Identification and Functional Analysis of Alternatively Spliced Isoforms of Soluble Adenylyl Cyclase in Human Bronchial Epithelial Cells

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IDENTIFICATION AND FUNCTIONAL ANALYSIS OF ALTERNATIVELY SPLICED ISOFORMS OF SOLUBLE ADENYLYL CYCLASE IN HUMAN BRONCHIAL EPITHELIAL CELLS

By

Xi Chen

A DISSERTATION

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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A dissertation submitted in partial fulfillment of
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Effective ciliary beating is one of the key factors that regulate mucociliary clearance. Ciliary beat frequency (CBF) is partly regulated by changes in intracellular calcium and cyclic adenosine monophosphate (cAMP). While transmembrane adenylyl cyclases (tmACs) are well known for their ability to produce cAMP upon activation, soluble adenylyl cyclase (sAC) is another source of cAMP production mediated specifically by HCO$_3^-$/CO$_2$. It contributes to CBF regulation and the prevention of lung diseases due to mucociliary dysfunction. The three known alternatively spliced isoforms of sAC were identified in mice and rats, but there was no systematic study aimed at identifying additional alternatively spliced transcripts of human sAC. Our laboratory previously reported that a ~50 kDa sAC variant was expressed in cilia of normal human bronchial epithelial (NHBE) cells. Since different alternatively spliced variants may have different localizations and function differently, in this comprehensive study, groups of sAC alternatively spliced transcripts were identified for the first time in NHBE cells. NHBE cells express multiple sAC alternatively spliced variants: full-length sAC (sAC$_{fl}$) and variants with partial deletion of the catalytic domain 1 (C$_1$). One variant contains two alternatively spliced sites creating new exons 5 (exon5v2) and 12 (exon12v2) as well as a new open reading frame encoding a ~45 kDa protein, similar in size to the variant found
in cilia. While not exhibiting sAC catalytic activity \textit{in vitro}, this sAC_{ex5v2-ex12v2} variant was targeted to cilia when expressed in NHBE cells. Further analysis revealed that the sequence corresponding to the new exon 5v2 was important for targeting the protein to axonemes while exons 2-4 prevented it. In wild type (WT) mouse tracheal epithelial cells (mTECs), the HCO\textsubscript{3}^-/CO\textsubscript{2}-mediated CBF decrease due to intracellular acidification was partially rescued by sAC since the sAC inhibitor KH7 led to a larger CBF decrease compared to cells not treated with KH7. Airway epithelial cells isolated from sAC C\textsubscript{1} knockout (KO) and C\textsubscript{2} KO mice showed no longer catalytic sAC activity since KH7 had no effect on the HCO\textsubscript{3}^-/CO\textsubscript{2}-mediated CBF decrease. Expression of the axonemal sAC_{ex5v2-ex12v2} variant, but not the cytoplasmic sAC_{ex2-ex12v2}, restored KH7 sensitivity of CBF rescue in sAC C\textsubscript{2} KO but not in C\textsubscript{1} KO mice. Thus, we show for the first time an axonemal targeting sequence that localizes a sAC variant to cilia where it is regulating CBF.
ACKNOWLEDGEMENTS

I appreciate my mentor, Dr. Matthias Salathe and his collaborator Dr. Fregien for their guidance and help these years in my project. Through this challenging process, I have broadened my skills, knowledge and experience in science. I am thankful for my committee members, including Dr. Salas, Dr. Lincoln and Dr. Kapiloff for their advice and understanding these years. Our collaborator, Drs. Buck and Levin have not only supplied me the precious sAC knockout mouse tracheae and antibodies but also offered discussion, shared the experience and data which I am extremely obliged to. Also I give thanks to the molecular and cellular pharmacology program, including Dr. Slepak and Dr. Zhai who helped me in my Ph.D. training. I am especially grateful for the great help and guidance from my former lab members in my project. Dr. Unwalla Hoshang and Dr. Philip Whitney have offered me a lot of guidance and scientific insights in my experiments, especially during the toughest time that lead to the breakthrough of my project. Nieves Falcon taught me the cytospin and immunostaining. Dr. Maria Elena Monzon guided me with the confocal microscope. Dr. Dahis Manzanares taught me cAMP assay and immunoprecipitation. Dr. Andreas Schmid taught me the CBF measurement, the cilia separation and discussed with me about my project. John Stephen Dennis gave help in some of my molecular biology experiments. Monica Valencia supplied me the RNA samples for real-time PCR. Nathalie Baumlin helped with the mouse airway epithelial cell culture and CBF measurement. Radia Forteza offered the normal mouse tracheae for me to practice. Overall, I appreciate all the encouragement and support from my former colleagues, friends and parents throughout this Ph.D. journey.
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<td>Apical-Junctional Complex</td>
</tr>
<tr>
<td>ALI</td>
<td>Air Liquid Interface</td>
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<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>ATPase</td>
<td>Adenosine Triphosphatase</td>
</tr>
<tr>
<td>BEGM</td>
<td>Bronchial Epithelial Growth Media</td>
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<td>cAMP</td>
<td>Cyclic Adenosine Monophosphate</td>
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<tr>
<td>CBF</td>
<td>Ciliary Beat Frequency</td>
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<tr>
<td>CFTR</td>
<td>Cystic Fibrosis Transmembrane Conductance Regulator</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic Guanosine Monophosphate</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon Dioxide</td>
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<tr>
<td>CTS</td>
<td>Ciliary Targeting Sequence</td>
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<td>C₁/C₂</td>
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<td>DAPI</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene-Diamineteraetacetic Acid</td>
</tr>
<tr>
<td>EST</td>
<td>Expressed Sequencing Tags</td>
</tr>
<tr>
<td>HA</td>
<td>Human Influenza Hemagglutinin</td>
</tr>
<tr>
<td>IFT</td>
<td>Intraflagellar Transport</td>
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<td>IP</td>
<td>Immunoprecipitation</td>
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<td>KO</td>
<td>KnockOut</td>
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<td>PCL</td>
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Chapter 1 Introduction

1.1 Mucociliary clearance and lung diseases

The human conducting airway is organized as a tree-like structure which extends mainly from the trachea to alveoli. It is principally responsible for transporting oxygen into the body to facilitate further gas exchange. On average, an adult inhales about 11,000 liters of air in a day along with dust, particles, virus, etc. Thus, the airway is constantly exposed to all sorts of environmental challenges and requires an effective system to protect itself from being damaged. Besides adaptive immunity, including T cell activation and production of cytokines and chemokines, the innate immunity of airway provides direct and instant protection against hazardous invasions. This includes the epithelial barrier, mucociliary clearance (MCC), and local phagocytosis. The airway epithelium provides the first line of defense against noxious inhaled substances. Epithelial barrier integrity depends on apical-junctional complexes (AJCs) that are located at the lateral membranes of adjacent cells. AJCs are composed of highly specialized structures: tight junctions and adherens junctions (Rezaee and Georas 2014). For local phagocytosis, macrophages and neutrophils are the predominant phagocytes that migrate and clean the airway during inflammation (Donnelly and Barnes 2012). Mucociliary clearance provides the most effective protection in the host defense system of human airway. The three components of the MCC apparatus are mucus layer, periciliary liquid layer (PCL), and beating cilia (Figure 1.1). These components work together to remove inhaled pathogens and pollutants from the airway. Large glycoprotein molecules (mucins) are the major components in the mucus which are produced by goblet cells at the surface epithelia and by mucous and serous cells at the submucosal glands. The presence of mucins, water, and
electrolytes (Houtmeyers, Gosselink et al. 1999) gives viscoelastic characteristic to the mucus layer in order to trap inhaled substances and prevent them from contacting the epithelium. The ciliary tips interact with the viscous mucus layer, which enables them to move the mucus towards the pharynx where it is usually swallowed or expectorated. Effective beating of cilia is essential for the prevention of airway diseases. The loss of adequate ciliary beating is observed in primary ciliary dyskinesia and other airway diseases with significant inflammation causing secondary ciliary dyskinesia. Adequate mucus hydration is another important factor to maintain ciliary beating. If inadequate, the mucus collapses onto cilia and beating becomes ineffective. The latter situation is encountered in cystic fibrosis.

![Diagram of the components of mucociliary clearance.](image)

**Figure 1.1 Components of human mucociliary clearance.**
Diagram of the components of mucociliary clearance (MCC) as the principal host defense system in human airway. The tracheal epithelium is mainly composed of ciliated columnar cells, goblet cells, and basal cells. Besides these, there are submucosal glands in the airway with serous and mucous cells also contributing to the mucus secretion (not shown in the graph). Periciliary liquid layer (PCL) is right on top of epithelia which immerses motile cilia and allows them to beat freely. The uppermost layer is the mucus that traps invasive pathogens and propel them away with the beating cilia. Apical-junction is one of the factors contributing to epithelial barrier.
1.2 Ciliary structure and beating

Motile cilia protrude from the apical side of columnar cells and line the human airway surface, with the length decreasing from 5-7 µm in the trachea to 2-3 µm in the seventh airway generation (Stannard and O'Callaghan 2006). There are about 100-150 cilia on each ciliated cell (Boysen 1982) and each cilium is connected to the cellular cytoskeleton with a basal body. The core of the cilium is the detergent-insoluble, membrane-free axoneme. The axoneme is mainly composed of microtubules arranged in a “9+2” pattern as seen in the cross sections of cilia by electron microscopy (Figure 1.2). Nine microtubules are doublets that encircle two central single microtubules. Microtubules are polymers of α and β-tubulin monomers. These two proteins form basic dimers where α-tubulin is expressed at the minus end of microtubules and β-tubulin is at the plus end with guanosine triphosphate (GTP) bound to it. Acetylated α-tubulin is enriched in cilia and can be used as a ciliary marker in immunostaining. Inner and outer dynein arms are attached to each doublet (Figure 1.2). Nexin links the neighbor microtubule doublets to each other and the central pair of microtubules is connected to the peripheral ones via radial spokes.

**Figure 1.2 Schematic diagrams of cillum axonemal components.**
Left: Longitudinal view of the axoneme. The outer dynein arms (green) and inner dynein arms (light and dark orange) attach to the microtubules periodically. Right: Cross sectional view of axoneme. The central pair of microtubules coated by the sheath is
circled by nine microtubule doublets (microtubules A and B). Nexins and dynein arms link the neighboring microtubules to each other and radial spokes connect the microtubule doublets with the central pair. The figure and the legend refer to Ibanez-Tallon, 2003 (Ibanez-Tallon, Heintz et al. 2003). Permission to use this image was received from the author and Oxford University Press for the dissertation of Xi Chen.

The motor protein in the axoneme is dynein. Dynein is an adenosine triphosphatase (ATPase) that powers ciliary beating by hydrolyzing adenosine triphosphate (ATP), which causes the adjacent microtubules to slide relative to each other. Because of the crosslink of nexins, the relative sliding of microtubule doublets is converted into bending (Warner and Satir 1974). In mammalian tracheal cilia, the outer dynein arm is a Y-shaped molecule. Each head contains a heavy chain which has ATPase activity (Pfister and Witman 1984). The inner and outer dynein arms function differently. The inner dynein arm is responsible for the beating pattern and the outer dynein arm regulates ciliary beat frequency (CBF) (Brokaw and Kamiya 1987, Hard, Blaustein et al. 1992).

1.3 Regulation of ciliary beat frequency

As mentioned, CBF is mainly controlled by outer dynein arms. In fact, CBF is a reflection of sliding velocity which is assumed to be equivalent to microtubule translocation rate in vitro (Satir, Barkalow et al. 1995). Regulation of CBF in mammalian cells is complex and includes changes in intracellular $[\text{Ca}^{2+}]_i$, phosphorylation of ciliary targets, and intracellular pH (pH$_i$) (Salathe 2007). Elevation of $[\text{Ca}^{2+}]_i$ enhances CBF and vice versa (Kelley, Evanson et al. 1980, Villalon, Hinds et al. 1989, Lansley, Sanderson et al. 1992, Schmid and Salathe 2011). Calcium can be released from intracellular storage or influx from outside of the cell upon various stimulations and bind with calmodulin to activate PKG or PKA, leading to the increase of CBF (Schmid and Salathe 2011). But the more complicated mechanism by which calcium regulates CBF is still not well
understood. $\text{pH}_i$ directly regulates CBF probably through the outer dynein arm of axoneme (Sutto, Conner et al. 2004). Decrease of $\text{pH}_i$ lowers CBF and in turn intracellular alkalization increases it (Sutto, Conner et al. 2004).

Several lines of evidence support that CBF is regulated by cyclic adenosine monophosphate (cAMP)-dependent phosphorylation of the outer dynein arm light chain called p29 in *Paramecium* (Satir, Barkalow et al. 1993, Hamasaki, Barkalow et al. 1995). Phosphorylation of p29 leads to a mechanochemical change of 22S dynein, resulting in a faster sliding of microtubules (Satir, Barkalow et al. 1993). The functional ortholog of p29 in ovine airway epithelia, a 26 kDa protein, was found to be phosphorylated in cAMP-dependent way (Salathe, Pratt et al. 1993). Bending frequency was increased in isolated bovine axonemes in contact with cAMP *in vitro* (Wyatt, Forget et al. 2005). Also, A-kinase anchoring protein was found in human airway cilia (Kultgen, Byrd et al. 2002). All these evidence suggest that cAMP increases CBF through PKA activation to phosphorylate a dynein arm light chain. cGMP can also increase CBF (Figure 1.3). In the presence of cGMP, axonemes beat faster in a cell-free preparation (Wyatt, Forget et al. 2005). However the mechanism by which cGMP increases CBF is not clear. It may be via the activation of PKG, phosphorylation or even dephosphorylation of some ciliary targets (Gertsberg, Hellman et al. 2004). Protein kinase C (PKC) has the opposite effect: it causes a decrease in CBF through phosphorylation of a 37 kDa polypeptide in sheep trachea (Salathe, Pratt et al. 1993).
1.3 Integration of signaling pathways in regulating ciliary beating.
This is a summary of the signaling pathways of ciliary beat frequency (CBF) regulation. 
Ca$^{2+}$ and cyclic adenosine monophosphate (cAMP) are the major second messengers in 
the whole pathway. cAMP leads to the activation of PKA which phosphorylates the 
ciliary targets to increase CBF. Intracellular Ca$^{2+}$ can be released from the endoplasmic 
reticulum or being brought into the cytoplasm via influx. Ca$^{2+}$ can either increase CBF 
directly or activate PKG to phosphorylate an axonemal target. pH$_i$ can also change CBF. 
The arrows in the graph indicate stimulation while blunt lines represent inhibition. The 
graph and legend are modified from Salathe, M., 2007.

1.4 Proteins trafficking to cilia

Since there is no biosynthetic machinery in cilia, all membrane and soluble proteins 
must be transported from the cell body to cilia. Though the cilium is not a strictly 
membrane-enclosed compartment, there is evidence of a barrier that separates the ciliary 
section from the cytoplasm (Hu, Milenkovic et al. 2010). It is evidenced that some 
membrane proteins, such as polycystin-2 and retinitis pigmentosa GTPase regulator, are 
concentrated in the periciliary area (Hong, Yue et al. 2001, Nauli, Alenghat et al. 2003, 
2009). This is also demonstrated by the observation that a genetically engineered 
fluorescent protein is specifically localized to the apical plasma membrane but fails to 
diffuse to the ciliary membrane (Vieira, Gaus et al. 2006). Besides these examples, it is 
also believed that there is a ciliary pore complex that acts as a gate for proteins to enter
the ciliary lumen (Satir and Christensen 2007). It is possible that targeted vesicles from the Golgi complex are transported to the base of the cilium, enter the cilium, and fused with the ciliary membrane (Nachury, Seeley et al. 2010). Alternatively, transporting protein would bind to the “fence” (actin-based skeleton structure) element proteins while loosening the “fence” pore and penetrating the ciliary barrier or these proteins could actively pass through the diffusion barrier by being physically pulled through (Nachury, Seeley et al. 2010). Another theory is that ciliary proteins require a specific sequence that can be identified by ciliary membrane proteins. Those specific sequences are called ciliary targeting sequences (CTSs) and they are sufficient as well as necessary for sending proteins to cilia. The first ciliary targeting sequence was identified in rhodopsin (Tam, Moritz et al. 2000). Fibrocystin also localizes to cilia via the CTS of 18 amino acids near the N-terminal end of the cytoplasmic tail (Follit, Li et al. 2010). Polycystin-2 traffics to cilia using an N-terminal rvxp motif (Geng, Okuhara et al. 2006). With the discovery of more CTSs, it is clear that there is no unique consensus CTS and there might be more than one mechanism involved in recognizing such sequences (Hsiao, Tuz et al. 2012).

