All-trans retinoic acid downregulates CCAAT/enhancer binding proteins in human bronchial epithelial cells

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DEDICATION

To my loving wife, Suha and my daughter, Seba for enduring.

and

To my mother and father.
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INTRODUCTION

In order to supply the organism with oxygen the inner surface of the lung (the lung epithelium) is in direct contact with the outside environment. This also results in constant contact of the epithelium with damaging and transforming agents in the inhaled air. Thus mechanisms such as repair, regeneration and cell growth are normally occurring events in the adult pulmonary epithelium. By studying the differentiation of the normal bronchial epithelial cells (BECs), and more specifically the role of the retinoic acid on the transcription factors CCAAT/enhancer binding proteins (C/EBPs) on cultured human BECs, we hope to gain a higher understanding of the processes regulating gene expression, cell growth and differentiation in the lung. Retinoic acid is the active metabolite of vitamin A that regulates multiple biological processes and plays key roles in embryonic development and in tissue remodeling in the adult. This hormone also displays distinct anticarcinogenic activities and has been used in clinical trials as a therapeutic and preventive agent in several types of cancers. Retinoic acid activities are mediated by retinoic acid receptors that are ligand-inducible transcription factors that are members of the superfamily of nuclear hormone receptors. Transcription factors play an important role in cellular proliferation and differentiation. Among those transcription factors are the basic-leucine zipper CCAAT/enhancer binding proteins transcription factors that have been shown to play pivotal roles in control of cellular growth and differentiation, inflammatory response, liver regeneration, metabolism and numerous other cellular responses. Here we have investigated the effects of retinoid (all-trans retinoic acid) on bronchial epithelial cell proliferation and differentiation in culture
and the involvement of C/EBPs in retinoid signaling through evaluating the effect of RA on genes that are involved in differentiation and apoptosis including, MUC1, FOXA1, Spr1 and Bcl-2. In this study, we have used normal BECs exposed to 30 nM concentration of All-trans-RA to investigate its effect on C/EBPs and other key factors involved in BECs differentiation.

Hypothesis:

All trans RA contributes to the differentiation of human bronchial epithelial cells through upregulation of CEBPA transcription factor, and that this in turn regulates genes involved in controlling cell proliferation, differentiation and apoptosis.
LITERATURE

Lung physiology and histology

The lung is optimized for supplying the organism with oxygen. The architecture of the highly branched respiratory tree that leads the air into alveoli provides a very large contact area between the inside of the body and the outside air, and is used to efficiently perform gas exchange. The respiratory tree is lined with a specialized epithelium consisting of several well-differentiated cell types. The upper bronchi are lined with ciliated pseudo-stratified epithelium containing scattered mucous producing goblet cells, which together form the mucociliary defense system. More distally, the ciliated cells and goblet cells decrease in number and serous secretory nonciliated bronchiolar (Clara) cells appear. The Clara cells are proposed to have a protective role against inhaled toxins and oxidative stress (Singh and Katyal, 2000; Stripp et al., 1995), and are stem cells for the bronchiolar epithelium during regeneration (Evans et al., 1978; Stripp et al., 1995). The Clara cells products include the Clara cells secretory protein (CCSP/CC16), several Secretoglobin 1A1 (SCGB1A1) (Broers et al., 1992; Singh et al., 1986), and several cytochrome p450 enzymes (Hukkanen et al., 2002) that are involved in detoxification of inhaled substances. In the most distal part of the lung, the alveoli are lined with two other types of epithelial cells, type I and type II cells. The flat alveolar type I cells completely cover the alveolar luminal surface and provide a thin surface for gas exchange, and the cuboidal alveolar type II cells that sit on the basal lamina of the epithelium and are responsible for the production of surfactant. The surfactant is a mixture of phospholipids and proteins, i.e. the surfactant protein (SP) A, B,C and D (Cardoso, 2000; Warburton et al., 2000). The primary function of the surfactant is to
facilitate inhalation of air by lowering the surface tension in the air/liquid interface in the alveoli to prevent collapse during expiration, but also to facilitate expansion of the alveoli during inspiration.

