organs. For example, in adult mammals, ETS family of transcription factors are widely expressed in a variety of tissues including hematopoietic tissue, vasculature, brain and central nervous system, mammary gland, endometrium, ovaries, testes, kidneys and lungs (140). Responding to stimuli, these transcription factors turn on/off the transcription of the appropriate genes which in turn allows changes of cell morphology or activity needed for cell fate determination and cellular differentiation. Although most ETS-domain proteins have been shown to be transcriptional activators, a considerable number have been shown to be repressors, including ERF, YAN and TEL. For example, ETS factor TEL1 has been shown to be a transcriptional activator and repressor, requiring homo-dimerization or oligomerization (156). It also interacts with another ETS factor Fli1, inhibiting Fli1 transactivation activity and thereby preventing the activation of target genes (157,158). Therefore, the cellular specificity required for tissue specific phenotypic of functional processes likely involves multiple, unique transcription factor combinations to confer a coordinated gene expression network. Here we show

that multiple transcription factors from ETS (ETV1, ESE1, SPIC, SPDEF, ETV5), RFX (RFX1, RFX5) and GATA (GATA2) family can regulate cilia gene expressions together with FOXJ1 in ciliogenesis. A diagram illustrating the overlapping and unique transcription factor-gene promoter pairs is depicted in Figure 24.

Besides transcriptional regulation, increasing evidence indicates that post-transcriptional regulation plays a very important role in regulating steady-state mRNA abundance or mRNA translation and, in turn, determining cell or tissue specificity. After a gene is transcribed, the stability and distribution of the different transcripts is regulated (post-transcriptional regulation) by means of RNA binding proteins (RBP) that control the various steps and rates of the transcripts. Small RNA, either encoded as polycistronic RNA elements expressed and processed into 21-24 bp microRNAs (miRNAs), or small RNAs processed from the introns of expressed protein-encoding genes is another type of post-transcriptional regulation of genomic expression.

Much information regarding the expression of miRNA has been elucidated in recent years. First, a primary miRNA transcript (pri-miRNA), which can be many kilobases long and forms a stem-loop secondary structure, is transcribed from the genome. This pri-miRNA is the recognized by a protein complex including the enzyme Drosha in nucleus, and excised as a ~60nt hairpin called pre-miRNA. This pre-miRNA is transported into cytoplasm and processed by an enzyme called Dicer, leaving a 22nt double-stranded RNA duplex which is then taken up by a protein complex RISC. Within RISC, the double strand RNA duplex will be separated, and a

guide miRNA strand will remain bound to RISC and be the element recognized by target RNAs. Depending on the degree with which the guide miRNA strand matches the target mRNA, the target RNA is inhibited through two different mechanisms. If the match is imperfect, protein translation of that RNA will be inhibited by RISC complex. On the other hand, if the match is perfect, the target RNA will be cleaved by Aragonaute (Ago) protein which is a component of RISC complex. MiRNAs play key roles in mammalian gene expression and cellular processes, especially differentiation and development. In Dicer knock-out mice in which all miRNA productions are inhibited, developing embryos die at an early embryonic stage and are depleted of pluripotent stem cells (159),supporting miRNA is crucial in proper embryogenesis and stem cell development. Conditional knockout of Dicer in a tissue or organ of interests has demonstrated that miRNAs play roles in morphogenesis of several organs including the lungs, limbs, and muscles, in T cell differentiation, and in skin development (160-165). Considering the crucial roles of miRNAs in regulation of gene expression and cellular differentiation, it is not surprising that misregulation of miRNAs are linked with a variety of human diseases such as cancers. In cilia dysfunction polycystic kidney disease (PKD), the functional patterns of predicted miRNA targets and differentially expressed mRNAs are similar, suggesting an important role of miRNAs in specific pathways underlying PKD (155). It is noteworthy to mention that in mice within which Dicer is conditionally knocked out in lung epithelium, normal branching is arrested and a few large epithelial pouches appear in these mutant lungs. Significantly, these initial phenotypes are apparent

before an increase in epithelial cell death is observed, proposing that miRNAs play specific roles in lung epithelial morphogenesis independent of its requirement in cell survival (160). In the ciliogenesis of lung epithelial cells, some cilia genes may be also regulated by post-transcriptional regulation, especially miRNA mechanism. In the future study, techniques such as RNA immunoprecipitation on a chip can be applied to investigate the expression patterns of predicted miRNAs targeting cilia genes, and their role in regulating cilia gene expressions, which may shed lights on elucidating the whole process of lung epithelial differentiation during development.

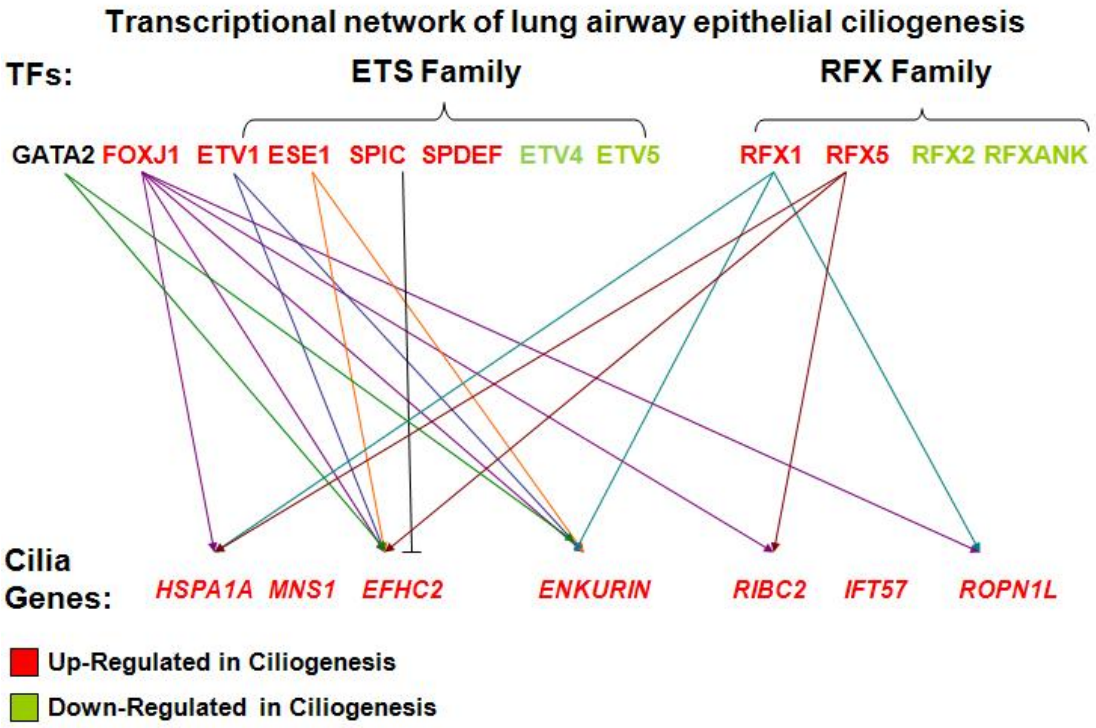


Figure 24. Transcriptional network in ciliogenesis: The transcriptional factors and their cilia gene targets are connected. (arrow: transactivation; bar: inhibition)

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