1.5 Soluble adenylyl cyclase

1.5.1 Soluble adenylyl cyclase in general

Transmembrane adenylyl cyclase (tmAC) has been widely accepted as the predominant source of cAMP in cells. An alternative source of cAMP, soluble adenylyl cyclase (sAC), was first purified and cloned from rat testes by Dr. Buck’s group (Buck, Sinclair et al. 1999). This was also annotated as the 10th adenylyl cyclase isoform (ADCY10) (Buck, Sinclair et al. 1999). sAC differs from the original nine isoforms of
tmACs because it does not possess a transmembrane domain. Contrary to what the name suggests though, sAC is not soluble but is usually tethered to cellular structures and can be found in mitochondria, centrioles, mitotic spindles, mid-bodies, and nuclei (Zippin, Chen et al. 2003). Uniquely, this enzyme is insensitive to heterotrimeric G proteins or forskolin, but can be specifically activated by bicarbonate and calcium. Like tmACs, full-length sAC (sAC\textsubscript{fl}) contains two heterologous catalytic domains (C\textsubscript{1} and C\textsubscript{2}), which show significant homology to bicarbonate sensing adenylyl cyclases from cyanobacteria (Chen, Cann et al. 2000, Kobayashi, Buck et al. 2004, Mime Kobayashi 2004). However, sAC in cyanobacteria only contains one catalytic domain. sAC activity requires two divalent metal cations in the catalytically active site of the enzyme to coordinate binding and hydrolysis of ATP (Tresguerres, Levin et al. 2011). \textit{In vivo}, Ca\textsuperscript{2+} increases sAC activity by increasing the affinity for ATP of mammalian sAC. HCO\textsubscript{3}\textsuperscript{-} releases substrate inhibition and increases Vmax (Litvin, Kamenetsky et al. 2003). Calcium and bicarbonate have a synergistic effect on the activation of sAC. The catalytic mechanism of cAMP production by sAC and its activation by HCO\textsubscript{3}\textsuperscript{-} have been elucidated for CyaC, an adenylyl cyclase from \textit{Cyanobacterium Spirulina platensis} (Steegborn, Litvin et al. 2005). In the crystal structure study of the sAC cyanobacteria model, Ca\textsuperscript{2+} binds to the phosphate of ATP and occupies the first ion-binding site (Steegborn, Litvin et al. 2005). This leads to an “open sAC state” meaning substrate binding may result in a conformational change of the enzymatic active site. Then, the second divalent metal, a Mg\textsuperscript{2+} ion, binds to the phosphate of ATP, leading to a distinct set of catalytic residue interactions referred to as the “closed state” (Steegborn, Litvin et al. 2005). Bicarbonate stimulates sAC activity by fostering the allosteric change that leads to active site closure,
recruiting the second bound ion and rearranging the phosphates of the bound substrate (Steegborn, Litvin et al. 2005). The limitation of this study is that cyanobacteria contains only one catalytic domain and forms a symmetrical homodimer with two identical nucleotide binding sites. Recently, X-ray crystallography of human sAC demonstrated that the catalytic domains in human sAC are heterodimers with only one ATP binding site and a catalytically inactive site (Saalau-Bethell, Berdini et al. 2014). This crystal structure study of human sAC is based on the cloned and purified human sAC expressed in insect cells (Saalau-Bethell, Berdini et al. 2014). The limitation of this study is that it does not identify alternative conformational changes and movements, which precludes other interaction of sites inside the cells. Therefore, the mechanisms of how human sAC interacts with activators and performs catalytic activity inside living cells are not completely understood yet.

sAC is found in various tissues and species contributing to different functions. It was discovered in bone, pancreas, brain, and digestive tract tissues where it serves as a source of cAMP. It was originally found in testes where it plays a role in spermiogenesis and mature sperm motility (Esposito, Jaiswal et al. 2004, Hess, Jones et al. 2005). sAC C1 domain knockout (KO) mice—where sAC and truncated sAC (sACt) were removed by deleting murine sAC exons 3, 4 and 5—showed a phenotype of infertility because of the loss of motility of the sperm flagellum (Farrell, Ramos et al. 2008). In proximal convoluted tubules of kidneys, sAC was responsible for regulating salt and fluid absorption (Zhou, Bouyer et al. 2006). In cow’s eyes, sAC was found in the corneal endothelium where sAC activated CFTR to regulate Cl−, HCO3−, and ATP secretion (Sun,
Thus, sAC activity and function in tissues rely on the physiological performance of the cAMP inside the cells.

1.5.2 Alternative splicing

Since the 1970’s, people gradually accepted the existence of alternative splicing: a single pre-mRNA precursor can generate multiple mRNA messages due to different combinations of splice sites and these mRNAs code for functionally different protein isoforms. This altered the original “one gene, one protein” concept. Splicing is facilitated by spliceosome which is formed by a group of ribonucleoproteins. The regulatory units of alternative splicing are broadly classified as cis-acting sequences (the nucleotides) and trans-acting (the spliceosome) factors. The well-accepted process of splicing is that trans-acting factors recognize splice sites by indirectly stabiling snRNA-pre-mRNA interaction or directly contacting the cis-acting sequences (Will and Luhrmann 2011).

There are mainly four types of alternative splicing (Figure 1.4). (1) exon skipping (cassette alternative exon): this is the most prevalent type of alternative splicing in vertebrates and invertebrates (38.4%) (Kim, Magen et al. 2007). Exons can be spliced out together with flanking introns. (2) Alternative 5’ or 3’ splice sites: two or more splice sites are found at one end of the exon (26%) (Kim, Goren et al. 2008). (3) Intron retention: the original defined intron sequences are retained in the mature mRNA (2.8%) (Kim, Goren et al. 2008). By using general techniques like microarrays, reverse transcription-polymerase chain reactions (RT-PCR), and analysis of expressed sequencing tags (EST), it was demonstrated that many alternatively spliced transcripts were of “low abundance.” Likewise, the proteins coded by these transcripts are probably low in abundance. But these proteins may have transacting alternative ways that can amplify their effect, for
instance, by sequestering nucleic acid binding proteins and reducing their function in gene expression (Blencowe 2006).

**Figure 1.4 Types of alternative splicing.**
The diagram of four major types of alternative splicing. Green squares represent constitutive exons; alternative exons are in yellow; introns are solid lines; and dashed lines indicate splicing options. This figure is modified from Kim E., *et al.*, 2008 with permissions of the author and John Wiley & Sons, Inc. for dissertation of Xi Chen.

Alternative splicing can not only create protein isoforms with distinct biological activities or functions but also can be tissue-specific. Abnormal regulation of alternative splicing has been implicated in several human diseases (Tazi, Bakkour et al. 2009). Aberrant alternative splicing can lead to the incorporation of premature termination codons that are degraded by nonsense-mediated mRNA decay (Lareau, Brooks et al. 2007). Alternatively, they may code for non-functional proteins. While the splicing pathway has been carefully delineated, there is much to be discovered as to how these pathways are regulated in a cell type-specific, developmental stage-specific, gender-
specific, and external stimuli-specific manner (Pajares, Ezponda et al. 2007). To some extent, nonsense-mediated mRNA decay eliminates aberrant transcripts while misregulation of splicing process can also lead to shorter or mis-localized proteins that are rapidly degraded by proteasomes (Pajares, Ezponda et al. 2007). There are also some mutations that disrupt the pre-mRNA splicing and cause human diseases (Faustino and Cooper 2003). Some strategies, like antisense oligonucleotides, have been used to restore wild type splicing in cell culture and animal models which lead to great anticipation for the treatment of human diseases (Dunckley, Manoharan et al. 1998, Friedman, Kole et al. 1999, Sazani, Gemignani et al. 2002, Garcia-Blanco, Baraniak et al. 2004).

1.5.3 Alternatively spliced variants of soluble adenylyl cyclase

The primary sAC transcript, coded by a single gene, undergoes extensive alternative splicing and uses different promoters (Jaiswal and Conti 2001, Geng, Wang et al. 2005, Schmid, Sutto et al. 2007, Farrell, Ramos et al. 2008). Human sAC₃ contains 33 exons and encodes a ~180 kDa protein. Besides the two catalytic domains, the C-terminus of sAC₃ comprises several putative regulatory domains: a canonical P-loop, an autoinhibitory region, and a leucine zipper domain (Buck, Sinclair et al. 1999, Chaloupka, Bullock et al. 2006, Kolodecik, Shugrue et al. 2012). A 50 kDa sAC₁ isoform only contains two catalytic domains. It was initially found in rat testes and demonstrated to be a product of alternative splicing by RT-PCR and RNase protection assay (Jaiswal and Conti 2001). This transcript skipped murine sAC exon 13, shifting the open reading frame and introducing an early stop codon at exon 14. This isoform is more abundant than full-length sAC in rat testes and is 20 times more active in vitro (Wuttke, Buck et al. 2001). Another sAC isoform was discovered in Sacytm1Lex/Sacytm1Lex “KO” mice
While this alternatively spliced isoform starts translation upstream of murine sAC exon 6, the termination site of this variant has yet to be determined. This isoform of sAC is called “somatic sAC” and only contains catalytic domain 2. Despite the missing catalytic domain 1, this isoform is active as tested in the mouse brain. Other groups also reported short alternatively spliced transcripts in different organs and species: For instance, Dr. Geng reported one transcript completely missing exon 5 and one with an extra 37 nucleotides retention from the 3’ end of intron 4 of human sAC in testes (Geng, Wang et al. 2005); Jaiswal reported a complete deletion of exon 11 sAC transcript in mice (Jaiswal and Conti 2001). Despite these variants, there are no reports on a systematic analysis of alternative splicing for sAC in human tissues.

1.5.4 Soluble adenylyl cyclase in normal human bronchial epithelial cells

Given that cAMP can be easily hydrolyzed by phosphodiesterases (PDEs), the diffusion of cAMP is restricted along the apical side of epithelial cells. Furthermore, PDE hydrolysis is not the only determinant for cAMP compartmentalization. Other factors like cell shape, cytoplasm viscosity, and structural impediments may also contribute to it (Murray 2012). Our lab focused first on determining if sAC was expressed in cilia of normal human bronchial epithelial (NHBE) cells and if sAC activity could be detected by HCO₃⁻/CO₂ stimulation.

Three alternatively spliced sAC transcripts were identified by RT-PCR in NHBE cells, re-differentiated at the air-liquid interface (ALI) with a forward primer hybridizing to exon 3 and a reverse primer spanning the exon 5 to exon 6 junction (Schmid, Sutto et al. 2007). Sequencing alignment confirmed that all of the three RT-PCR amplimers were human sAC (Schmid, Sutto et al. 2007). One variant demonstrated the canonical pattern
of splicing while the other two retained different portions of the originally named intron 4. One of the alternatively spliced transcripts contained 49 nucleotides of the 3’ end of intron 4 and the other one 37 nucleotides. Both of these transcripts introduce a new translation initiation codon in intron 4 that encodes the amino acid sequence MSLSE, but does not cause shift in the reading frame of exon 5. To detect the expression of human sAC protein in NHBE cells, Western blot analysis was conducted using a sAC specific antibody (a gift from Dr. Geng) raised against a peptide sequence encoded in original intron 4 and exon 5 (SLSEGDALA) (Geng, Wang et al. 2005). Three specific bands of 180 kDa, 75 kDa, and 50 kDa were observed. After separating cilia from the cell body, the 50 kDa band was found specifically in cilia. Immunochemistry with another sAC antibody (R41) targeting the C2 domain found sAC localized to cilia of NHBE cells (Schmid, Sutto et al. 2007). These observations confirmed the presence of sAC in NHBE cells.

Next, the function of sAC in regulating CBF was examined by changing [HCO₃⁻], in the presence or absence of sAC specific inhibitor KH7. Fully differentiated NHBE cells cultured at ALI were exposed to apical changes in HCO₃⁻/CO₂. An initial decrease in CBF was detected due to acidification inside the cells from CO₂. However, this decrease was partially ameliorated by sAC activation, evidenced by the further decrease of CBF in the presence of KH7 (Schmid, Sutto et al. 2007). These data confirmed the expression of sAC in NHBE cells and involvement of sAC in the regulation of CBF. We also found the presence of other alternatively spliced sAC transcripts in NHBE cells, as yet unreported in other species and human tissues.
Based on these evidences, we hypothesize that alternatively spliced sAC isoforms can be found in NHBE cells where some may localize at the axoneme of cilia, regulating ciliary beating in human airways. To determine the ciliary specific sAC isoform and its role in CBF regulation, the following aims were performed:

1) To systematically identify sAC alternatively spliced variants in human airway epithelial cells and

2) To identify specific sAC variants that localize to cilia and regulate ciliary beating in human airways.
Chapter 2 Materials and Methods

2.1 Human and murine primary airway epithelial cell cultures

NHBE cells were isolated from healthy lung donors whose lungs were rejected for transplant by the University of Miami Life Alliance Organ Recovery Agency and conformed to the declaration of Helsinki. Airway epithelial cells were isolated by protease, expanded in serum-free bronchial epithelial growth media (BEGM) in collagen I coated petri dish. Passage 1 cells were re-differentiated into ciliated, goblet, and basal cells in ALI medium on 12 mm/24 mm Transwell-clear filter inserts (Corning costar Corporation, Corning, NY) coated with human placental collagen type IV (Bernacki, Nelson et al. 1999, Nlend, Bookman et al. 2002). These cultures started transporting mucus across the surface within four weeks of air exposure.

Mouse tracheal epithelial cells (mTECs) were isolated and cultured according to published protocol (You and Brody 2013). Briefly, tracheas from wild type (WT) (C57/BL6), sAC C\textsubscript{1} KO, and C\textsubscript{2} KO mice (Chen, Martinez et al. 2013) were isolated and plated on collagen IV coated Transwell filters in the presence of Rho-associated protein kinase (ROCK) inhibitor Y27632 (5µM) (Horani, Nath et al. 2013). Some of sAC C\textsubscript{1} or C\textsubscript{2} KO mTECs (Chen, Martinez et al. 2013) were transduced with different sAC constructs, expressed through lentiviruses, on the day of plating on filters. These cells were proliferated on the filters in mouse tracheal epithelial cell basic media until becoming confluent when media were changed to serum free mTEC media. About two weeks after being exposed to air, the cells were fully differentiated.
2.2 mRNA extraction, reverse transcription-polymerase chain reaction, and sequencing

Total RNA was extracted from fully differentiated NHBE cells with the RNeasy plus mini kit (Qiagen, Valencia, CA) followed by poly (A)^+ Tract mRNA isolation (Promega, Madison, WI). Reverse transcription was carried out from mRNA with oligo (dT)\textsubscript{20} primer using the Superscript III first-strand synthesis system (Life Technologies, Grand Island, NY) according to the manufacturer’s manual. cDNA was then purified with the Qiaquick PCR purification kit (Qiagen, Valencia, CA) due to low expression levels of sAC in NHBE cells. PCR reactions were performed with platinum Taq DNA polymerase high fidelity (Life Technologies, Grand Island, NY) using specific primer pairs designed by primer premier 5 software (Premier Biosoft, Palo Alto, CA) complimentary to exons of the full-length human sAC sequence (Genbank/EMBL/DDBJ accession no. NM_018417). The sequences of all primers used in this study are listed in Table 2.1. Amplified PCR fragments were visualized by electrophoresis on Seakem LE agarose (Lonza, Rockland, ME). PCR products were purified by the Qiaquick PCR purification kit (Qiagen, Valencia, CA), subcloned into the pGEM-Teasy vector (Promega, Madison, WI) and sequenced at the University of Miami Oncogenomic core facility. The sequencing results were analyzed by vector NTI (Invitrogen, Grand Island, NY).