**Gene regulation and transcription**

The specificity of gene expression and transcription is controlled by regulatory transcription factors. By binding to sequence-specific binding sites in the promoter, the control region of transcription, the transcription factors influence the rate of transcription both positively and negatively (Kornberg, 2005; Roeder, 2005). In the cell, DNA is tightly packed around a core of histone proteins in chromatin fibers. The chromatin has an intrinsic condensation property, which is dynamic and is driven by DNA charge neutralization or mediated by histone or non-histone proteins (Hansen, 2002). For transcription to occur, chromatin remodeling is required so that the transcriptional machinery can gain access to the DNA. Binding of transcriptional regulators to the promoter and their interaction with chromatin remodeling functions makes the DNA accessible for transcription. Histones are subject to an enormous number of posttranslational modifications, including acetylation and methylation of lysines and arginines, phosphorylation of serines and threonines, ubiquitination and sumoylation of lysines as well as ribosylation (Peterson and Laniel, 2004). Acetylation, carried out by histone acetyltransferases (HAT) that reduce the positive charge of the histone tails and weaken the interaction between histones and DNA. This facilitates binding of additional transcription factors and cofactors. The process is reversible and the removal of the acetyl group by histone deacetylases (HDACs) compacts the DNA and silences transcription (Peterson and Laniel, 2004). A variety of co-activators such as cAMP binding protein
(CBP) and a 300-kDa nuclear protein (p300), CBP/p300, have been found to have intrinsic HAT activity, and many co-repressors, such as Rpd3, have been found to have histone deacetylation (HDAC) activity. Unliganded and DNA bound retinoid receptors interact with HDAC complexes, such as the nuclear receptor corepressor (NcoR) and the silencing mediator for retinoid and thyroid-hormone receptors (SMRT), which direct histone deacetylase activity to target gene repression (Aranda and Pascual, 2001; Peterson and Laniel, 2004). Other coactivators such as CARM-1 act through their histone methyltransferase (HMT) activity which upon methylation of specific arginine or lysine residues also change histone-DNA and histone-histone contacts (Roth et al., 2001; Zhang and Reinberg, 2001). Regulatory information from transcription factors and cofactors assembled at the promoter is transmitted through the Mediator, a giant multiprotein complex, to the general transcription machinery (Dilworth and Chambon, 2001; Malik and Roeder, 2000). The general transcriptional machinery, also called the preinitiation complex (PIC), includes the general initiation factors TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIF, which together with RNA polymerase II are responsible for initiation of transcription and synthesis of messenger RNA (Kornberg, 2005; Roeder, 2005). The contact between regulatory transcription factors and the general transcription machinery, the Mediator and chromatin remodeling co-activators can occur at many different levels, directly or indirectly. For example the C/EBP transcription factors have been shown to interact with the Mediator (Mo et al., 2004) and with two essential components of the general transcription machinery, the TATA box binding protein (TBP) and TFIIB (Nerlov and Ziff, 1995; Pedersen et al., 2001). Retinoid receptors interact with TFIIF (Rochette-Egly et al., 1997). The transcription factor Forkhead A1(FoxA1) has been
shown to bind compacted DNA and to open up the chromatin structure (Cirillo et al., 2002). C/EBPs have been shown to interact directly with HATs and HDAC (Chen et al., 2001; Mink et al., 1997; Pedersen et al., 2001). In addition, FOXA1 facilitates the binding of C/EBPs to their consensus site within the DNA (Cirillo et al., 2002). Together these interactions provide specificity in gene expression by recognition and binding of multiple transcription factors.

**Retinoic acid and gene expression**

Retinoic acid (RA) is a potent metabolite of vitamin A (Kastner et al., 1995) and acts as a growth and differentiation factor in many tissues (Chambon, 1996; Chen and Ross, 2004). RA results from sequential oxidation of vitamin A from retinol to retinaldehyde and to the acid forms, all-\textit{trans} RA, 9-cis and 13-cis RA (Chambon, 1996). Retinoids are essential for the control of normal cell differentiation and proliferation in a wide variety of cell types (Kastner et al., 1995). Among those cells that are growth inhibited by RA are bronchial epithelial cells (BECs) and RA in the physiological range, (0.001-1 µM) inhibits BEC proliferation (Albright et al., 2002). Most of the biological actions of RA are mediated by two families of nuclear retinoid receptors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs). All-\textit{trans} RA is the direct ligand for RARs, whereas 9-cis RA is the ligand for RXRs (Bohnsack and Hirschi, 2004). RARs are ligand-activated transcription factors responsible for regulating the expression of RA-responsive genes (Altucci and Gronemeyer, 2001; Bastien and Rochette-Egly, 2004; Petkovich, 1992). The binding of RA to RAR and RXR nuclear transcription factors enables the formation of RAR/RXR heterodimers that bind to two direct repeats (DR) of a core hexameric motif separated by five (DR5) base pairs (Leid et al., 1992; Mangelsdorf and Evans, 1995). The
functional receptor unit is a heterodimer (RXR/RAR) and dimerization is mediated primarily by the interacting surface of the ligand binding domain (LBD) on each receptor (Egea et al., 2000; Kurokawa et al., 1995). RXR occupies the 5` hexameric motif, whereas the RAR partner occupies the 3` motif (Chambon, 1996; Gronemeyer and Miturski, 2001). Ligand binding is facilitated by cellular retinoic acid-binding protein II (CRABPII) which upon shuttling into the nucleus and interaction with RAR/RXR heterodimers (Delva et al., 1999) channels retinoic acid to RARs (Budhu and Noy, 2002). RAR/RXR heterodimers then orchestrate the transcription of a wide range of retinoid responsive genes through binding to specific DNA sequences within the promoters of retinoid-responsive genes or RA response elements (RARE) and recruit cofactor complexes that act to modify local chromatin structure and/or engage the basal transcription machinery to the promoter (Bastien and Rochette-Egly, 2004; Chambon, 1996; Dilworth and Chambon, 2001; Gronemeyer and Miturski, 2001; Kastner et al., 1997; Mark et al., 1999; Wei, 2003). RARs also can be activated by signals other than retinoid ligands, such as those involving protein kinases and metabolic products (Chakravarti et al., 1996; Tahayato et al., 1993). RAR may act as either an activator or repressor of gene transcription (Petkovich, 1992). RARs are able to repress the activity of oncogenic β-catenin-mediated gene transcription, adaptor protein-1 (AP-1), PI3K/Akt pathway, and NF-kappa-B and therefore have potent antitumor, anti-proliferative and anti-inflammatory properties (Altucci and Gronemeyer, 2001; Easwaran et al., 1999; Nicholson et al., 1990; Salbert et al., 1993; Xiao et al., 2003). Differential usage of promoters and alternative splicing give rise to different isoforms (Chambon, 1994; Leid et al., 1992). Two major isoforms have been identified for RARα (α1 and α2) and for
RXRγ (γ1 and γ2), while five major isoforms are known for RARβ (β 1, 2, 4, 5 and 1’) (Chambon, 1994; Leid et al., 1992). Due to the antiproliferative and apoptosis-inducing effects of retinoids, both natural isomers of RA and various synthetic retinoids have been used therapeutically in human disease such as leukemia and other cancers including, Kaposi’s sarcoma, neuroblastoma, renal cell cancer and squamous cell cancers of the skin and cervix (Chao et al., 1997; Lippman et al., 1997; Nagy et al., 1998; Todesco et al., 2000). In addition, it has been shown recently that RA can activate the “orphan” nuclear receptor PPARβ/δ, which, in turn induce the expression of prosurvival genes (Schug et al., 2007). Recent studies have demonstrated that three intracellular lipid binding proteins (iLBPs), CRABP-II, fatty-acid binding protein 5 (FABP5) and FABP4 selectively cooperate with the nuclear receptors RARα, PPARβ/δ, and PPARγ, respectively (Budhu and Noy, 2002; Schug et al., 2007; Sessler and Noy, 2005). However, many questions remain about the molecular mechanism behind the effects of retinoids.