Table 2.1 sAC primer pair sequences of PCR for alternatively spliced transcripts analysis

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>Primer Name</th>
<th>5’ position</th>
<th>Primer Sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fex2~Rex33</td>
<td>Fexon2</td>
<td>386</td>
<td>TCCCCAGAGCGACCCTTTATG</td>
</tr>
<tr>
<td></td>
<td>Rexon33</td>
<td>5068</td>
<td>GTTTACCGCCTGCTACAAT</td>
</tr>
<tr>
<td>Fex2A~Rex32A</td>
<td>Fexon2A</td>
<td>379</td>
<td>ACATTTCTCCCCAGAGCGACCCCT</td>
</tr>
<tr>
<td></td>
<td>Rexon32A</td>
<td>4916</td>
<td>GCCGCAAGGTGTGTTCAGGA</td>
</tr>
<tr>
<td>Fex2L~Rex27</td>
<td>Fexon2L</td>
<td>387</td>
<td>CCCAGAGCGACCCCTTTATG</td>
</tr>
<tr>
<td>Fex2L~Rex23</td>
<td>Rexon27</td>
<td>4184</td>
<td>CCACGATTTCAATGCCCCT</td>
</tr>
<tr>
<td></td>
<td>Rexon23</td>
<td>3515</td>
<td>GGGCCAGAGGCAAGATG</td>
</tr>
</tbody>
</table>
2.3 Sybr green real-time PCR

Total RNA was extracted from NHBE cells cultured at the ALI condition during different differentiation stages on air. cDNAs were synthesized with random hexamer primers using the iscript select cDNA synthesis kit (Bio-Rad, Hercules, CA). Sybr green real-time PCR (iTaq Sybr Green Universal, Bio-Rad, Hercules, CA) was performed with the forward primer designed specifically for the retained intron 4 sequence 5’-GGCATGTCTCTCTCTGAAGGT-3’ and reverse primer targeting at exon 6 sequence 5’-GTCCACTGCCTGACCAATCA-3’, annealed at 56°C for 40 cycles.

2.4 Cloning of sAC variants into lentivirus vectors

Human sAC variants were expressed using the pCDH-EF1-MCS-T2A-copGFP (CD526A-1, System Bioscience, Mountain View, CA) lentivirus vector. Origene hsAC\_fl plasmid CMV\_6-sAC-flag/DDK (RC214876, Origene, Rockville, MD) was used as the PCR template and cloned into the lentivirus vector with human influenza hemagglutinin (HA) (N-terminus) and flag/DDK (C-terminus) tags. sAC\_fl was amplified by platinum Taq DNA polymerase high fidelity (Life Technologies) with primer pairs listed in Table 2.2.
Table 2.2 Primer sequences for cloning of sAC alternatively spliced variants

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>sACex5v2-ex12v2 1st F</td>
<td>CGATGTGCCGGATTATGCTATGCTCTCTCTCTGAAGG</td>
</tr>
<tr>
<td>sACex5v2-ex12v2 1st R</td>
<td>CGTCATCTTTGTAAATCCTTCTCTCTCTGAAGG</td>
</tr>
<tr>
<td>sACex5v2-ex12v2 2nd F</td>
<td>CGCTTAGAGCCACCATGTATCCATACCGATGTGCCGGATTAT</td>
</tr>
<tr>
<td>sACex5v2-ex12v2 2nd R</td>
<td>CGCGGATCCCCTATCGTCGTCATCCCTTTGTAATC</td>
</tr>
<tr>
<td>sACex2-ex5 1st F</td>
<td>GATGTGCCGGATTATGCTATGACACTCCAAAAGAAGG</td>
</tr>
<tr>
<td>sACex2-ex5 (NheI) R</td>
<td>GGGGCTAGCAGTGCATCACCTGCAAATTTC</td>
</tr>
<tr>
<td>sACex2-ex5 2nd F</td>
<td>CGCTTAGAGCCACCATGTATCCATACCGATGTGCCGGATTAT</td>
</tr>
<tr>
<td>sACfl 1st F</td>
<td>CGATGTGCCGGATTATGCTATGACACTCCAAAAGAAGG</td>
</tr>
<tr>
<td>sACfl 2nd F</td>
<td>CGCTTAGAGCCACCATGTATCCATACCGATGTGCCGGATTAT</td>
</tr>
<tr>
<td>sACfl R</td>
<td>CGCGGATCCCTATCGTCGTCATCCCTTTGTAATC</td>
</tr>
<tr>
<td>sACex2-ex7 F</td>
<td>CGCTTAGAGCCACCATGTATCCATACCGATGTGCCGGATTAT</td>
</tr>
<tr>
<td>sACex2-ex7 1st R</td>
<td>CGTCATCTTTGTAAATCCTTCTCTCTCTGAAGG</td>
</tr>
<tr>
<td>sACex2-ex7 2nd R</td>
<td>CGCGGATCCCTATCGTCGTCATCCCTTTGTAATC</td>
</tr>
</tbody>
</table>

F, forward primer; R, reverse primer
Bold letters: hemagglutinin (HA) tag sequence; Bold italic letters: flag/DDK tag sequence
Underlined letters: restriction site sequences

PCR products were digested with BamHI and XbaI enzymes, ligated with the vector, and transformed into NEB 5-αF'βIq competent E.coli. The HA-sACex5v2-ex12v2-flag/DDK was generated by nested PCR reactions using primer pairs listed in Table 2.2 and RT-PCR products were used as the template for the insert. Following double digestions with BamHI and XbaI, PCR products were ligated into lentivirus vectors. sACex2v2-ex12v2 insert was amplified firstly with the sACex2-5 1st F and sACex2-5 R primer pair, using the Origene sACfl plasmid as template. A second round of PCR was performed with primer pair sACex2-5 (NheI) 2nd F and sACex2-5 R. Then this PCR product was digested by
BamHI and NheI and ligated to the NheI and XbaI double digested pCDH-EF<sub>1</sub>-HA-sAC<sub>ex5v2-ex12v2</sub>-flag/DDK-T<sub>2</sub>A-copGFP construct. The pCDH-EF<sub>1</sub>-HA-sAC<sub>ex2-in7</sub>-flag/DDK-T<sub>2</sub>A-GFP construct was produced by nested PCR with primers sAC<sub>ex2-7</sub> F, sAC<sub>ex2-7</sub> 1<sup>st</sup>, 2nd R and ligated to the BamHI as well as XbaI digested lentivirus vector. All cloned plasmids were confirmed by sequencing with the EF<sub>1</sub> promoter primer and GFP primer individually.

2.5 In vitro cyclase assays

HEK293T cells were transfected with recombinant sAC variants by calcium phosphate precipitation. Forty eight hours after transfection, cells were lysed with cold buffer: 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, and protease inhibitors (Roche, Indianapolis, IN). After sonication, crude extract of the cell lysate was centrifuged at 10,000g for 10 minutes to get rid of cellular debris. Cell lysate (25 µg) was added in the 100 µl reaction mixture containing 200 mM Tris-HCl, pH 7.5, 20 mM Creatine Phosphate, 3 mM DTT, 0.5 mM IBMX, 100 U/ml phosphocreatine kinase, 2.5 mM ATP, and protease inhibitors with or without either 40 mM NaHCO<sub>3</sub> or 50 µM KH7. The reactions were incubated at 30°C for 30 minutes and stopped by adding 100 µl of 0.2 N HCl. cAMP produced from the reaction was measured with the Correlate-EIA Direct cAMP Enzyme Immunoassay kit (Enzo life science, Farmingdale, NY) following manufacturer’s protocol. Data were analyzed using Prism, Version 5.0 b (GraphPad Software, Inc., La Jolla, CA).
2.6 Lentivirus production and transduction of human and mouse bronchial epithelial cells

Third generation, replication-deficient HIV-pseudotyped lentivirus was packaged in HEK293T cells. Forty-eight hours after transfection, virus was collected at twenty-four hours intervals for two days. Virus was concentrated with 40% PEG precipitation and transduced to NHBE cells in BEGM media directly unto filter inserts. Twenty-four hours post transduction, BEGM media was replaced by ALI media and cells were re-differentiated at the ALI as described above.

2.7 Immunoprecipitation and catalytic activity assay in NHBE cells

Cell lysate from fully differentiated NHBE cells transduced with sAC lentivirus constructs was precleared with protein G sepharose 4 fast flow (GE Health, Pittsburgh, PA) for 2 hours. The cleared cell lysate was incubated with 2.5 µg per sample of the Origene mouse flag/DDK antibody by rotating overnight at 4°C. Mouse IgG antibody was used as a control. After centrifugation and washing, the beads were incubated with the reaction buffer for 30 minutes, followed by adenylyl cyclase activity measurements as described above.

2.8 Western blot

HEK293T cells transfected with recombinant sAC variants were lysed after the in vitro cyclase assay. Proteins were separated by SDS-PAGE with 7.5% MiniProtean precast gels (Bio-Rad, Hercules, CA) and transferred to PVDF membrane. The membrane was blocked with 5% non-fat dry milk power in 0.05% TTBS buffer, followed by blotting with mouse anti-sAC R21 antibody at 1:1000 (CEP biotech, Orlando, FL) in TTBS buffer at 4°C overnight. Chemiluminescence was used to detect the protein with a
Chemidoc XRS system (Bio-Rad, Hercules, CA). The membrane was then stripped with the Restore Western blot stripping buffer (Thermo Fisher Scientific, Rockford, IL) and blotted with mouse anti-β-actin antibody (1:5000, Sigma-Aldrich, St. Louis, MO). Images were quantified by QuantityOne analysis software (Bio-Rad, Hercules, CA).

2.9 Cytospin and immunostaining

Fully differentiated NHBE cells or mTECs at ALI culture were gently washed once with DPBS and trypsinized for 5 minutes to dissociate the cells. Following the addition of soybean trypsin inhibitor to stop trypsinization, cells were diluted 30 fold with PBS before being applied to a slide using cytospin at 1000 rpm for 1 minute. Once the slides were dry, cells were fixed with 4% formaldehyde for 30 minutes followed by permeabilization with 0.1% Triton X-100 for 10 minutes. After blocking cells with 1% BSA at room temperature for 1 hour, the slides were incubated with a monoclonal mouse HA antibody (1:500, Cell Signaling, Danvers, MA) and a rabbit anti-acetylated tubulin antibody (1:800, Cell Signaling, Danvers, MA) at 4 °C overnight. Secondary antibody (goat) coupled to Alexa 555 (1:1000, Invitrogen, Grand Island, NY) was used to develop HA. Alexa 647 (1:2000, Invitrogen, Grand Island, NY) was used to develop acetylated tubulin. DAPI (1:500, Invitrogen, Grand Island, NY) was applied after the last wash and incubated at room temperature for 20 minutes. Images were taken on a Zeiss LSM700 confocal microscope with a 63x oil objective for NHBE cells and C2 KO mTECs. Images for C1 KO mouse cells were taken with a Zeiss Axiovert 200M microscope with a 40x objective.
2.10 Ciliary beat frequency measurement

Fully differentiated mTECs on Transwell filters were mounted in a closed chamber (Warner Instruments RC20H, Hamden, CT), perfused apically first with HEPES buffered Hanks’ balanced salt solution (Table 2.3) and then 25 mM HCO\text{3}\textsuperscript{−}/5% CO\texttextsubscript{2} with or without KH\texttextsubscript{7} (25 µM). CBF was measured on the stage of a Nikon E600fn microscope with a 63x water immersion objective. The light path was directed to a charge-coupled device (CCD) video camera. A box of three by three pixels from the live, digitized, contrast-enhanced video image was selected (Salathe and Bookman 1999). The light intensity of each pixel signal was computed with a fast Fourier transform and the magnitude spectrum was displayed (Salathe and Bookman 1999). Eight regions in one filter were selected to get enough samples for statistical analysis. Data were filtered with IgorPro software (WaveMetrics, Inc., Lake Oswego, OR, USA) and statistically analyzed with Prism (GraphPad Software, Inc., La Jolla, CA). Multiple groups were compared using one-way ANOVA followed by Neuman Keuls. Two groups were compared using Student’s T-test.

Table 2.3 Composition of solutions for CBF measurement (in mM)

<table>
<thead>
<tr>
<th></th>
<th>0 mM HCO\text{3}\textsuperscript{−}</th>
<th>25 mM HCO\text{3}\textsuperscript{−}</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>117</td>
<td>117</td>
</tr>
<tr>
<td>KCl</td>
<td>5.3</td>
<td>5.3</td>
</tr>
<tr>
<td>Na-gluconate</td>
<td>25</td>
<td>-</td>
</tr>
<tr>
<td>NaHCO\text{3}</td>
<td>-</td>
<td>25</td>
</tr>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>----------------</td>
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</tr>
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<td>CaCl₂</td>
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<td>1.3</td>
</tr>
<tr>
<td>MgCl₂</td>
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Chapter 3 Identification of Alternatively Spliced sAC Transcripts in NHBE Cells and Determination of Cyclase Activity of sAC Isoforms in Vitro

3.1 Introductory Remarks

Several alternatively spliced sAC variants have been identified in various organisms and organs, suggesting that sAC has undergone extensive alternative splicing. The three well-known alternatively spliced isoforms of sAC were found in mice but nothing has been reported in human tissues. In NHBE cells, the three identified alternatively spliced sAC transcripts (Schmid, Sutto et al. 2007) are different from the ones in other tissues and have not been reported previously. Moreover in western blot with the specific sAC antibody (epitope SLSEGDALLA), there are three different sized bands shown in fully differentiated NHBE cell lysate. Therefore we hypothesize that there are alternatively spliced variants of sAC in NHBE cells. This hypothesis also brings the novelty of investigating alternatively spliced sAC variants in NHBE cells. However being amplified by RT-PCR with primer pairs from exons 3 to 6, these transcripts are only short fragments of the full-length sAC message which could not provide enough information to predict the presence of sAC isoforms in NHBE cells. Therefore in this study we aim to acquire a further understanding of sAC alternative splicing events in NHBE cells. In general, there are several approaches to analyzing the alternative splicing in a tissue, including RT-PCR, microarray, expressed sequencing tags (EST), rapid amplification of cDNA ends (RACE), and next generation sequencing. This study focused on using RT-PCR by extracting RNA from ALI cultured, fully differentiated NHBE cells. Meanwhile, the recombinant alternatively spliced sAC transcripts were expressed in HEK293T and NHBE cells in order to examine the cyclase activity in vitro.
3.2 Results

3.2.1 Identification of alternatively spliced sAC transcripts in NHBE cells

3.2.1.1 Short length sAC RT-PCR products with cDNA synthesized from total RNA of NHBE cells

Full-length human sAC initiates translation from exon 2 and stops at exon 33. Since little has been known about alternative splicing of human sAC, RT-PCR was performed with primer pairs from exon 2 coupled with different reverse primers targeting at exons 6, 8, 10, and 12, which produced amplimers less than 1.0 kb. There were some different variants discovered in lungs from different donors, which made the analysis more complex. Consistent splice sites, however, were found in exons 3 and 5 with different combinations at the 3’ ends of the transcripts.

![Diagram of sAC RT-PCR products](image)
Figure 3.1 Identification of alternatively spliced soluble adenylyl cyclase transcripts in normal human bronchial epithelial cells with primer pairs of exons 2-6 and exons 2-8.

A. 1% DNA agarose gel image of PCR products with primer pairs at exons 2-6 and exons 2-8 using cDNA synthesized from total RNA of fully differentiated normal human bronchial epithelial (NHBE) cells. The cloned full-length soluble adenylyl cyclase (sAC\textsubscript{FL}) plasmid was used as a positive control. The expected size of the plasmid control for primer pair exons 2-6 is ~550 bp and for exons 2-8 is 700 bp. Three bands were detected at the following sizes from exons 2-6: 480 bp, 440 bp, and 300 bp. With primer pair of exons 2-8, three bands were detected at sizes of 386 bp, 448 bp, and 700 bp. The bands larger than the plasmid amplifier were not sAC by sequencing. B. Schematic exon map showing sequencing alignment of PCR products with primer pairs exons of 2-6 and exons 2-8. Purple box indicates a combination of exon\textsubscript{5v1} and a portion of previously identified 3’ end of intron 4 sequence, now named exon\textsubscript{5v2}. Included exons are represented by the green boxes and excluded exons are denoted by the yellow dashed boxes. Black arrows below the images indicate the open reading frame of the predicted protein when there is no stop codon found within the range. White dashed bars indicate deleted amino acids encoded by corresponding excluded exons above.

Figure 3.2 Identification of alternatively spliced sAC transcripts in NHBE cells with a primer pair spanning exons 2-10.

A. 1% DNA agarose gel image of PCR products with primer pair of exons 2-10 using cDNA synthesized from total RNA of fully differentiated NHBE cells. Four major bands
were found at the following sizes: 713 bp, 818 bp, and 893 bp. The bands at 400 bp and 1000 bp are not sAC by sequencing. B. Schematic map of primer pair from exons 2 to 10 based on the sequencing results of the cloned PCR products in *E. coli*. Boxes and bars are designated the same as in Figure 3.1.

Figure 3.3 Identification of alternatively spliced sAC transcripts in NHBE cells with primer pairs of exons 2-10 and exons 2-12 from different lung donor.
A. NHBE cells from a different lung donor as in Figures 3.1 & 3.2 were analyzed by reverse transcription-polymerase chain reaction (RT-PCR). This is 1% DNA agarose gel image of PCR products from exons 2 to 10 and from exons 2 to 12. The expected size of full-length sAC plasmid control from exons 2 to 12 is ~1.2 kb. PCR products were used for TA-cloning and sequencing. Some sAC bands are almost invisible in the gel image. B. Schematic map showing sequencing results of PCR products. Two alternatively spliced transcripts were detected from exon 2 to exon 10 and four transcripts from exon 2 to exon 12. Boxes and colors are the same as in Figure 3.1. Black bars above the boxes represent the catalytic domains.

The sequencing analysis suggested that exons 3 and 5 were spliced out in most of the transcripts, encoding sAC isoforms without a complete $C_1$ domain. One alternatively
spliced transcript had the previously identified 3’ sequence of intron 4, creating a new translation start codon. The resultant isoform contains only a partial C1 domain.

Considering the efficiency of RT-PCR for sACn (5.0 kb), RACE was attempted to investigate alternatively spliced transcripts in NHBE cells (Appendix A). However, sequencing alignments of RACE products did not reveal any sAC transcripts. This could be due to technical limitations of the primer pairs for PCR supplied by the Marathon RACE kit. Hence, RT-PCR was used as the means to identify alternatively spliced transcripts with primer pairs designed to amplify increasingly longer sequences (Figure 3.6).

More RT-PCR reactions were performed with a 5’ primer designed to hybridize to exon 2 as well as different 3’ primers designed to hybridize to exons 12, 13, and 20. However, it was increasingly difficult and no amplification products were observed when the expected sAC products were longer than 1.0 kb (Appendix B). This problem occurred in multiple lungs and became an obstacle to investigating long alternatively spliced sAC transcripts in NHBE cells. An alternative strategy was to check separate fragments of sACn by PCR with primer pairs designed to hybridize to exons 12-17, exons 20-26 (Figure 3.4), and exons 26-32 (Figure 3.5). All expected sizes of RT-PCR products from the above primer pairs were less than 1.0 kb. This strategy spanned almost all exons of human sAC. However, it was impossible to reliably predict different alternatively spliced isoforms of sAC from these combinations. Therefore, it was imperative to optimize the RT-PCR protocol to get longer products (Appendix C).
Figure 3.4 Identification of alternatively spliced sAC transcripts in NHBE cells with primer pairs of exons 12-17 and exons 20-26.
A. 1% DNA agarose gel image of PCR products from primer pairs of exons 12-17 and exons 20-26 with cDNA synthesized from one lung donor. There are three bands shown from exons 12 to 17 at sizes of 736 bp, 457 bp, and 302 bp. Only one band was detected with primers pair of exons 20-26 at 962 bp. Human sAC² plasmid was used as the PCR positive control. B. Schematic map of sequenced transcripts from bands shown in A. Designation of boxes and colors are the same as in Figure 3.1.
Figure 3.5 Identification of alternatively spliced sAC transcripts in NHBE cells with primer pair of exons 26-32.
A. 1% DNA agarose gel image of PCR products from primers at exons 26-32 using cDNA synthesized from total RNA of three lung donors. There is only one band detected in the gel. This transcript is full-length sAC within the frame by sequencing. The amplification of this product is consistent in different lung donors. Human sAC₇₅ plasmid was used as the positive control for PCR. B. Schematic map of sequenced transcript from the band in DNA gel image. Boxes and colors are indicated the same as in Figure 3.1.

3.2.1.2 Full-length sAC RT-PCR with cDNA synthesized from mRNA of NHBE cells

With the drastic improvement of sAC RT-PCR in NHBE cells after optimization (Appendix C), this approach was used to amplify longer RT-PCR products, including the 5.0 kb sAC₇₅ (Figure 3.6).

Figure 3.6 Full-length human sAC RT-PCR products in NHBE cells.
1% DNA agarose gel images of RT-PCR products. A. cDNA from two lung donors (NHBE384, NHBE423) were column purified before PCR. There is only one band shown in the gel that is the size of sAC₇₅. Sequencing also confirmed full-length human sAC. B. The same purified cDNA from A was amplified with primer pair of exons 2-31 and resulted in only one full length sAC transcript after sequencing. C. PCR products with purified cDNA from a lung donor (NHBE387) are shown in the agarose gel with primer pair of exons 2-32. Besides sAC₇₅, the lower 4.0 kb band was sequenced and found to be
an alternatively spliced sAC transcript. With primer pair of exons 2-30, only sAC\textsubscript{n} was amplified. The other bands seen in the gel (not pointed by the arrows) are not sAC by sequencing.

3.2.2 Summary of alternatively spliced human sAC transcripts in NHBE cells

After optimization of RT-PCR for full-length sAC, more primer pairs were designed at different positions to amplify PCR fragments from exons 2 to 27, exons 2 to 23, exons 2 to 20, etc using cells from different lung donors. The sequencing results were analyzed and grouped according to splicing patterns.

**Figure 3.7 Groups of alternatively spliced sAC transcripts identified in NHBE cells.** The exon composition of alternatively spliced sAC mRNAs is diagrammed showing the included exons (green boxes), excluded exons (yellow boxes), and newly identified exons (containing previously identified intron sequences retention; purple boxes). Top: sAC\textsubscript{n} mRNA containing coding exons from 2 to 33 is shown with the locations of the two catalytic domains (C\textsubscript{1} and C\textsubscript{2}; black bars above their coding exons 2-7 and 9-12,
respectively). Full-length or C1 and C2 containing splice variant represent 24% of the found forms. The black line below the mRNA indicates the open reading frame. Alternatively spliced transcripts identified by RT-PCR with primers from exon 2 to exons 20 or 23 are summarized into two groups. Group I contains mRNAs that delete portions of the C1 coding region. Thus, the predicted proteins do not contain a complete C1 but maintain the correct reading frame through C2 domain as indicated by the black arrow below. Group I variants made up 63% of the found forms. The dotted black line indicates the putative remainder of the mRNA. Group II contains transcripts that retain a portion of previously annotated intron 4 (now exon 5v2), introducing a stop codon and new translation initiation codon that is in frame with the sAC coding sequence and thus encodes a truncated C1 and complete C2. Group II variants made up 13% of the found forms. One of these mRNAs (bottom) also includes a portion of what was formerly annotated as intron 12 (new exon 12v2), introducing a new stop codon and encoding a ~45 kDa protein. Figure and legend are reprinted with permission of the American Thoracic Society. Copyright © 2014 American Thoracic Society (Chen, Baumlin et al. 2014).

Among the previously identified three alternatively spliced sAC transcripts in NHBE cells using primers specific for exons 3 and 6, there are two retained portions of the 3’ end of the previously identified intron 4, creating new versions of exon 5 (ex5v2 and ex5v3). These two introduce a new in-frame translation start codon which is 16 bases upstream of the originally identified exon 5, adding the amino acid sequence MSLSE to the N terminus and encoding a protein that misses part of C1. Using an extensive RT-PCR approach with different combinations of specific primers through 33 exons of human sAC, more alternatively spliced transcripts were found in fully differentiated NHBE cells.

Sequencing analysis of RT-PCR products indicated that the C1 coding region was the major site of alternative splicing. From over eighty sequencing results (Appendix D) using various combinations of exon-specific primers (Table 2.1) and eight different human lung donors, alternatively spliced variants could be assigned to sACβ along with two additional groups. Group I contained variants with C1 deletions (i.e., skipping exon 5 or exons 3 and 5) and Group II started translation from a partially retained intron 4 (16
nucleotides from the 3’ end of previously annotated intron 4). Given our expression data and the previous Western blots, this sequence is expressed and we therefore label it exon5v2. One of these variants stopped at a premature stop codon in a second intron retention in the previously labeled intron 12, now called exon 12v2 (sAC_{ex5v2-ex12v2}, Figure 3.7). The predicted molecular size of the protein encoded by sAC_{ex5v2-ex12v2} is ~45 kDa, similar to the molecular size of the form identified in cilia using an anti-sAC antibody that recognizes the unique start site encoded by exon 5v2.

### 3.2.3 Construction and expression of alternatively spliced sAC isoforms

The sAC_{ex5v2-ex12v2} variant represents the typical features of alternatively spliced sAC variants found in NHBE cells: an incomplete C1 domain with a new translation start codon within the 3’ end of a previously labeled intron 4 sequence and a complete C2 domain. This isoform has not been reported before. Thus, its catalytic activity was examined by cloning it into the lentivirus vector, pCDH-EF1-MCS-T2A-coGFP. Lentiviruses were used because NHBE cells are recalcitrant to transfection. sAC_{ex2-ex12v2} is the isoform that starts translation from the original start codon in exon 2 and ends in the newly identified stop codon in exon 12v2. This sAC isoform comprises only complete C1 and C2 domains, similar to the sACt reported in rat testes. The C1 only variant (sAC_{ex2-in7}) was constructed also for catalytic activity comparison. To facilitate immunoprecipitation (IP) and/or detection by Western blot or immunofluorescence, all constructs were tagged with HA at the 5’ end and flag/DDK at the 3’ end. These constructs were transfected into HEK293T cells followed by Western blotting using the previously reported sAC R21 antibody (epitope at human sAC exon 6) (Figure 3.8). Lentiviruses were packaged in HEK293T cells and then transduced to de-differentiated
NHBE cells. The cells were allowed to differentiate on Transwell filters using ALI conditions. Due to the low expression levels of such proteins in NHBE cells, these sAC variants had to be concentrated by IP before being detected by Western blot (Figure 3.9). The relative intensity of these bands on Western blot was later used for enzymatic activity normalization.

Figure 3.8 Expression of recombinant sAC isoforms in HEK293T cells. Diagrams of lentivirus constructs of sAC variants are shown on the left. The expression of these sAC variants was tested in HEK293T cells. Forty-eight hours after transfection, equal amounts of protein from whole cell lysates were separated via SDS-PAGE, transferred to a PVDF membrane, and probed with anti-sAC R21 antibody. The blot was stripped and re-probed for β-actin (bottom blot) to quantify relative amount of expression of each variant. Corresponding band of each variant in Western blot is shown on the right. Purple boxes indicate a combination of the original exon and a portion of the previously identified intron retention. Included exons are in green boxes. Grey box indicates the
originally called intron 7 sequence, which also initiates a stop codon. Black bars show the catalytic domains of sAC while white dashed bars indicate the missing part of catalytic domains. Figure and legend are modified and reprinted with permission of the American Thoracic Society. Copyright © 2014 American Thoracic Society (Chen, Baumlin et al. 2014).

Figure 3.9 Expression of recombinant sAC isoforms in NHBE cells.
Undifferentiated NHBE cells were transduced with lentiviruses driving the expression of human sACfl and other sAC variants (shown on the left). When fully differentiated, cells were lysed with RIPA buffer containing protease inhibitors and immunoprecipitated with a mouse flag/DDK antibody coated (cell lysates alone had insufficient sAC activity for analysis) sepharose beads. Aliquots of the immunoprecipitated protein were run on SDS-PAGE, transferred to a PVDF membrane, and probed with a biotinylated anti-sAC R21 antibody followed by HRP-streptavidin as background control after stripping. Colors for the boxes on the left are indicated the same as in Figure 3.8. Figure and legend are modified and reprinted with permission of the American Thoracic Society. Copyright © 2014 American Thoracic Society (Chen, Baumlin et al. 2014).

3.2.4 In vitro cyclase activity of recombinant sAC alternatively spliced isoforms

3.2.4.1 In vitro cyclase activity of recombinant sAC alternatively spliced isoforms expressed in HEK293T cells

In order to understand whether human sAC isoform in NHBE cells with incomplete C1 and complete C2 is active or not, the catalytic activities of these constructed sAC
variants were compared in HEK293T cells by in vitro cyclase activity assay. As described earlier, transient transfection of these sAC variants into HEK293T cells leads to readily detectable expression by Western blot. Without enrichment of sAC by IP (Method 2.5), in vitro cyclase activity assay allows direct measurement of cAMP in the presence of the reaction reagents. In separate experiments, the specific sAC inhibitor, KH7 (Bitterman, Ramos-Espiritu et al. 2013) was added to the reaction to confirm the specificity of sAC activity (Method 2.5). Figure 3.10 shows the catalytic activity of sAC variants expressed in HEK293T cells.

**Figure 3.10 In vitro cyclase activity assay of recombinant sAC isoforms expressed in HEK293T cells.**
Cyclase activity was measured in vitro using a cAMP assay of the transfected cell lysates. White bars show the basal level of activity, black bars show activity in the presence of 40 mM HCO$_3^-$ and grey bars show activity in the presence of 40 mM HCO$_3^-$ plus 50 μM KH7. Activity was normalized to the expression levels of sAC variants by measuring the intensity of each band in the Western blot image with QuantityOne software (Figure 3.8). Data are shown as mean ± S.E.M. from 3 experiments done as triplicates. Different variants were compared with the mock transfected negative control using one-way ANOVA followed by Dunnett’s multiple comparison test. * indicates significantly difference of catalytic activity under HCO$_3^-$ stimulation to mock transfected HEK293T cells. The figure and legend are modified and reprinted with permission of the American Thoracic Society. Copyright © 2014 American Thoracic Society (Chen, Baumlin et al.)
HA-sAC<sub>ex5v2-ex12v2</sub>-flag/DDK variant in HEK293T cells showed almost no catalytic activity and so did the variant of HA-sAC<sub>ex2-in7</sub>-flag/DDK. Truncated sAC (HA-sAC<sub>ex2-ex12v2</sub>-flag/DDK) and sAC<sub>fl</sub> demonstrated significant catalytic activity, shown by HCO<sub>3</sub>⁻ stimulation and its inhibition by 50 μM KH7 (Figure 3.10). The catalytic activity of sAC<sub>ex2-ex12v2</sub> was about three times higher than that of sAC<sub>fl</sub>, consistent with the previous report that truncated sAC is more active than the full-length sAC (Chen, Cann et al. 2000).

3.2.4.2 In vitro cyclase activity of recombinant sAC alternatively spliced isoforms in NHBE cells

To determine the catalytic activity of sAC isoforms in NHBE cells, lentiviral constructs expressing these isoforms were used to transduce de-differentiated NHBE cells and fully differentiated at the ALI culture. Since the expression level of sAC isoforms was low in NHBE cultures, they were enriched by immunoprecipitation with the mouse flag/DDK antibody from combined several NHBE cultures. Because the detergents used in the immunoprecipitation protocol were known to suppress the inhibitory effect of KH7 on sAC (Farrell, Ramos et al. 2008), KH7 was not used in these activity assays. However, the specificity of sAC catalytic activity was deduced from its unique property of being stimulated by bicarbonate. sAC<sub>fl</sub> lentivirus was difficult to package and the transduction efficiency was also low though a large amount of NHBE cells were used. There are only two complete biological experiments performed with all the constructed sAC variants tested together which is not applicable for statistical analysis. But the catalytic activity of
immunoprecipitated sAC isoforms in NHBE cells has the similar tendency of the data obtained with lysates from HEK293T cells (Figure 3.11).