**Domain features of RARs and RXRs**

All the nuclear receptors, including RARs and RXRs, share common modular domains (A to F) (Fig. 1) that can be interchanged without loss of function (Aranda and Pascual, 2001; Bastien and Rochette-Egly, 2004; Moras and Gronemeyer, 1998; Renaud and Moras, 2000; Tenbaum and Baniahmad, 1997). The N-terminal A/B domains are the least conserved and contain an autonomous activation function, named activation function 1 (AF-1). The C domain, DNA-binding domain (DBD), is the most highly conserved domain and contains nine cysteins and two zinc finger modules responsible for DNA binding (Aranda and Pascual, 2001; Wei, 2003). The region at the base of the first finger is named the P box and is responsible for discriminating the DNA sequence, whereas the
region at the base of the second finger, D box, participates in receptor dimerization. The D domain, or hinge region, is variable and connects the zinc finger DBD to the variable E region, which encompasses the ligand-binding domain (LBD). The hinge region contains a nuclear receptor localization signal and is responsible for the interaction with certain nuclear coregulators (Abbondanza et al., 1998; Yu et al., 1998). The LBD is a multifunctional domain that is responsible for ligand binding, receptor dimerization, and interaction with proteins such as coactivators (Moras and Gronemeyer, 1998; Renaud and Moras, 2000). The structures of the LBDs of RARs and RXRs are similar as demonstrated by the crystallographic studies (Moras and Gronemeyer, 1998; Renaud and Moras, 2000; Wurtz et al., 1996). The LBD contains consensus phosphorylation sites that, upon activation of protein kinase A (PKA) signaling, is phosphorylated at a conserved serine residue (Rochette-Egly et al., 1995). The RXRs LBD also can be phosphorylated by mitogen-activated protein kinases (p38MAPKs) (Adam-Stitah et al., 1999; Lee et al., 2000). The activation function-2 (AF-2) domain is composed of an amphipathic α helix that also is highly conserved and is responsible for the agonist-induced conformational change of receptors that results in changes in protein interaction and stimulates the recruitment of coactivators, leading to the activation of gene transcription (Wei, 2003). The F region is absent in RXRs and its role in RARs is still unknown.

Fig. 1: major functional domain of RAR and RXR (Li-Na Wei, 2002)
Coregulators of RARs and RXRs

Numerous coregulatory proteins interact with the RARs and/or RXRs and play important roles in the ultimate control of the activity of a gene promoter regulated by RA (Wei, 2003). In the absence of ligands, or upon binding by antagonist, the receptor recruits corepressors including, N-CoR (Horlein et al., 1995) and SMRT. While in the presence of an agonist, the receptor changes its conformatinal structure to favor the interactions between RAR and RXR (Rastinejad et al., 2000) and release corepressors and recruit coactivators such as the steroid receptor coactivator (SRC/p160) family (which includes SRC-1, SRC-2 and SRC-3) (Chen, 2000; Leo and Chen, 2000), p300 (Vo and Goodman, 2001; Whyte et al., 1989), CBP and p300/CBP-associated factor (P/CAF) (Arias et al., 1994; Kwok et al., 1994).

Regulation of RAR/RXR-mediated transcription through phosphorylation

RARs and RXRs are substrates for a multitude of kinases (Fig. 2) (Rochette-Egly, 2003). Subsequent to their interaction with TFIIH, RARs (RARα and RARγ) are phosphorylated in their N-terminal A/B region by cdk7 subunit of TFIIH which has a cyclin dependent kinases activity (Bastien et al., 2000; Rochette-Egly et al., 1997). This phosphorylation process plays a critical role in the retinoid response. Phosphorylation can facilitate the recruitment of components of the transcription machinery and thereby stabilize the formation of nuclear receptor transcription complex. In addition, phosphorylation may facilitate the dissociation of RARα from transcription inhibitors or help the dissociation of RARα from the transcription machinery in order to allow elongation to proceed (Bastien and Rochette-Egly, 2004). Phosphorylation of the AF-1
domain of RARα does not influence the ubiquitylation and the proteosomal degradation of RARα (Kopf et al., 2000), while phosphorylation of RARγ by both TFIIH and p38MAPK is crucial for both the transcriptional activity and the degradation of RARγ (Gianni et al., 2002). Transcriptional activities of RARα and RARγ can also be modulated upon phosphorylation by other kinases in response to a variety of signals such as Protein kinas A (PKA) and Protein kinase C (PKC) (Bastien et al., 2002; Delmotte et al., 1999; Gianni et al., 2003; Lee et al., 2000; Matsushima-Nishiwaki et al., 2001; Rochette-Egly et al., 1995). In addition, coactivators and corepressors also can be phosphorylated. Phosphorylation of SMRT correlates with an inhibition of their interaction with RARs and their redistribution from the nucleus to the cytoplasm (Hong and Privalsky, 2000). In contrast, the phosphorylation of p300/CBP, SRC-1, and TIF-2 by a variety of kinases, including MAPK or PKA, enhances their enzymatic activity as well as their efficiency to interact with retinoid receptors and/or the HAT complexes (Font de Mora and Brown, 2000; Lopez et al., 2001; Rowan et al., 2000; Vo and Goodman, 2001).
Control of RAR/RXR transactivation by the ubiquitin-proteasome system

The transcriptional activity of retinoid receptors, as that of most transcription factors, also is regulated by the ubiquitin-proteasome pathway (Fig. 3) (Conaway et al., 2002; Muratani and Tansey, 2003). One main role of the ubiquitin-proteasome system is to degrade transcriptional activators. In this process, following a signal, the substrate protein is multiubiquitylated at a lysine group and then targeted for destruction by 26S proteasome.
proteasome (DeMartino and Slaughter, 1999). Within RARγ/RXR heterodimers bound at response elements, both receptors are degraded by the proteasome in response to retinoid (Gianni et al., 2002; Gianni et al., 2003; Kopf et al., 2000; Zhu et al., 1999). This process involves the ubiquitylation of RARγ and the recruitment of the proteasome at the AF-2 domain through SUG-1 which is one of six ATPase in the base of the 19S regulatory complex of the 26S proteasome. The ubiquitin-proteasome machineries may play a dual role, controlling on the one hand the functionality of RARγ/RXR heterodimers through helping the recruitment of the transcription machinery (Lin et al., 2002), and on the other hand the ubiquitylation and the subsequent degradation of the heterodimers. This degradation process may provide a mechanism to control the magnitude and the duration of retinoid-mediated transcription (Bastien and Rochette-Egly, 2004). This mechanism is not applicable for RARα since inhibition of the proteasome by specific inhibitors does not abrogate, but amplifies RARα-mediated transcription (Bastien and Rochette-Egly, 2004).
CCAAT/Enhancer Binding Proteins

CCAAT/enhancer binding proteins (C/EBPs) are a family of regulatory transcription factors whose expression has been linked to development, cellular differentiation, and regulation and expression of tissue specific genes. The cloning of the family’s original member, C/EBPα (Johnson et al., 1987) from rat liver nuclei which led to characterization of the basic leucine zipper (bZIP) domain (Graves et al., 1986; Johnson et al., 1987). Five other C/EBPs have been identified that interact with each other and transcription factors in other protein families. The six members include; C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, C/EBPε and C/EBPζ (Ramji and Foka, 2002; Roman et al.,
1990). C/EBPs have structural as well as functional homologies (Lekstrom-Himes and Xanthopoulos, 1998). The different members exhibit different modes of regulation and have individual stage-specific expression patterns. C/EBPα, C/EBPβ and C/EBPδ are expressed in several organs such as lung, liver, adipose tissue, intestine, adrenal gland, placenta, breast, and white blood cells of the myelocytic lineage (Antonson and Xanthopoulos, 1995; Birkenmeier et al., 1989; Buck and Chojkier, 2003; Cao et al., 1991; Darlington, 1999; Flodby et al., 1996; Poli, 1998). C/EBPε is primarily expressed in myeloid and lymphoid cells (Antonson et al., 1996; Chumakov et al., 1997; Yamanaka et al., 1997) whereas C/EBPγ and C/EBPζ are ubiquitously expressed (Roman et al., 1990; Ron and Habener, 1992). In adult human lung; C/EBPα, C/EBPβ and C/EBPδ are expressed in alveolar type II pneumocytes and C/EBPδ and lower levels of C/EBPα are expressed in Clara cells of the bronchiolar epithelium (Cassel et al., 2000b; Halmos et al., 2002; Nord et al., 1998). C/EBPs regulate the expression of several genes directly or indirectly during lung differentiation, including surfactant protein A (SP-A) (Rosenberg et al., 2002), SP-B and Clara cell-SP (CCSP/SCGB1) (Li et al., 1995; Nord et al., 1998), SP-D (He and Crouch, 2002) and P450-enzymes (CYP2B1) (Cassel et al., 2000a).

Several studies have demonstrated a major role of C/EBPs in human lung development. Antisense knock-down of C/EBPδ decreased expression of the endogenous SP-A gene in a human lung cancer cell line (Cassel and Nord, 2003). In transient transfections in lung epithelial cells, reporter gene activity driven by 1,350 bp of the CYP2B1 promoter was increased by both C/EBPα and C/EBPδ, and inactivation of the C/EBP-binding site caused a significant reduction of the transactivation efficiency from both factors (Cassel et al., 2000a). Knockout mice for C/EBPα have defects in liver and white and brown
adipose tissues, and die within a few hours after birth due to hypoglycemia (Wang et al., 1995). However, a few mice die from respiratory failure in C/EBPβ and C/EBPδ knockout mice, and it has been shown that C/EBPα knockout mice exhibit under-developed lung with hyperproliferation of alveolar type II cells at birth, indicating a role for C/EBPα in the control of proliferation-differentiation processes in the lung epithelium (Cassel and Nord, 2003; Flodby et al., 1996). In contrast to the C/EBPα knockout mice, double knockout mice for C/EBPβ and C/EBPδ have no demonstrated lung phenotype, but have defects in white adipose tissue and lack brown adipose tissue (Tanaka et al., 1997).