![NHBE Cells](image)

**Figure 3.11** *In vitro* cyclase activity assay of recombinant sAC isoforms expressed in NHBE cells.

*In vitro* cyclase activity was measured from sAC isoforms expressed in NHBE cells after immunoprecipitation with mouse flag/DDK antibody coated sepharose G beads (cell lysates could not be used directly since expression levels were insufficient to reliably measure cyclase activity). White bars show the basal level of activity and black bars indicate activity in the presence of 40 mM HCO$_3^-$ . The activity was normalized to the expression levels of sAC variants according to the Western blot data (Figure 3.9). Data are shown as mean ± S.E.M. from triplicates in one biological replicate due to the difficulty of transducing full-length sAC lentivirus in NHBE cells (two biological experiments were performed with all variants tested together). Figure and legend are modified and reprinted with permission of the American Thoracic Society. Copyright © 2014 American Thoracic Society (Chen, Baumlin et al. 2014).

### 3.3 Conclusion

sAC isoforms encoded by alternatively spliced transcripts in NHBE cells are characterized by comprising of partial C$_1$ and complete C$_2$ domains. These results are different from the reported C$_2$ only variants as the somatic sAC found in Sacytm1Lex/Sacytm1Lex “KO” mice. The major distinction lies in the retained part of
sAC $C_1$ domain, especially the newly identified translation start codon encoded by exon5v2 in group II. *In vitro* cyclase activity assay shows that even with part of $C_1$ domain remains, sAC isoforms still lose their activities. This indicates that both complete $C_1$ and $C_2$ domains are required for maintaining catalytic activity of human sAC *in vitro*.

### 3.4 Discussion

The analysis of identifying alternatively spliced sAC transcripts in NHBE cells is the first time researchers have systematically examined the alternative splicing events of sAC in human tissues. Besides sACfl, sACt and somatic sAC found in mice or rats, there were only a few short alternatively spliced transcripts reported in other tissues which were not long enough to predict the proteins. Furthermore, the optimization process of RT-PCR process confirmed the low copy number of sAC message in somatic tissues except testis that PCR at 35–40 cycles became a common standard (Farrell, Ramos et al. 2008) in Dr. Buck’s group. In this study, besides choosing the appropriate approach for exploring alternatively spliced sAC transcripts, the inhibitory effect of reverse transcriptase on *Taq* polymerase in rare message was found to be the major issue of RT-PCR (Appendix C). It is known that reverse transcriptases can directly inhibit *Taq* polymerase activity and cDNA cannot be detected when low abundance transcripts are involved (Rossen, Norskov et al. 1992, Sellner, Coelen et al. 1992, Wilson 1997). While heat denaturation is employed at the end of reverse transcription, superscript III may be more recalcitrant to heat inactivation and could inhibit *Taq* polymerase in the subsequent PCR step. It was also demonstrated that the ratio of RT: *Taq* greater than 3:2 can inhibit *Taq* (Sellner, Coelen et al. 1992). However, if specific RNA is abundant enough, reverse transcriptase is sequestered by binding to the RNA, thereby protecting *Taq* polymerase from inhibition.
(Sellner, Coelen et al. 1992). Thus, mRNA of NHBE cells together with purification of the synthesized cDNA was performed to improve the RT-PCR reactions.

We found that sAC with partial $C_1$ and complete $C_2$ is not catalytically active in HEK293T and NHBE cells in vitro. This result is consistent with the finding that the artificially constructed $C_2$ domain only sAC isoform is catalytically inactive in insect sf9 cells (Geng, Wang et al. 2005), confirming the requirement of both catalytic domains to maintain the enzymatic activity of sAC. But the somatic sAC which only contains $C_2$ domain was found to be active when immunoprecipitated from the mice brain (Farrell, Ramos et al. 2008). This contradictory result leads us to consider that there might be more distinctions of the intracellular environment between mammalian and insect cells that result in different catalytic activity of sAC $C_2$ only isoform.
Chapter 4 Identification of Ciliary sAC Isoform in Airway Epithelial Cells

4.1 Hypothesis

The ubiquitous second messenger, cAMP, is compartmentalized in the microdomains of the cells (Zaccolo 2009) where the signal is delivered to targeted effectors and terminated in a spatial and temporal manner by traditionally believed specific PDEs (Tasken and Aandahl 2004). Due to the diffusion limitation, it requires the source of cAMP to be present in its close proximity. There was no tmAC found in the cilia of NHBE cells (Nlend, Schmid et al. 2007) but instead a 50 kDa sAC was found to be localized there. The alternatively spliced sAC_{ex5v2-ex12v2} is predicted to be 45 kDa which is close in size to the ciliary sAC found in western blot, especially the newly identified exon5v2 sequence contains the peptide sequence of the antibody used. It is possible that the sAC_{ex5v2-ex12v2} is localized in cilia and functions as the local source of cAMP in NHBE cells. Thus we hypothesized and explored the ciliary localization of sAC_{ex5v2-ex12v2} in NHBE cells.

4.2 Results

4.2.1 Subcellular localization of recombinant sAC isoforms in NHBE cells

The Group II isoform, sAC_{ex5v2-ex12v2}, matches the size of the sAC variant observed in cilia using Western blot. It contains only part of the C1 domain and a complete C2 domain which is typical of what is discovered in NHBE cells. To determine the localization of this isoform, it was initially expressed in NHBE cells followed by immunostaining. The sAC_{ex2-12v2} isoform, which showed strong catalytic activity in vitro, was used for localization comparison.
Lentiviral constructs of all these variants were used to transduce undifferentiated NHBE cells that were then re-differentiated at the ALI. Cells were impacted on slides using cytospin and stained with an HA antibody. The staining showed that sAC\textsubscript{ex2-ex12v2} stayed in the cytoplasm whereas sAC\textsubscript{ex5v2-ex12v2} migrated to cilia (Figure 4.1).

Figure 4.1 Cytospin immunostaining of NHBE cells transduced with recombinant sAC isoforms.

NHBE cells were transduced with lentiviruses of EF\textsubscript{1}-HA-sAC\textsubscript{ex2-ex12v2}-flag/DDK-T\textsubscript{2}A-GFP and EF\textsubscript{1}-HA-sAC\textsubscript{ex5v2-ex12v2}-flag/DDK-T\textsubscript{2}A-GFP. After fully differentiated, cells were trypsinized and processed by cytospin. Top, EF\textsubscript{1}-HA-sAC\textsubscript{ex2-ex12v2}-flag/DDK-T\textsubscript{2}A-GFP lentivirus transduced NHBE cells were stained with mouse HA antibody (1:500) and Alexa 555. Rabbit acetylated tubulin antibody (1:800) and Alexa 647 were used for cilia staining. The merged image shows that sAC\textsubscript{ex2-ex12v2} is expressed in the cytoplasm of NHBE cells. Middle, EF\textsubscript{1}-HA-sAC\textsubscript{ex5-ex12v2}-flag/DDK-T\textsubscript{2}A-GFP lentivirus transduced NHBE cells were stained with the same antibodies as sAC\textsubscript{ex2-ex12v2}. In the merged image, sAC\textsubscript{ex5v2-ex12v2} is localized at cilia. Bottom, no insert lentiviral vectors transduced NHBE cells were incubated with mouse IgG and Alexa 555 for negative controls. Scale bars indicate 10 µm.
Comparing the sequence of $sAC_{ex2-ex12v2}$ and $sAC_{ex5v2-ex12v2}$, the major differences lay in the additional 37 nucleotides from originally identified intron 4 and exons 2, 3 and 4. In order to find out whether the additional intron 4 sequence or 3’ end of sAC were important for the ciliary localization, more truncated sAC variants were cloned and expressed in NHBE cells. These isoforms are $sAC_{ex5v1-ex12v2}$ (isoform without original 3’ end of intron 4 sequence) and $sAC_{ex5v2-ex7}$ (C-terminal truncated isoform of $sAC_{ex5v2}$).

While HA-$sAC_{fl}$-flag/DDK, HA-$sAC_{ex2-ex12v2}$-flag/DDK (C1 and C2 only), and HA-$sAC_{ex2-in7}$-flag/DDK (C1 only variant) remained cytoplasmic, HA-$sAC_{ex5v2-ex12v2}$-flag/DDK was found almost exclusive in cilia (Figure 4.2). In fact, a variant that initiated within exon 5v1 was also targeted, to a certain degree, to cilia. These data suggest that the MSLSE N-terminus, encoded from sequences previously thought to be intronic, is important for ciliary targeting. But deleting part of the exon 5v2 sequence reduced, but did not eliminate, ciliary targeting suggesting that other sAC sequences contribute to ciliary targeting. In addition, our data suggest that exons 2 to 4 prevent ciliary targeting.
Figure 4.2 Localization of recombinant sAC isoforms expressed in NHBE cells.
Lentivirus constructs expressing different portions of sAC (N-terminal HA and C-terminal flag/DDK tagged) were transduced to undifferentiated NHBE cells. After the cells were differentiated at air-liquid interface (ALI) conditions, the localizations of the expressed sAC isoforms were determined by immunostaining with HA antibody using cytopsin preparations. Left: Diagrams of sAC constructs expressed in NHBE cells. Green boxes represent the coding exons and purple boxes indicate newly classified exons (containing previously known intron 4 sequences). White dashed bars indicate the
missing catalytic domain and black bars represent the contained catalytic domains. Gray box represents the original intron 7 retention. Right panels: Immunofluorescence of cytospin preparations of fully differentiated NHBE cells transduced with lentiviruses expressing different sAC variants, stained with mouse HA antibody (red), cilia with rabbit anti-acetylated tubulin (Ac-tubulin) antibody (white), and DAPI for nuclei (blue). HA-sACfl-flag/DDK, HA-sACex2-ex12v2-flag/DDK and HA-sACex2-in7-flag/DDK are localized in the cytoplasm of ciliated cells. HA-sACex5v2-ex12v2-flag/DDK and HA-sACex5v2-in7-flag/DDK are localized to cilia. HA-sACex5v1-ex12v2-flag/DDK is found in both cilia and cytoplasm. Lower right panels: NHBE cells transduced with the no insert lentivirus vector served as a negative control. Scale bar: 20 μm. Figure and legend are reprinted with permission of the American Thoracic Society. Copyright © 2014 American Thoracic Society (Chen, Baumlin et al. 2014).

4.2.2 Expression of sACex5v2 containing variant during differentiation of NHBE cells

The expression pattern of the ciliary variant (exon5v2 sequence) during differentiation of NHBE cells was examined. Quantitative sybr green real-time PCR was performed using RNA isolated from NHBE cells at different times during differentiation with a forward primer designed to hybridize with the retained intron 4 of sACex5v2-ex12v2 mRNA and a reverse primer in exon 6 (Method 2.3). FoxJ1 mRNA expression was used as a marker for ciliated cell differentiation. The results show that while sACex5v2-ex12v2 variant mRNA is expressed in undifferentiated cells (day 0 on air), its level of expression does not increase significantly during differentiation after statistical analysis (Figure 4.3).
Figure 4.3 Expression of exon5v2 containing sAC variant during differentiation.
A. 1.5 % agarose gel analysis of PCR products using exon5v2 specific primers (see Method 2.3). Lane 2, cDNA synthesized with RNA from fully differentiated NHBE cells; lane 3, a plasmid with exon5v2 variant; lane 4, a plasmid with exon5v1 variant (i.e. without the retained intron 4 sequence); lane 5, no reverse transcriptase negative control; lane 6, H₂O only no template control. The expected 266 bp band is observed in the human NHBE cells cDNA and exon5v2 plasmid, but not in the ex5v1 plasmid, indicating that the primers are specific. DNA size markers are in lane 1. B. Graph of the exon5v2 expression (black circles, left Y-axis) during NHBE cell differentiation relative to GAPDH mRNA measured by sybr green real time PCR. The level of FoxJ1 mRNA (black squares, right Y-axis) is shown as a marker for ciliated cell differentiation. Since day 11 FoxJ1 mRNA shows significant increase compared with the expression level at day 0 after analyzing with one-way ANOVA followed by Dunnett's Multiple Comparison Test, indicated by *. The exon5v2 mRNA is present throughout NHBE cell differentiation. Data are shown as mean ± S.E.M and there is no significant difference of sAC exon5v2 mRNA expression during differentiation, analyzed by one-way ANOVA followed by Tukey's Multiple Comparison Test. Figure and legend are reprinted with permission of the American Thoracic Society. Copyright © 2014 American Thoracic Society (Chen, Baumlin et al. 2014).

4.2.3 Subcellular localization of recombinant human sAC isoforms in sAC C₁ or C₂ KO mTECs

To confirm that HA-sAC_{ex5v2-ex12v2-flag/DDK} localizes to cilia and HA-sAC_{ex2-ex12v2-flag/DDK} to the cytosol also in sAC C₁ or C₂ KO mTEC, transduced mouse cells were stained with the HA antibody. In fact, HA-sAC_{ex5v2-ex12v2-flag/DDK} was mainly found in cilia and HA-sAC_{ex2-ex12v2-flag/DDK} in the cytosol of both KO mice cells.
Figure 4.4 Localization of human sAC isoforms in C₁ KO and C₂ KO murine airway epithelial cells.

Human HA-sAC<sub>ex5v2-ex12v2</sub>-flag/DDK or HA-sAC<sub>ex2-ex12v2</sub>-flag/DDK were transduced into undifferentiated sAC C₁ KO and C₂ KO murine airway epithelial cells. Cytospin preparations were made from fully differentiated airway epithelial cells and stained with the HA antibody (red). Cilia were identified with the rabbit acetylated tubulin (Ac-tubulin) antibody (white). DAPI shows nuclei (blue). These are representative of ≥ 2 independent experiments. Upper panels: human HA-sAC<sub>ex5v2-ex12v2</sub>-flag/DDK variant is localized to cilia, analogous to NHBE cells. Middle panels: human HA-sAC<sub>ex2-ex12v2</sub>-flag/DDK variant is not localized to cilia, but remains in the cytoplasm, analogous to NHBE cells. Lower panels: non-transduced C₁/C₂ KO murine cells stained with mouse IgG antibody. Scale bars are 10 μm. Figure and legend are modified and reprinted with permission of the American Thoracic Society. Copyright © 2014 American Thoracic Society (Chen, Baumlin et al. 2014).

The data show that sAC<sub>ex5v2-ex12v2</sub> localizes to cilia both in human and mouse airways. This result is consistent with the previous finding that a 50 kDa sAC is specially localized in cilia. This is also a representative isoform found in NHBE cells that contains partial C₁ domain and full C₂ domain. Since this sAC variant is not active <i>in vitro</i> but is localized in cilia, our next step was to determine if this isoform with partial C₁ was catalytically active <i>in vivo</i> and played a role in regulating CBF.
4.3 Conclusion

Among the recombinant sAC isoforms (sACfl, sACex2-ex12v2, sACex5v2-in7 and sACex2-in7) expressed in fully differentiated NHBE cells, sACex5v2-ex12v2 and sACex5v2-in7 were the only ones that were expressed in the cilia axoneme. The common feature of these two isoforms is the retained MSLSE sequence encoded by originally named intron 4. Further constructed truncated isoform sACex5v1-ex12v2, which deleted the MSLSE sequence, was also localized in cilia though some were seen in the cytoplasm. This indicates that besides MSLSE, the sequences in sAC exons 5, 6 and 7 are also important for the ciliary targeting. In addition, the sACex5v2-ex12v2 was also found to be localized in the cilia of sAC C1 KO and C2 KO mice airway epithelial cells. These results confirm the requirement of the MSLSE sequence in targeting the sAC isoform in cilia. Real-time PCR result also proves that the exon5v2 sequence is present in NHBE cells during ciliogenesis.