C/EBP structure and function

C/EBPs are classified as basic leucine zipper (bZIP) transcription factors based on the basic-leucine zipper domain in their C-terminal (Fig. 4). The leucine zipper domain is a highly positively charged domain that directly interacts with DNA and enables dimerization between family members and both homo- and heterodimers can be formed (Agre et al., 1989; Cassel and Nord, 2003; Landschulz et al., 1988; Ramji and Foka, 2002; Vinson et al., 1993). The C/EBPα, C/EBP-β, C/EBP-γ and δ genes are intronless, whereas C/EBPε and C/EBP-ζ contain two and four exons respectively (Ramji and Foka, 2002). All members of the C/EBPs family have similar basic region DNA-binding motifs except C/EBPζ which lacks DNA-binding activity (Cassel and Nord, 2003). The basic region, approximately 20 amino acids (Johnson, 1993), is highly homologous within the family and, as a consequence, the dimers exhibit similar target gene specificities and interact with virtually identical DNA sequences (5’ ATTGCACAAT 3’) (Cassel and Nord, 2003; Osada et al., 1996; Ramji and Foka, 2002). The N-terminus of the C/EBPs
harbors the transactivation domain, which interacts with, or recruits, co-activators and co-repressors. In contrast to the DNA-binding domain, the transactivation domain is less well conserved leading to different transactivation and/or repression potential of the different C/EBPs and dimers (Ramji and Foka, 2002). C/EBPγ lacks an activation domain and represses gene transcription by forming inactive heterodimers with other members of C/EBP family (Cooper et al., 1995). Different sized polypeptides can be produced for C/EBPs, C/EBPα mRNA can give rise to two polypeptides, 42kDa and 30kDa (Lin et al., 1993; Ossipow et al., 1993). Whereas C/EBPβ mRNA can produce three polypeptides; transcriptional activators 38 kDa and 35 kDa liver activating protein (LAP), and transcriptional repressor 20 kDa liver inhibitory protein (LIP), (Descombes and Schibler, 1991). In addition, C/EBPε mRNA can produce four polypeptides (32 kDa, 30 kDa, 27 kDa and 14 kDa) (Yamanaka et al., 1997). The complexity of the response to C/EBP is further increased by the fact that the C/EBPs also have the ability to dimerize with other transcription factors belonging to the bZIP family such as FOS/JUN (AP-1) (Hsu et al., 1994) and CREB (Vallejo et al., 1993), as well as to interact with non-bZIP factors such as p50 of NF-κB, glucocorticoid receptor (GR), p21 and the retinoblastoma (RB) protein (Boruk et al., 1998; Chen et al., 1996; Ramji and Foka, 2002).

Fig. 4: Basic structure of CCAAT/enhancer binding protein (Tobias N. Cassel, 2003)
Cell cycle regulation

In order to understand the role of RA and C/EBPs in normal human bronchial epithelial cells (BEC) differentiation, it is imperative to understand cell cycle regulation. For a living organism to grow, their individual cells must increase in size, make exact replicas of all their genetic material, and then go through a process of division. This results in two daughter cells, each with one complete copy of the genome. In order to transit the cell cycle and produce more or less identical daughter cells, essential regulators of the cell cycle contribute to sequential and controlled phases: genomic replication during S (synthesis) phase, segregation of chromosomes during M (mitotic) phase and two gap (G1 and G2) phases that separate S and M phases (Fig. 5). The decision of cells to differentiate is commonly made in G1 phase of the cell cycle, and the induction of differentiation requires cell cycle arrest. Further, phase checkpoints exist within the cell cycle, which serve to survey the cell and DNA integrity. Passage through the restriction point and entry into S phase is coordinated by the D-type cyclin and G1 cyclin-dependent kinases 4/6 (cdks4/6) (Morgan, 1995; Sherr, 1993; Stevens and La Thangue, 2003). Formation of complexes between cyclins and cyclin-dependant kinases causes the phosphorylation of the retinoblastoma tumor suppressor protein (pRb) (Ewen et al., 1993; Kato et al., 1993; Zetterberg et al., 1995) which enables release and activation of E2F family of transcription factors which in turn, leads to transactivation of numerous E2F downstream target genes that are required for cell cycle progression, particularly DNA synthesis proteins such as, DNA polymerase α, thymidine kinase, dihydropholate reductase (DHFR), and the genes involved with apoptosis (Chellappan et al., 1991; DeGregori et al., 1995; Lees et al., 1993; Weinberg, 1995).
The E2F family consists of six structurally related transcription factors (E2F1-6). The E2F consensus binding site is “TTTCGCGC,” present in the promoters of cellular genes required for cell division and apoptosis (Dyson, 1998). Specifically, the E2F family can be divided into three subgroups: E2F1-3, E2F4-5 and E2F-6. E2F1-3 bind exclusively with pRb, activate E2F-responsive genes and derive cellular proliferation (Stevens and La Thangue, 2003). E2F-4 and E2F-5 are predominantly cytoplasmic and need to assemble with a pocket protein to be transported into nuclei to act as repressors of E2F-responsive genes. E2F-6 differs from the other E2Fs; it lacks the C-terminal transactivation and pocket protein binding domains (Morkel et al., 1997). This has led to suggestion that E2F-6 may act as a repressor of E2F-responsive genes. Activation of E2F1, which is often associated with DP (DRFT1-plypeptide), is important for cell proliferation, progression into S phase and transactivation of cyclin E and c-myc (Rhee et al., 1994) as well as itself (DeGregori et al., 1995; Girling et al., 1993; Stevaux and Dyson, 2002; Stewart et al., 1995). c-myc is an oncogenic basic-helix-loop-helix-leucine zipper (bHLH-Zip) transcription factor that transactivates cyclin E and cdk4 (Hermeking et al., 2000; Jansen-Durr et al., 1993). c-myc dimerizes with another bHLH-Zip protein, Max, and recognizes the specific DNA sequence CACGTG, referred to as E-box (Henriksson and Luscher, 1996; Kretzner et al., 1992). Consequently, the phosphorylation of RB may result in the activation and upregulation of E2F1, upregulation of c-myc transcription by E2F1 and upregulation of cyclin E and cdk4 transcription by c-myc. Each of these events may contribute to a loss cell proliferation control if a compensatory negative feedback mechanism is absent or inactivated. Compensatory feedback signals including activation and/or upregulation of p53 by
p14/MDM pathway. P53 then acts to slow cell proliferation through the upregulation of the cyclin dependent kinase inhibitor (p21) (Harper et al., 1993; Xiong et al., 1993; Zhu et al., 1998). As another feedback signal, E2F1 transactivates p73 (Rodicker et al., 2001; Stiewe and Putzer, 2000), and p73 transactivates p21 (Zhu et al., 1998) which acts to inhibit the release of E2F1 from Rb. E2F1 is able to transactivate p21 (Hiyama et al., 1998), which inhibits the activity of cyclin-cdk complexes at G1 and G2 and results in cell cycle arrest (Vousden, 2000). Further, p21 can inhibit c-myc’s ability to activate the transcription of the proliferating cellular nuclear antigen (PCNA), an enzyme that binds to DNA polymerase and leads to inhibition of DNA replication (Kitaura et al., 2000).