4.4 Discussion

Till now only a few proteins have been identified in the cilium while the exact mechanism for delivering protein into cilium is not well-known. As mentioned before, CTS has been accepted as an efficient way to transport proteins to cilia. However, all the publications are only limited to membrane proteins (Geng, Okuhara et al. 2006, Follit, Li et al. 2010) and have never been reported in non-membrane bound ones. The new exon5v2 encoded amino acid sequence “MSLSE” could serve as the CTS for sAC isoform in cilia when accompanied with exons 5, 6, and 7 sequences at the N-terminus. Therefore, the findings of this study contribute to a better understanding of trafficking non-membrane bound protein targeting to cilia.
Chapter 5 CBF Regulation by Alternatively Spliced sAC Isoforms

5.1 Background

It was demonstrated that KH7, along with HCO$_3^-$/CO$_2$, led to a further decrease in CBF compared with HCO$_3^-$/CO$_2$ alone, suggesting that sAC in NHBE cells was activated by HCO$_3^-$ and involved in regulating CBF (Schmid, Sutto et al. 2007). sAC$_{ex5v2-ex12v2}$ contains the complete C$_2$ domain only. Another C$_2$ only sAC variant found in sAC C$_1$ KO mouse brain shows same active as sAC in wild type mice (Farrell, Ramos et al. 2008). On the contrary, artificially constructed sAC C$_2$ only variant showed no activity in insect sf9 cells (Geng, Wang et al. 2005), suggesting that various cellular environment would influence differently in sAC catalytic activity. Due to the special ciliary localization of sAC$_{ex5v2-ex12v2}$ in NHBE cells, we hypothesize that this sAC isoform is different in activity in CBF regulation compared when it is expressed in HEK293T cells in vitro. It would be valuable to understand the role of this sAC isoform in regulating CBF in the sAC KO murine airway epithelial cells. In order to study the murine CBF regulation by human sAC isoforms, we need to confirm firstly that sAC regulation of CBF in mice is the same as in NHBE cells. HCO$_3^-$/CO$_2$ perfusion with or without KH7 was performed in ALI cultured mTECs. As shown in Figure 5.1, sAC plays a role in regulating murine CBF similar to that observed in NHBE cells.

5.2 Results

5.2.1 CBF regulation by recombinant human sAC alternatively spliced isoforms in sAC KO mTECs

Based on the above results, further experiments were carried out in ALI cultured sAC C$_1$ or C$_2$ KO mTECs. After dissection and separation, mTECs were grown in the
presence of a ROCK inhibitor, which showed to “enhance proliferation and maturation of epithelial basal cells” (Horani, Nath et al. 2013). HA-sAC_{ex2-ex12v2-flag/DDK} (C_1 and C_2 only) or HA-sAC_{ex5v2-ex12v2-flag/DDK} (partial C_1 and complete C_2) were transduced into murine sAC C_1 KO and C_2 KO airway epithelial cells, which were then differentiated in air. CBF was measured in fully differentiated cells growing on Transwell filters that were mounted in a closed chamber, firstly perfused with HEPES buffered Hanks’ balanced salt solution apically (Table 2.3) and then with 25 mM HCO_3^-/5% CO_2 in the presence or absence of KH7 (25 μM). In WT cells, CBF decreased from baseline upon 25 mM HCO_3^-/5% CO_2 perfusion due to cytosolic acidification from the rapid CO_2 diffusion into the cells (Figure 5.1). CBF decreased further when 25 mM HCO_3^-/5% CO_2 was perfused together with 25 μM KH7, confirming that sAC activity in WT cells regulates CBF. In contrast, CBF was insensitive to KH7 in both C_1 KO and C_2 KO cells, i.e., KH7 addition did not lead to a further decrease in CBF (Figure 5.1). Given the results from two different KO mice, these data demonstrate that functional ciliary sAC activity was absent in C_1 KO and C_2 KO cells. When C_1 KO cells were transduced with the HA-sAC_{ex5v2-ex12v2-flag/DDK} or HA-sAC_{ex2-ex12v2-flag/DDK} construct, CBF responses were identical to the non-transduced KO cells, indicating that sAC activity was not restored. In contrast, C_2 KO cells transduced with the HA-sAC_{ex5v2-ex12v2-flag/DDK} variant demonstrated CBF regulation identical to WT cells. These data indicate restored sAC activity in C_2 KO cells only when transduced with the human HA-sAC_{ex5v2-ex12v2-flag/DDK} variant that localizes to cilia (Figure 5.1).
Figure 5.1 CBF in sAC C₁ and C₂ knockout murine airway epithelial cells expressing human sAC variants.

Depicted here are the baseline and ΔCBF values from sAC C₁ and C₂ knockout (KO) mice upon HCO₃⁻/CO₂ exposure in the presence or absence of KH7, a sAC specific inhibitor. Data are shown as mean ± S.E.M. from at least eight cell culture experiments seeded from ≥ 2 different animal tracheas. Upper panels show CBF baselines and lower panels show ΔCBF in response to HCO₃⁻/CO₂ ± 25 μM KH7. * indicates statistically significant difference to pooled wild type (WT, upper panels) CBF baselines or CBF decreases upon exposure to HCO₃⁻/CO₂ in the presence and absence of KH7 (lower panels). Left: Data from C₁ KO mouse tracheal epithelial cells (mTECs). Upper left: Baseline CBF is not different in all groups. Lower left: Quantitative comparisons of CBF decreases as a fraction of baseline in WT and sAC C₁ KO mTECs upon 25mM HCO₃⁻/5% CO₂ perfusion ± 25 μM KH7. CBF decreases when changing the perfusate to HCO₃⁻/CO₂, mainly due to acidification of the cells. When cells were perfused with HCO₃⁻/CO₂ ± KH7, CBF of WT mTEC decreased further, suggesting that sAC partially rescued the
pH induced decrease in CBF. This was not the same for sAC C\textsubscript{1} KO or C\textsubscript{2} KO mTEC transduced with the indicated human variants, demonstrating that sAC activity was absent in these cells. Right: Data from C\textsubscript{2} KO mTECs. Upper right: CBF baseline in C\textsubscript{2} KO mTEC transduced with HA-sAC\textsubscript{ex2-ex12v2-flag/DDK} and non-transduced are lower than CBF baseline of the WT and HA-sAC\textsubscript{ex5v2-ex12v2-falg} transduced mTEC. Lower right: There is no significant difference in C\textsubscript{2} KO CBF when perfused with HCO\textsubscript{3}\textsuperscript{-}/CO\textsubscript{2} in the presence and absence of KH7, suggesting the lack of sAC like activity when missing C\textsubscript{2} domain. While in C\textsubscript{2} KO mTECs transduced with HA-sAC\textsubscript{ex5v2-ex12v2-falg} variant, CBF further decrease can be seen after HCO\textsubscript{3}\textsuperscript{-}/CO\textsubscript{2}+KH7, suggesting the recovered sAC activity in these cells. Figure and legend are reprinted with permission of the American Thoracic Society. Copyright © 2014 American Thoracic Society (Chen, Baumlin et al. 2014).

5.3 Conclusion

Though inactive \textit{in vitro} when expressed in HEK293T and NHBE cells, recombinant human sAC\textsubscript{ex5v2-ex12v2} isoform was found to be involved in the increase of CBF only in sAC C\textsubscript{2} KO murine airway epithelial cells but not in C\textsubscript{1} KO mice. This result suggests that both C\textsubscript{1} and C\textsubscript{2} domains are required to maintain the catalytic activity of sAC. In sAC C\textsubscript{2} KO mouse airway epithelial cells, it is highly possible that there is a C\textsubscript{1} like protein that can bind to the C\textsubscript{2} only sAC\textsubscript{ex5v2-ex12v2} isoform and help it to recover the catalytic activity \textit{in vivo}.

5.4 Discussion

As mentioned above, there are two sAC C\textsubscript{2} only isoforms that have been examined for their catalytic activities in different organisms. One is the artificially constructed sAC C\textsubscript{2} only isoform expressed in insect sf9 cells. This sAC isoform proves to be inactive in these cells (Geng, Wang et al. 2005). The other one is the somatic sAC discovered in sAC C\textsubscript{1} KO mice that shows to be active just like the full-length sAC in this mouse brain (Farrell, Ramos et al. 2008). Comparing the catalytic activities of both sAC C\textsubscript{2} isoforms in these cells, sAC\textsubscript{ex5v2-ex12v2} shows to be inactive \textit{in vitro}, like in insect cells but there
exists a high activity when expressed in C₂ KO mTECs as somatic sAC. The contradictory results indicate that there are different intracellular environments in mammalian cells from insect cells. The identification of the complimentary C₁ like protein in C₂ KO mouse airway epithelial cells is an essential factor that leads to the difference in vitro and in vivo.
cAMP is an important second messenger that modulates many physiological process in living organisms. “The cAMP-protein kinase A (PKA) pathway is one of the most common and versatile signal pathways in eukaryotic cells and is involved in the regulation of cellular functions in almost all tissues in mammals” (Tasken and Aandahl 2004). cAMP signaling often occurs in a compartmentalized fashion where the cyclases and the corresponding PDEs are localized in near proximity and act to ensure that the signal is localized. sAC is likewise distributed at discrete intracellular loci and contributes to localized cAMP generation (Zippin, Chen et al. 2003). In NHBE cells, our lab found that sAC was involved in regulating CBF under the stimulation of HCO₃⁻/CO₂ (Schmid, Sutto et al. 2007). This result suggests that sAC or one of its isoforms could be localized to cilia. Thus, this research of sAC in NHBE cells aims at investigating alternative splicing and identifying the physiological role of ciliary specific sAC isoform.

6.1 Alternative splicing of sAC

6.1.1 Identification and sequence diversity of sAC alternatively spliced variants in NHBE cells

Alternatively spliced transcripts of sAC have been discovered in rat testes (Chen, Cann et al. 2000), mouse testes, brain (Farrell, Ramos et al. 2008), and human testes (Jaiswal and Conti 2001, Geng, Wang et al. 2005), etc. This suggests that sAC variants could have different spatial distributions or could execute different functions. Using RT-PCR, alternatively spliced sAC variants were identified specifically expressed in NHBE cells. Most of the alternative splicing occurred in C₁ domain, thus, creating variants that lack a complete C₁ but maintain C₂ domain. Interestingly, one of the transcripts retained
part of the canonical intron 4 (now exon5v2) sequence which introduced a new translation start codon and terminated in intron 12 (exon12v2). This variant has never been reported before. A similar variant with only complete C₂ was thought to define somatic sAC isoform and found specifically in sAC C₁ KO mouse brain (Farrell, Ramos et al. 2008). Thus, the identified alternatively spliced sAC isoforms from NHBE cells are tissue specific.

Since sAC was expressed at very low levels, RT-PCR was required to proceed up to 40 cycles in minimal. Even in testes, where sAC is expressed at a relatively higher level, endogenous sAC protein was detected by Western blot only after prior immunoprecipitation. Lower levels of transcripts likewise translate to very low levels of sAC protein making detection of endogenous sAC very difficult. The original report by Dr. Buck required 950 rat testes to yield less than 3ug of sAC₁. Most technical difficulties encountered in the experiments can thus be attributed to the low abundance of sAC in NHBE cells, even for the recombinant sAC variants. However, low levels of sAC might still be functional if the enzyme in microdomains achieves a relevant concentration at these sites. When the signaling machinery is compartmentalized into microdomains, physiological effects require only small changes in local cAMP concentrations (Chen, Martinez et al. 2013). Therefore, the low levels of sAC expression found in NHBE cells may result from its expression in specific microdomains where it might be functionally relevant.

6.1.2 Catalytic activity of sAC C₂ domain isoform

The somatic sAC isolated from the C₁ KO mice brain, containing only C₂ domain, was found to be as active as sAC found in WT mice by IP and in vitro activity assay
(Farrell, Ramos et al. 2008). On the other hand, an artificially constructed sAC variant with C2 domain only was found to have no adenylyl cyclase activity when expressed heterologously in insect Sf9 cells (Geng, Wang et al. 2005). Consistent with this finding, the sAC variant without complete C1 but complete C2 domain (sAC_{ex5v2-ex12v2}) found in NHBE cells had no detectable cyclase activity in vitro. This was the same result as CBF response in C1 KO and C2 KO mTECs: knocking out either catalytic domain led to no further decrease of CBF upon KH7 treatment. These results suggest that both catalytic domains are required for catalysis and different catalytic activities in different in vivo tissues make the mechanism of sAC catalytic regulation more complicated. The correlated finding is that point mutations in either the C1a or C2a domains of type I adenylyl cyclase, which can abolish nearly all the enzymatic activity since catalytic domains are the highly conserved regions among different adenylyl cyclases (Sunahara, Dessauer et al. 1996). However, in vivo C2 domain only somatic sAC is active in mouse brain as mentioned. In the CBF measurement, at least one of these variants was functional: when localized to cilia in C2 KO mouse airway epithelial cells, where it rescued sAC-dependent beat regulation. Since this variant does not have catalytic activity in vitro, it seems that the lack of a complete C1 may be complimented in certain cells by proteins that can act as C1 “donors” or through other mechanisms. We, therefore, hypothesize that there might be C1 only containing variant that has yet to be identified, which complements the C2 only variant in trans to generate a complete sAC molecule in NHBE cells (Figure 6.1). This could explain why sAC-regulated CBF changes could be rescued in C2 KO but not in C1 KO mice.
Figure 6.1 Model of ciliary sAC in human and sAC C2 KO mouse airway epithelial cells.
A. In NHBE cells, alternatively spliced isoform, sAC\textsubscript{ex5v2-ex12v2}, interacts with an unknown C1 donor (C\textsubscript{x}). Both of them transport to cilia and regulate CBF in NHBE cells. Targeting depends on appropriate sequences: exons 5v2-7 only will allow ciliary transportation; if exons 2-4 are present, as in sAC\textsubscript{fl}, no ciliary localization will occur. The putative associating sites between the C1 donor and sAC\textsubscript{ex5v2-ex12v2} are purely speculative.
B. In sAC C2 domain KO mTECs, recombinant expressed human sAC\textsubscript{ex5v2-ex12v2} is binding with the mouse unknown C1 donor and regaining the catalytic activity of sAC to regulate CBF. Figure and legend are modified and reprinted with permission of the American Thoracic Society. Copyright © 2014 American Thoracic Society (Chen, Baumlin et al. 2014).

The structures of tmAC and a sAC-like bacterial cyclase (Linder and Schultz 2003, Kamenetsky, Middelhaufe et al. 2006), along with homology alignments and modeling, reveal that mammalian nucleotidyl cyclases are active as dimers of two catalytic units which can be found in three distinct modular arrangements (Katsushika, Chen et al. 1992). The crystallized bacterial sAC-like cyclase and transmembrane guanylyl cyclases are active as homodimers of proteins containing a single catalytic domain, either C1 or C2. Soluble guanylyl cyclases (sGC) are active as heterodimers between two distinct proteins (sGC\textalpha and sGC\textbeta), each containing a single catalytic domain. tmACs and the well-characterized sAC isoforms (sAC\textsubscript{i} and sAC\textsubscript{n}) are active due to intramolecular “dimerization” between two related, but distinct C domains (C\textsubscript{1a} and C\textsubscript{2a} in tmACs; C\textsubscript{1} and C\textsubscript{2} in sAC isoforms). The C\textsubscript{1} and C\textsubscript{2} interface and its asymmetry are crucial to the function of adenylyl cyclase and activators of adenylyl cyclase; in order to facilitate the interactions between the two catalytic domains and to stimulate catalysis. In the heterodimeric cyclases (whether they are intermolecular heterodimers or intramolecular “heterodimers”), nucleotide selectivity (guanylyl versus adenylyl) is defined by amino acid residues in only one of the catalytic domains. The other catalytic domain contributes
catalytic residues but no nucleotide specifying interactions due to the extension of β strands and the loop linking the catalytic domains which precludes the binding of a second substrate (Saalau-Bethell, Berdini et al. 2014). It is also accepted that one catalytic domain is predominantly or exclusively catalytic, while the other is regulatory in guanylyl cyclase (Thompson and Garbers 1995). In sAC1, C₁ provides catalytic residues while C₂ defines specificity for ATP over GTP; in tmACs, C₁a is catalytic and C₂a defines specificity for ATP; in sGC, the α subunit is catalytic while β is responsible for GTP selectivity. The sAC C₂ isoforms identified here do not possess all the residues necessary for both nucleotide specificity and catalysis, which is consistent with our inability to recover cyclase activity in vitro. It is tempting to hypothesize that they may heterodimerize with yet unidentified C₁ only containing sAC isoforms, C₁a containing tmAC isoforms, or sGCα subunits.