Fig. 6: Cell cycle
Numerous studies have illustrated the effects of RA on cell cycle control (Chao et al., 1997; Lippman et al., 1997; Nagy et al., 1998; Todesco et al., 2000). Treatment of immortalized human bronchial epithelial cells with RA in the presence of carcinogens, compared to cells only exposed to carcinogens, decreases cyclin D1 and cyclin E protein expression resulting in G1 arrest (Langenfeld et al., 1997; Langenfeld et al., 1996). In addition, in vascular smooth muscle cells, RA inhibits proliferation and promotes differentiation through the downregulation of cyclins D3 and E, and CDK2, CDK4, and CDK6, resulting in hypophosphorylation of pRb and the inhibition of G1/S phase progression (Bohnsack and Hirschi, 2004). Further, RA induces ubiquitination of cyclin D1, resulting in its proteolysis and cell cycle arrest (Bohnsack and Hirschi, 2004; Spinella et al., 1999). Thus RA may act to prevent cancerous transition by inhibiting cell cycle progression through different mechanisms.

In addition C/EBPs play an important role in cell cycle control. C/EBPα and C/EBPβ have opposing roles during proliferation. In contrast to C/EBPβ which supports proliferation, C/EBPα is a strong inhibitor of proliferation. It directly interacts with cell cycle regulators and chromatin-remodeling proteins (Cao et al., 1991; Flodby et al., 1993; Hendricks-Taylor and Darlington, 1995; Iakova et al., 2003; Slomiany et al., 2000) and induces growth arrest (Hendricks-Taylor and Darlington, 1995). The growth inhibitory effects of C/EBPα are mostly independent of gene activation and instead are mediated through protein-protein interactions with several proteins involved in cell-cycle regulation such as p21 which leads to stabilization of p21 and protects the protein from degradation (Harris et al., 2001; Johnson, 2005). Further, C/EBPα interacts with
retinoblastoma function through interaction with Rb/E2F1 protein complex (Slomiany et al., 2000; Timchenko et al., 1996). C/EBPα also has been shown to cooperate with p21 to inhibit the cell cycle activator CDK2 (Harris et al., 2001), or to directly bind to both CDK2 and CDK4 and inhibit their function (Wang et al., 2001). C/EBPα is down regulated in proliferating cells and knockout mice for C/EBPα show increased proliferation in lung and liver cells (Basseres et al., 2006; Flodby et al., 1996). C/EBPα plays a major role in the all-trans retinoic acid-induced differentiation of myeloid cell lines (Halmos et al., 2002; Orkin, 2000). In addition, many cancer forms lack expression of C/EBPα suggesting that C/EBPα might function as a tumor suppressor (Halmos et al., 2002; Shim et al., 2005).

Fundamentally, maintaining genomic integrity and the normal division of cells requires coordinated control of the cell cycle mediated precise regulation of both cell cycle facilitators (cyclins, cyclin-dependant kinases (CDK), proto-oncogenes) and inhibitors (CDK-inhibitors, tumor suppressors).
All-trans retinoic acid downregulates CCAAT/enhancer binding proteins in human bronchial epithelial cells

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**Abbreviations**

AP-1, activator protein-1; BCL2, B-cell leukemia/lymphoma 2; BEBM, bronchial epithelial cell basal medium; BECs, bronchial epithelial cells; bZIP, basic region-leucine zipper; CCSP, Clara cell secretory protein; CYP, cytochrome P450; C/EBP, CCAAT/enhancer binding protein; DR, direct repeat; FBS, fetal bovine serum; FOXA1, hepatocyte nuclear factor 3 alpha; LBD, ligand binding domain; MMP-1, collagenase; MUC1, type I transmembrane protein; NFκB, nuclear factor-kappa B; PI3K, phosphoinositide 3-kinase; RA, retinoic acid; RAR, retinoic acid receptor; RA-OH, retinoic acid hydroxylase; RARE, retinoic acid response element; RXR, retinoid X receptor; SP, surfactant protein; StaRT-PCR, standardized reverse transcription polymerase chain reaction; TA, transcript abundance; TGM2, tissue transglutaminase 2.