6.2 Localization of sAC alternatively spliced isoforms

sAC is ubiquitously present in different intracellular organelles (Zippin, Chen et al. 2003). sAC and its isoforms have also been identified in different species performing different functions. It is found in sea urchin sperm flagella, regulating flagellar mobility (Nomura and Vacquier 2006); it is found in mouse sperm where KO of sAC leads to infertility (Hess, Jones et al. 2005); it is expressed in the bovine corneal epithelial cells that regulates its secretory function (Sun, Cui et al. 2004); it is found in astrocyte-neuron (Choi, Gordon et al. 2012); and it is also reported to be in potato plants where cAMP is believed to be a secondary messenger in this system (Lomovatskaya, Romanenko et al. 2008). Previous data from our lab suggested that multiple forms of sAC exist in airway epithelia and that one ~50 kDa form localizes specifically to cilia. An antibody made
against a peptide sequence, SLSEGDALLA present at the N-terminus, recognized this latter form. The identified sAC_{ex5v2-ex12v2} splice variant by RT-PCR contains the same peptide sequence and has a calculated molecular weight of ~45 kDa, similar to the ciliary variant detected by Western blot using that special antibody. Our data also indicate that splice variants expressing the MSLSEGDALLA are targeted to cilia, suggesting that MSLSEGDALLA may act as a CTS. On the other hand, the presence of exons 2-4 prevented ciliary localization. Searching the Genbank sequence databases identified a large number of splice variants with predicted coding regions that encode sAC isoforms without a C\textsubscript{1} but a complete C\textsubscript{2} domain. These are predicted in variety of species including human, cattle, rodents, manatees, and walrus, suggesting some physiological relevance to these isoforms. It is interesting to note that the in-frame MSLSE coding sequence in the retained portion of canonically called intron 4 is conserved in primates but is altered by a single base deletion in rodents and other species. It suggests that this sAC isoform may have a unique function in primates. A few CTS have been identified for transmembrane ciliary proteins including rhodopsin (Tam, Moritz et al. 2000), fibrocystin (Nachury, Seeley et al. 2010), and polycystin-2 (Geng, Okuhara et al. 2006) but not for non-transmembrane proteins like sAC (requiring axonemal targeting). The mechanism of trafficking membrane proteins to cilia is hypothesized to be via vesicular targeting and crossing the diffusion barriers (Nachury, Seeley et al. 2010). The identification of a CTS for a non-membrane ciliary protein provides a new clue to the understanding of the targeting of non-membrane proteins to cilia. In addition, since sAC is localized to many different locations in the cell, these observations suggest that other alternatively spliced isoforms may target to different cellular locations. It is reported that
sAC is involved in CBF regulation of NHBE cells in response to changing HCO$_3^-$/CO$_2$. Here it is also shown that mouse airway epithelial cells possess the same regulatory mechanisms. However, cells from both C$_1$ KO and C$_2$ KO mice lost their ability to regulate CBF in a sAC-dependent manner, providing strong evidence that the HCO$_3^-$ and CO$_2$-mediated changes in CBF are truly mediated by sAC. In C$_2$ but not C$_1$ KO mice, this regulation is restored by the HA-sAC$_{ex5v2-ex12v2}$-flag/DDK variant that is localized to cilia. On the other hand, the cytosolic variant (sAC$_{ex2-ex12v2}$), even though showing strong adenylyl cyclase activity \textit{in vitro}, did not rescue CBF regulation. This confirms that cAMP production by sAC is compartmentalized and requires close proximity to the cilia and that cAMP product cannot diffuse freely into cilia.

6.3 Significance of sAC alternative splicing and its localization in NHBE cells

Previous studies of alternative splicing at the protein level have shown that the residues that are differentially present between the splice isoforms frequently fall in the intrinsically disordered protein regions (Romero, Zaidi et al. 2006). This can be a consequence of avoidance of structured protein domains, but could also imply a connection between the individual isoform and specific function. If alternative inclusion of protein segments with distinct binding motifs is used to modify the behavior of the protein in cellular pathways, then this process should be carefully regulated. Hence, protein segments encoded by finely regulated alternative splicing are more likely to be enriched in functionally significant regions compared to all the other alternatively spliced segments. The identified alternatively spliced region of sAC in NHBE cells in this study affects the C$_1$ domain. This suggests the C$_1$ domain is the functionally essential region of sAC in NHBE cells. This tissue specific isoform also determines its specific character,
like the sAC$_{ex5v2-ex12v2}$ isoform localized in cilia. Somatic sAC, which also comprises the complete C$_2$ domain but lacks a C$_1$ domain, clearly explains the importance of the partially retained C$_1$ domain and additional intron 4 sequence in targeting sAC$_{ex5v2-ex12v2}$ to cilia and its possible role in interaction with the other “C$_1$- like” protein.

### 6.4 Clinical implications

The clinical significance of alternatively spliced isoforms was found in some cancer genes. Such aberrant splicing has already been connected to cystic fibrosis (Chu, Trapnell et al. 1993), retinitis pigmentosa (Chakarova, Hims et al. 2002), spinal muscular atrophy (Lorson, Hahnen et al. 1999), neurofibromatosis type 1 (Ars, Serra et al. 2000), haemophilia B (Rees, Rizza et al. 1985), and several types of neoplasia (Stickeler, Kittrell et al. 1999, Pajares, Ezponda et al. 2007). In fact, it is reported that both Cancer Gene Census genes (genes that have been causally implicated in cancer) (Futreal, Coin et al. 2004) and genes from the COSMIC database (genes found to be somatically mutated in different cancer cells) (Forbes, Bhamra et al. 2008) were enriched with genes that have tissue-specific isoforms. This suggests a possible connection between the genes with tissue-specific isoforms and diseases. In therapy, antisense oligonucleotide using base pairing to target specific RNA sequences in order to correct splicing disorders has been applied to cystic fibrosis in cell cultures (Friedman, Kole et al. 1999, Dhir and Buratti 2010). Identification of the ciliary specific sAC isoform helps to understand not only the ciliary targeting mechanism, but also the ciliary specific sAC isoform in CBF regulation. Being activated specially by HCO$_3^-$/CO$_2$ and fitting the physiological circumstance of breathing, ciliary specific sAC isoform that regulates CBF provides a more specific target for drug development in specific tissues. The increase of the specificity could reduce the
side effects of medicine due to the ubiquitous expression of sAC in tissues. Overall, the identification and analysis of ciliary sAC isoform provides a target for the treatment of mucociliary dysfunction and secondary respiratory infection.

6.5 Limitations of this study

In this study we chose to use RT-PCR as an approach to examine the alternative splicing events of sAC in NHBE cells. After a tremendous amount of optimization in the protocol, we used about 8 normal lung donors from over 80 sequencing results to identify these transcripts. Due to the technical limitations, we could not find any correlation between certain transcripts and lung diseases among the samples we tested. Expanding the samples of RT-PCR and sequencing might help to discover whether the sAC isoforms in NHBE cells are related to respiratory diseases. Moreover, the ciliary sAC isoform examined in this study is a recombinant protein. If the antibody from Dr. Geng, which has identified a special 50 kDa band in Western blot, was available and if technically there have been enough samples for mass spectrometry, the analysis would help confirm the ciliary sAC variant identified by RT-PCR.

6.6 Future directions

We hypothesize that there is a C1 donor protein that helps regain the sACex5v2-ex12v2 catalytic activity in sAC C2 KO mouse airway epithelial cells. Unfortunately, we were not able to pull down the C1 donor protein by immunoprecipitation and Western blot in NHBE cells, which is supposed to bind with the ciliary sAC after chemically separating the cilia from the cell body (Appendix G). Due to the low abundance and sensitivity of the antibodies for IP and Western blot, we were even not able to identify the ciliary sAC
in Western blot. Future experiments would need to rely on another designed and more sensitive sAC antibody that is available for IP and Western blot.

In summary, this study presents an analysis of alternatively spliced transcripts of sAC in NHBE cells. Most of them contain only a part of C₁ but a complete C₂. One of these variants is specifically localized to cilia, suggesting a previously unappreciated axonemal targeting mechanism. Even though many of the investigated incomplete C₁ variants are not active in vitro, the ciliary variant rescues CBF regulation by sAC in C₂ KO mice and thus gives credence to previous findings that sAC variants with incomplete C₁ are possibly associated with helper proteins to become functionally active adenylyl cyclases in the cell. Further work will be needed to identify such proteins.
APPENDIX A

Identification of alternatively spliced transcripts of soluble adenylyl cyclase in human bronchial epithelial cells by rapid amplification of cDNA ends

Another alternative technique to identify alternatively spliced transcripts of soluble adenylyl cyclase (sAC) is the rapid amplification of cDNA ends (RACE). Marathon cDNA amplification kit (Clontech) was used with cDNA synthesized from the mRNA of fully differentiated normal human bronchial epithelial (NHBE) cells, following the manufacturer’s protocol.

cDNA samples adapted with AP1 primer were diluted and used as template in the PCR reactions with reverse primers at exon 10 and exon 8 separately (Figure A1) for 5’ RACE and forward primer at exon 26 for 3’ RACE (Figure A2). After sequencing, none of them were determined as sAC while the Human Placental Poly A⁺ (HPP) positive control demonstrated the right size in the gel. Considering the sensitivity of RACE in detecting the rare message of sAC, the second round PCR reactions were performed with nested primers for both 5’(AP2 & reverse exon 8) and 3’ (forward exon 26 &AP2) RACE (Figure A3). Unfortunately, there were no bands detected as sAC after sequencing analysis.
Figure A1 5’ rapid amplification of cDNA ends (RACE) of sAC reverse primers at exons 10 and 8.
1% DNA agarose gel image of 5’ RACE with reverse primers at exon 8 and exon 10. AP1 was used in the 5’ RACE as the forward primer. The expected PCR product for AP1 & exon10 is 1.3 kb and for AP1 & exon 8 is 1.0 kb. Lanes 1 & 3 are PCR products with the 1:100 diluted AP1 adapted cDNA templates. Lanes 2 & 4 are PCR products using 1:50 diluted AP1 adapted cDNA template. Lane 5 shows the PCR products using 1:250 diluted cDNA of Human Placental Poly A⁺ (HPP) positive control (expected size 2.6 kb). After sequencing, those bands shown in the gel from lane 2 to 5 for both primer pairs were not sAC.
Figure A2 3’ RACE with sAC forward primer at exon 24.
Forward sAC primer at exon 24 coupled with reverse primer AP1 were used for the 3’RACE. The expected size of PCR product for primers AP1 & exon 24 is 1.5 kb. In the 1% DNA agarose gel image, Lane 1 is the PCR product using 1:250 dilution of HPP cDNA positive control. The expected size of this PCR product for HPP with AP1 and 3’ Transferrin Receptor (TFR) is 2.9 kb. Lanes 2 to 4 are PCR products with 1:100 diluted cDNA template ligated with AP1. Lane 2 is the PCR amplimer with primer pair of exon 24 and AP1. Lane 3 is the PCR product using AP1 primer only and Lane 4 is the product with forward exon 24 primer only. No sAC sequence was identified.
Figure A3 Nested PCR for RACE.

Using the template of 5’RACE PCR products with primer pair AP1 and exon 10 (Figure A1), nested PCR was performed with AP2 and reverse primer at exon 8 (R8). Lanes AP2 and R8 primer alone serve as the background control. 3’ RACE product of forward primer exon 24 and AP1 (image shown in Figure A2) was used as template for nested PCR with primer pair exon 26 and AP2. AP2 primer only was also used as a background control for this nested PCR. Bands that were different in size with the background control were gel extracted and sequenced. None of them was aligned with sAC.
APPENDIX B

The problem in reverse transcription-polymerase chain reaction of sAC in NHBE cells

cDNA synthesized from total RNA of fully differentiated NHBE cells was used in the reverse transcription-polymerase chain reaction (RT-PCR). As shown previously (Chapter 3), only PCR products less than 1.0 kb were consistently amplified while PCR amplimers longer than 1.0 kb were less reproducible or not detectable. This was also seen in the PCR products of two other lungs (Figure B1). This problem was a major obstacle in this study and had drawn the attention for optimization (Appendix C).

Figure B1 Identification of alternatively spliced sAC transcripts in NHBE cells with primer pairs of exon 2 and serial reverse primer pairs to exon 20.
1% DNA agarose gel image of RT-PCR products from primer exon 2 to serial reverse primers to exon 20 with cDNA synthesized from 2 lung donors (315,344). The expected sizes of full length sAC for different primer pairs are: exons 2-6 (548 bp), exons 2-8 (696 bp), exons 2-10 (961bp), exons 2-12 (1225 bp), exons 2-13 (1336 bp), and exons 2-20 (2300 bp). PCR products less than 1.0 kb (forward primer at exon 2 with reverse primers at exons 6, 8 and 10) show clear bands and are close to the expected full length size. Longer RT-PCR products (> 1.0 kb) as exons 2-13 and exons 2-20 show no bands.
APPENDIX C

Optimization of full length RT-PCR for sAC in NHBE cells

Systematic approaches have been attempted to optimize the RT-PCR protocol step by step.

1. Examination of RNA integrity: To determine whether RNA degradation is the reason for failure of RT-PCR, total RNA extracted from NHBE cells that failed to get PCR products was sent for examination. The results confirmed that those RNA samples were not degraded (Figure C1). Due to the low copy number of sAC in NHBE cells, mRNA was used in the following experiments.

Figure C1 Examination of RNA integrity by angilent 2100 bioanalyzer expert software.
Electropherogram of total RNA samples from four different lung donors used for RT-PCR of sAC in the above mentioned experiments (Figures 3.1, 3.2, 3.3 and 3.4). Small amounts of RNA samples were separated in the channels of the microfabricated chips according to molecular weight and subsequently detected via laser-induced fluorescence detection. The result is visualized as an electropherogram where the amount of measured fluorescence correlates with the amount of RNA of a given size (Schroeder, Mueller et al. 2006). Each RNA integrity number (RIN) of these samples was >10, suggesting that the RNAs were intact. Two peaks of 18s and 28s were also shown at the right ratio.
(2) Efficiency of reverse transcriptases (RTs): different RTs including qscript mastermix (Quanta), AMV, superscript III (Invitrogen) with oligo (dT)₂₀ primer, random hexamer, as well as sAC gene specific primers were compared (Figures C2 and C3) for cDNA synthesis taking into consideration of secondary structure of RNA samples. All cDNA samples synthesized by these RTs failed to reach expected size (3.5 kb) of PCR amplimers even though some small-size bands were observed. Considering that superscript III was the latest engineered version of the enzyme from Moloney murine leukemia virus and the manufacturer’s protocol allowed the initial heating of RNA samples at 65 °C to denature secondary structures, superscript III with oligo (dT)₂₀ primer was kept for use in the following experiments.

Figure C2 Comparison of different reverse transcriptases for cDNA synthesis and PCR reaction of exons 2-26.
1% DNA agarose gel image of PCR reaction by sAC primers targeting at exons 2 and 26. cDNA was synthesized with the following reverse transcriptases according to each protocol: qscript, AMV, superscript III with random hexamer, and superscript III with oligo d(T)₂₀. The expected full-length size of sAC from exons 2 to 26 is 3.5 kb.
(3) Comparison of cDNA synthesized with RNA from different passages of NHBE cells. Considering different passages of NHBE cells might influence the PCR amplification results, cDNA synthesized from mRNA of passage 0 and 1 NHBE cells were compared in PCR reactions (Figure C3). Only bands at around 3.5 kb were observed in human brain samples. Faint bands in NHBE cells were not sAC after sequencing alignment.