**Background**

Most of the biological actions of RA are mediated by two families of nuclear retinoid receptors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs). All-trans RA is the direct ligand for RARs, whereas 9-cis RA is the ligand for RXRs (1). RARs are ligand-activated transcription factors responsible for regulating the expression of RA-responsive genes (2-4). The binding of RA to RAR and RXR nuclear transcription factors enables the formation of RAR/RXR heterodimers that bind to two direct repeats (DR) of a core hexameric motif separated by five base pairs (DR5) (5-6). The functional receptor unit is a heterodimer (RXR/RAR) and dimerization is mediated primarily by the interacting surface of the ligand binding domain (LBD) on each receptor (7-8). RXR occupies the 5` hexameric motif, whereas the RAR partner occupies the 3` motif (9-10).
Ligand binding is facilitated by cellular retinoic acid-binding protein II (CRABPII) which, upon shuttling into the nucleus and interaction with RAR/RXR heterodimers (11), channels retinoic acid to RARs (12). RAR/RXR heterodimers then orchestrate the transcription of a wide range of retinoid responsive genes through binding to specific DNA sequences within the promoters of these genes, or RA response elements (RARE), and recruit cofactor complexes that act to modify local chromatin structure and/or engage the basal transcription machinery to the promoter (13-19). RARs also can be activated by signals other than retinoid ligands, such as those involving protein kinases and metabolic products (20-21). RAR may act as either an activator or repressor of gene transcription (22). RARs are able to repress the activity of oncogenic β-catenin-mediated gene transcription, adaptor protein-1 (AP-1), PI3K/Akt pathway, and NF-kappa-B and therefore have potent antitumor, anti-proliferative and anti-inflammatory properties (23-27). Differential usage of promoters and alternative splicing give rise to different isoforms. Two major isoforms have been identified for RARα (α1 and α2) and for RXRγ (γ1 and γ2), while five major isoforms are known for RARβ (β1, 2, 4, 5 and 1’) (28-29). Due to the antiproliferative and apoptosis-inducing effects of retinoids, both natural isomers of RA and various synthetic retinoids have been used therapeutically in human disease such as leukemia and other cancers including, Kaposi’s sarcoma, neuroblastoma, renal cell cancer and squamous cell cancers of the skin and cervix (30-33). However, many questions remain about the molecular mechanism behind the effects of retinoids.

CCAAT/enhancer binding protein (C/EBPs) are a family of regulatory transcription factors whose expression has been linked to development, cellular differentiation, and regulation and expression of tissue specific genes. Since the cloning of the family’s
original member, C/EBPα from rat liver nuclei which led to characterization of the basic leucine zipper (bZIP) domain (34-35), five other C/EBPs have been identified that interact with each other and transcription factors in other protein families. The six members include; C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, C/EBPε and C/EBPζ (36-37). C/EBPs have structural as well as functional homologies (39). The different members exhibit different modes of regulation and have individual stage-specific expression patterns. C/EBPα, C/EBPβ and C/EBPδ are expressed in several organs such as lung, liver, adipose tissue, intestine, adrenal gland, placenta, breast, and white blood cells of the myelocytic lineage (40-46). C/EBPε is primarily expressed in myeloid and lymphoid cells (47-49) whereas C/EBPγ and C/EBPζ are ubiquitously expressed (50-51). In adult human lung; C/EBPα, C/EBPβ, C/EBPγ and C/EBPδ are expressed in alveolar type II pneumocytes and C/EBPδ and lower levels of C/EBPα are expressed in Clara cells of the bronchiolar epithelium (52-55). C/EBPs regulate the expression of several genes directly or indirectly during lung differentiation, including surfactant protein A (SP-A) (56), SP-B, SP-D, Clara cell secretory protein (CCSP/SCGB1) (57-59), and P450-enzymes (CYP2B1) (60). Several studies have demonstrated major role of C/EBPs in human lung development. Antisense knock-down of C/EBPδ decreased expression of the endogenous SP-A gene in a human lung cancer cell line (61). In transient transfections in lung epithelial cells, reporter gene activity driven by 1,350 base pairs of the CYP2B1 promoter was increased by both C/EBPα and C/EBPδ, and inactivation of the C/EBP-binding site caused a significant reduction of the transactivation efficiency from both factors (62). Knockout mice for C/EBPα have defects in liver and white and brown adipose tissues, and die within a few hours after birth due to hypoglycemia (63). However, a few mice die
from respiratory failure in C/EBPβ and C/EBPδ knockout mice, and it has been shown that C/EBPα knockout mice exhibit under-developed lung with hyperproliferation of alveolar type II cells at birth, indicating a role for C/EBPα in the control of proliferation-differentiation processes in the lung epithelium (64-65). In contrast to the C/EBPα knockout mice, double knockout mice for C/EBPβ and C/EBPδ have no demonstrated lung phenotype, but have defects in white adipose tissue and lack brown adipose tissue (66). In the present study, using standardized quantitative reverse transcription-PCR (StaRT-PCR), we found downregulation of C/EBPα, C/EBPβ, C/EBPγ and Spr-1 mRNA expression and upregulation of Bcl-2, FOXA1 and MUC1 mRNA in cultured bronchial epithelial cells exposed to 30 nM RA. These results indicate that RA enhances differentiation of BECs through downregulation of C/EBPα, C/EBPβ and C/EBPγ transcription factors.

**Results**

**Effect of All-trans RA on Cell Cycle Phase Distribution**

To establish the effects of all-trans RA on cultured bronchial epithelial cells, flow cytometric analysis was performed on control and RA-treated populations. Bronchial epithelial cells (D2) were plated on Transwell inserts for five days using serum free media in the presence or absence of 30 nM RA and harvested at 24, 48 and 72 hours intervals. Table 1 shows that there were no significant differences in G0/G1, S and G2/M cell cycle phases on (D2) RA-treated cells compared with control cells at 24, 48 and 72-hours. Further, no significant difference was observed in the H661 cell line. Although there was a decrease in percentage of apoptotic cells after 72 hours, there was no
significant difference between RA-treated and untreated controls, indicating that RA
treatment of BECs did not cause cell cycle arrest or induce apoptosis.