![Figure C3 Comparison of different primers for cDNA synthesis, passages of NHBE cells for RT-PCR.](image)

1% DNA agarose gel image of RT-PCR with primers at exon 2 and exon 26. Human brain total RNA was used as the positive control. Three different lung donors were used for analysis. One of them is from passage 0. Superscript III was used for cDNA synthesis with oligo d(T)$_{20}$ or random hexamer. Oligo d(T)$_{20}$ primer synthesized human brain cDNA template can amplify ~3.5 kb bands but not in cDNA of NHBE with oligo d(T)$_{20}$ or random hexamer, despite several bands being observed in these lanes. They were not sAC after sequencing alignment.

(4) Amplification efficiency of different Taq polymerases. Several Taq polymerases including pfu-ultra, Go Taq green mastermix, platinum Taq, phusion HF master mix and advantage 2 polymerase were compared by diluting the full length sAC plasmid template
and amplifying with primer pairs designed to hybridize in exon 2 and exon 26 (Figure C4). Human mAKAP was used in the touchdown PCR experiment as a positive control. All polymerases were able to amplify expected bands with mAKAP samples. But there were no bands even close to the expected size in all NHBE samples (Figure C4). Based on its adjustable conditions for PCR reactions, high sensitivity and convenience for TA cloning, Platinum Taq high fidelity polymerase was selected for the following PCR experiments.

Figure C4 Comparison of touchdown PCR with different polymerases using primers of mAKAP and human sAC.

cDNA was synthesized from mRNA of NHBE cells (for sAC) and total RNA of human heart (for mAKAP) with superscript III plus gene specific primers (GSP). Three different polymerases were compared for the same touchdown PCR protocol (Platinum Taq, Pfu-ultra and advantage 2 polymerase). mAKAP (8.5 kb of expected size); NHBE for sAC
(5.0 kb of expected size). "-" means no template negative control. mAKAP primer pair: C1+0;r100-4.2. sAC primer pair: exon 1 and exon 33.

**Figure C5 Test of the cDNA synthesis from the NHBE samples in Figure C4.**

With the NHBE cDNA samples from Figure C4, PCR reactions were performed by using the primer pairs of exon 2 to exon 20 and exon 26 to exon 32. For exon 2 and exon 20, full-length product should be 2.4 kb. For exon 26 and exon 32, the full-length PCR product is 986 bp. Sequencing results showed that they were all full-length sAC within the frame, confirming that the cDNA was synthesized and intact for the touchdown PCR showed in Figure C4. This indicated that different Taq polymerases and protocols for PCR were not the key points for failure of full-length RT-PCR.

**Figure C6 Sensitivity of Platinum Taq polymerase.**

The human full-length sAC plasmid (Origene) was used as the template for PCR with primers at exons 2-26. Platinum high fidelity Taq polymerase from Invitrogen was tested for its sensitivity. After serial dilution of the template with 30 cycles of PCR, the minimum starting amount of plasmid template that is visible in the agarose gel is 0.01 pg.

(5) Inhibition effect of reverse transcriptase on Taq polymerase. When the message is rare as sAC in NHBE cells, increased level of reverse transcriptase can be detrimental to
the subsequent Taq mediated PCR step (Sellner, Coelen et al. 1992). The spiking experiment was performed to determine this inhibition (Figure C7). During the experiment, diluted full-length sAC plasmid was used as the PCR template with primer pairs of exons 2-26 at 40 cycles. As shown in the gel, a clear 3.5 kb band was observed from the PCR with full-length sAC plasmid template. But there was no band at expected size from the cDNA template amplimers. Once adding the same amount of cDNA was added into the same plasmid template as they were used individually in PCR under employing the same conditions, the clear 3.5 kb band from the plasmid template became fainter. This suggests that there is an inhibitory effect of cDNA sample from NHBE cells on the PCR reaction of plasmid template.

Figure C7 Inhibitory effect of cDNA synthesized from NHBE cells on the PCR reactions of sAC plasmid template.
Diluted full-length sAC plasmid template was spiked with cDNA synthesized from NHBE cells. PCR products with primer pair exon 2 and exon 26 were run in 1 % DNA agarose gel. “P” stands for the human full-length sAC plasmid template. One band was detected matching the full-length sAC size within the range (black arrow). “-” stands for
no template negative control. “P+C” stands for the same amount of PCR plasmid template used in the first lane that was mixed with cDNA synthesized from NHBE cells. Compared with the plasmid template only positive control, the intensity of full-length band (black arrow) is fainter. “C” represents the PCR product of cDNA synthesized from the NHBE cells. Full-length band in this lane is hard to see. This suggests the inhibitory effect of cDNA from NHBE cells on full-length sAC PCR reaction is due to the low copy number of sAC in NHBE cell.

To mitigate the inhibitory effects, cDNA samples were purified with PCR purification columns (Qiagen). This approach led to a dramatic improvement of RT-PCR for sAC in NHBE cells (Figure C8).

Figure C8 Comparison of cDNA purification for PCR reactions.
The cDNA synthesized with superscript III kit from mRNA of NHBE cells was purified with Qiagen PCR purification columns. A. DNA agarose gel image of sAC PCR products with primer pair exon 2 and exon 20. “un” stands for unpurified cDNA samples while “pu” stands for the purified ones. Only after cDNA purification, PCR could amplify the expected size bands. B. PCR products with primer pair exon 18 and exon 32 from cDNAs of two lung donors. The results are the same as when using primer pair of exons 2 and 20. No band from unpurified cDNA sample was seen in the gel image while clear bands were observed in purified cDNA templates. This experiment confirms the inhibitory effect of cDNA sample on RT-PCR reaction for rare sAC message in NHBE cells.
APPENDIX D

Schematic map of alternatively spliced sAC transcripts in NHBE cells
**Figure D1 Schematic map of sAC alternatively spliced transcripts in NHBE cells.**
Sequence analysis results of RT-PCR products from mRNA of fully differentiated NHBE cells in ALI culture. The data were grouped according to the primer pair sites. Ratio of each transcript in individual lungs was counted according to the colony PCR results. Purple and red boxes indicate intron retentions which make new exons. Included exons are represented by the green boxes and excluded exons are denoted by the yellow dashed boxes. Black arrows below the images indicate the open reading frame of the predicted protein when there is no stop codon found within the range. White dashed bars indicate deleted amino acids encoded by corresponding excluded exons above. Blue bars denote the range of catalytic domains.
APPENDIX E

Identification of alternative start site upstream of exon 2 of sAC transcripts in NHBE cells

The human full-length sAC starts translation from exon 2. All the above PCR primer pairs are designed targeting at exon 2 which assumes that the alternative spliced transcripts all initiate translation from exon 2. As known, sAC transcription can also initiate from an alternative promoter possibly leading to a different pattern of splicing. Thus, the primer pair designed to hybridize to exon 1 was used in RT-PCR reactions (Figure E1). Two out of the three lungs showed bands of the PCR products. However, sequence analysis determined that these were not sAC, suggesting that at least in NHBE cells sAC transcription does not initiate from the alternative promoter.

Figure E1 Identification of alternative translation start upstream of exon 2 of sAC in NHBE cells.
1% DNA agarose gel image of PCR products with primer from exon 1 to exon 33(full-length product is expected to be over 5.0 kb). No bands are sAC after sequencing from this primer pair. cDNA was purified after being synthesized from lung 383, 200, 406. With the same purified cDNA, PCR products with primer exon 2 to exon 32 were seen in the gel.
APPENDIX F

Immunostaining of recombinant sAC isoforms in NHBE cells by R21 antibody

sAC isoforms which were cloned in propagation deficient lentiviral vectors (Chapter 4) were packaged in HEK293T cells and used to transduce de-differentiated NHBE cells on filters in suspension and then allowed to re-differentiate at air-liquid interface (ALI) filters. Cells were fixed and stained with R21 (sAC antibody). GFP fluorescence was an indicator of transduction efficiency. Staining for cilia shows that transduced cells are less ciliated compared with the non-transduced cells. It was also difficult to see the cilia localization in Z-stack. Due to this visualization problem, cytospin preparation before immunostaining was performed as described in 2.9.
Figure F1 Immunostaining of R21 for NHBE cells transduced with sAC isoforms.
A. 10x magnification of R21 stained NHBE cells grown on filters were detected under Zeiss microscope. Anti-mouse IgG Alexa 555 is used as the secondary antibody. Cilia were labeled with rabbit anti-acetylated tubulin and the secondary antibody was Alexa647 coupled anti-rabbit IgG. Co-localization of expressed sAC isoforms in NHBE cells was shown by GFP and R21. This 10x magnification also shows the pattern that GFP fluorescent cells are not ciliated. This is also seen in the 40x magnification in images B. B. 40x confocal merged images of NHBE cells transduced with sAC_{ex5v2-ex12v2} and sAC_{ex2-ex12v2}. R21 is coupled to anti-mouse Alexa 555 and acetylated tubulin is coupled to anti-rabbit Alexa 647. C. Merged confocal image of empty vector transduced NHBE cells shows that these cells grow cilia. The Z-stack images confirm that NHBE cells transduced with the sAC isoforms lack cilia compared with the empty vector transduced NHBE cells that are ciliated. D. Non-transduced NHBE cells stained with mouse IgG antibody and Alexa 555 serve as negative control.
APPENDIX G

Alternative Attempts to Identify and Characterize Ciliary sAC and the Domain Compositions in NHBE Cells

An alternative approach besides immunostaining to identify ciliary sAC in NHBE cells is Western blot after biochemically separation of cilia and cell body. This also helps identifying the catalytic domain composition of ciliary sAC and potentially C₁ “donor” protein. Though our lab has previously identified a ciliary sAC in Western blot by the polyclonal sAC antibody from Dr. Geng (peptide sequence: SLSEGDALA), due to its unavailability, another sAC antibody has to be used instead to determine it. Two other sAC antibodies from Dr. Buck, namely R21 antibody (sAC antibody targeting at human exon 6) and R40 antibody (recognizing a peptide sequence of human sAC in exon 2) were applied in this method (Figure G1).

Figure G1 Localizations of sAC antibody peptides in human sAC schematic map.
The antigen localizations of sAC antibodies mentioned in this dissertation are listed beneath the schematic diagram of human sAC. sAC antibody from Dr. Geng is a polyclonal rabbit antiserum with the peptide sequence encoded by part of the newly identified human sAC exon5v2 sequence. R40 mouse monoclonal antibody is generated by antigen of the amino acid sequences encoded by human sAC exon 2. R21 is a mouse monoclonal antibody produced by antigens at human sAC exon 6 encoded amino acid sequences. Two catalytic domains of sAC are denoted as light blue boxes in the graph.

R40 antibody was previously used by Dr. Buck and was only successful in sAC IP from mouse testes but not in Western blot (data not published). In NHBE cells, neither of these antibodies could detect endogenous sAC from the cell lysate in Western blot. Thus
the sensitivity of R21 antibody for sAC in Western blot was firstly evaluated. By serial dilution of the HEK293T cell lysates expressing recombinant sAC_ex2-ex12v2, R21 antibody detected the recombinant sAC variant in Western blot.

**sAC_ex2-ex12v2 transfected HEK293T cells**

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Figure G2 Determination of the R21 antibody sensitivity in HEK293T cell lysate expressing recombinant sAC_ex2-ex12v2.

Cell lysate of HEK293T cells transfected with sAC_ex2-12v2 was blotted with R21 antibody. Lysate was serially diluted and the concentrations start from lane 2 (20 ug), lane 3 (10 ug), lane 4 (5 ug), lane 5 (2.5 ug), lane 6 (1.25 ug), lane 7 (0.625 ug), lane 8 (0.31 ug), and 9 (0.15 ug). sAC_ex2-ex12v2 can be detected minimally in 0.31ug protein (lane 8) from the transfected cell lysate. Lane 1 is the protein ladder of 50 kDa.

Since NHBE cells express too little amount of endogenous sAC to be detected by R21 antibody in Western blot, an optimization for this approach was to enrich sAC by IP with R40 antibody firstly from a large amount of cell lysates before blotting with R21 antibody. R40 antibody for IP was firstly tested in HEK293T and NHBE cells transduced with sAC_ex2-ex12v2 isoform.
Figure G3 Immunoprecipitation of recombinant sAC_{ex2-ex12v2} by R40 antibody in HEK293T and NHBE cells.

A. The left panel is biotinylated R21 blot of R40 antibody immunoprecipitated sAC_{ex2-ex12v2} from HEK293T cells. The right membrane is probed with streptavidin alone as background control. Lane 1, preIP lysate with 150 mM NaCl in lysis buffer. Lane 2, preIP lysate with 250 mM in NaCl lysis buffer. Lane 3 and 4 are R40 and IgG pulled down with 150 mM NaCl in lysis buffer. Lanes 5 and 6 are R40 and IgG IP with 250 mM NaCl in lysis buffer. Lanes 7 and 8 are postIP lysate from R40 and IgG with 150 mM NaCl in lysis buffer. Lane 9 is post IP lysate from R40 with 250 mM NaCl in lysis buffer. 

B. Western blot by biotinylated R21 antibody of recombinant sAC_{ex2-ex12v2} reconcentrated by R40 antibody from transduced NHBE cell lysate. Lane 1, preIP lysate; Lane 2, R40 antibody immunoprecipitated beads; Lane 3, IgG immunoprecipitated beads; Lane 4 is post IP lysate from R40 antibody. Right panel is the streptavidin blot only.

Recombinant sAC_{ex2-ex12v2} expressed in HEK293T and NHBE cells can be detected by R21 antibody in Western blot after being immunoprecipitated with R40 antibody in the presence of 150 mM or 250 mM NaCl in lysis buffer (Figure G3). This result confirms the feasibility of R40 antibody for IP in NHBE cells. Then endogenous sAC in
NHBE cells was examined by R40 IP followed by biotinylated R21 antibody Western blot.

**Figure G4 R40 antibody immunoprecipitation from NHBE cells.**

A. Fully differentiated NHBE cells were de-ciliated biochemically and the cilia lysate was separated from the cell body lysate. R40 IP with 150 mM NaCl containing lysis buffer was used to enrich sAC before blotted by biotinylated R21 antibody. Lane 1, cilia lysate (IP from 200 ug total protein) precipitated with R40 coated beads. Lane 2, cilia lysate (IP from 200 ug of total protein) precipitated with IgG coated beads. Lane 3, cell body lysate (IP from 10 mg total protein) precipitated with R40. Lane 4, cell body lysate (IP from 10 mg total protein) precipitated with IgG. Right membrane is the streptavidin only blot as background control. B. Left panel is Western blot of biotinylated R21 antibody for R40 IP with lysis buffer starting with 1M NaCl. Lane 1, NHBE whole cell lysate. Lane 2, cell body debris. Lane 3, cilia debris. Lane 4, cell body supernatant. Lane 5, cilia supernatant. Lane 6, cell body lysate (10 mg total protein) precipitated with IgG coated beads. Lane 7, cell body lysate (10 mg total protein) precipitated with R40 coated beads. Lane 8, cilia (400 ug total protein) lysate precipitated with IgG. Lane 9, cilia (400 ug total protein) lysate precipitated with R40. Right panel is the blot with streptavidin only.

Based on the previous R40 IP results in sACex2-ex12v2 detection, lysis buffer containing 150 mM NaCl was initially applied for NHBE cells (10 mg total protein to start) (Figure
G4 A). Fractionation of cilia and cell body was performed as described above and cilia pellet was re-suspended in IP lysis buffer. Biotinylated R21 antibody could not detect sAC in Western blot from the enriched lysate by R40 antibody. Then lysis buffer starting with increased salt concentration (1M NaCl) was used for R40 IP of cilia and cell body. After incubation in 1M NaCl lysis buffer for 20 minutes, NaCl was later diluted to final concentration of 250 mM (Figure G4 B).

Neither low nor high salt concentrations in the IP buffer could detect any endogenous sAC from the NHBE cell lysate, even though recombinant sAC_{ex2-ex12v2} in both HEK293T and NHBE cells can be easily detected by R21 after R40 antibody immunoprecipitation. This negative result leads to the question of the sensitivity of this approach compared with immunostaining and becomes a hindrance for the further detection of possible C_{1} “donor” protein in NHBE cells.
References