**Analysis of known retinoid-regulated genes**

cDNA prepared from all-trans RA treated or untreated cells for StaRT-PCR analysis
was used to verify that known retinoid-regulated genes were modulated by RA after 24
hours of all-trans retinoic acid treatment using StaRT-PCR. Known retinoid-regulated
genes include RARβ, retinoic acid hydroxylase (RA-OH), tissue transglutaminase 2
(TGM2), and collagenase (MMP-1). The gene that was chosen for investigation was
tissue transglutaminase 2 (TGM2). The expression of TGM2 was up-regulated by RA
treatment in all BECs (Fig. 1 and Table 3).

**All-trans RA Decreases C/EBPα, C/EBPβ and C/EBPγ Gene Expression**

Since C/EBPα, C/EBPβ and C/EBPγ are closely related bZIP transcription factors that
are expressed in respiratory epithelial cells, they may serve as target genes for RA
induced differentiation of BECs. We sought to determine the gene expression changes on
C/EBPs transcription factors after RA treatment. We measured the transcript abundance
levels of C/EBPs isoformes by StaRT-PCR. Mean values presented in units of
molecules/ 10E6 β-actin molecules are summarized in Table 3. In the case of cultured
primary bronchial epithelial cells (17684X1)

which was initially cultured on a collagen and fibronectin layer for five days and then transferred onto Transwell inserts, expression of C/EBPα was decreased (19 fold) after RA treatment but it did not reach the statistical significance (p= 0.077). In the case of the
other culture condition when cells were plated directly onto Transwell inserts for five
days before RA treatment, the transcript abundance levels for C/EBPα was decreased in
cultures from both individuals (13 fold for 17684X2 (p= 0.083) and 6 fold in D2 (p=
0.066) respectively but it did not reach the statistical significance (Fig.2a and Table 3).
Transcript abundance level for C/EBPβ was significantly decreased in 17684X1 BEC (6
fold) and it was not significant decrease in (D2) BEC (5 fold) (p=0.092) and (17684X2)
BEC (1 fold) (p=0.27) (Fig. 2b and Table 3). Transcript abundance for C/EBPγ was also
measured on two individuals. 17684X2 BEC showed significant reduction on C/EBPγ
transcript abundance levels after RA treatment (p< 0.05), while D2 did not reach the
statistical significance (Fig. 2c and Table 3). These data suggest that C/EBPα, C/EBPβ
and C/EBPγ transcription factors may not be involved in RA induced cellular
differentiation of cultured BECs.

**All-trans RA Enhances MUC1 and FOXA1 Gene Expression**

To test whether RA treatment of BECs can induce the expression of MUC1 and
FOXA1mRNA differentiation genes, transcript abundance levels for MUC1 and FoxA1
were assessed by StaRT-PCR before and after RA treatment. Expression of MUC1
mRNA was induced significantly (8.5 fold) after RA treatment for the first culture
condition (p< 0.05) whereas, for the second culture condition, the transcript abundance
level of MUC1 was induced in both individuals but it was statistically insignificant (p>
0.05) (Fig. 3a and Table 3). Similarly, transcript abundance levels for FOXA1 were
induced significantly after RA treatment with the first culture condition (43 fold, p< 0.05),
and, although the transcript abundance levels of FOXA1 were induced on both individuals
with the second culture condition (0.7 and 10 fold respectively), it was statistically insignificant (p > 0.05) (Fig. 3b and Table 3).

**All-trans RA Decreases Spr-1 Gene Expression**

To test whether RA treatment of BECs will enhance normal differentiation and inhibit squamous metaplasia in cultured cells, the transcript abundance levels of small, proline rich (Spr1) squamous differentiation marker were measured on RA treated and untreated BECs by StaRT-PCR. We found significant reduction of the transcript abundance levels in 17684X2 RA treated BEC compared with the untreated controls, and, although the transcript abundance levels of Spr1 was reduced on D2 BEC, it was statistically insignificant (p > 0.05). These results indicate that RA treatment of cultured BECs enhances normal cell differentiation by downregulating Spr1 (Fig. 4 and Table 3).

**All-trans RA Increases Bcl2 Gene Expression**

To test whether RA treatment protects BECs from spontaneous apoptosis, we measured the transcript abundance levels of anti-apoptosis Bcl-2 gene by StaRT-PCR before and after RA treatment. RA treatment significantly induced the transcript abundance levels of Bcl-2 in 17684X2 individual (p < 0.05) and although there was an induction on Bcl-2 transcript abundance level on the second individual, D2 BEC, the increase was statistically insignificant (Fig. 5 and Table 3).
Discussion

The purpose of this study was to investigate the role of All-trans Retinoic Acid (RA) in control of differentiation and proliferation in cultured normal bronchial epithelial cells (NBEC) by assessing transcript abundance of C/EBPs transcription factor genes and genes responsible for differentiation and apoptosis before and after RA treatment. RA is known to play vital role in various physiological processes such as embryogenesis, growth, differentiation and reproduction (67). A lot of studies have demonstrated that the effects of RA are mediated by two families of nuclear retinoid receptors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs) which are ligand activated transcription factors that are members of nuclear receptor superfamily (68). In addition, expression of C/EBPs have been linked to development, cellular differentiation, and regulation and expression of tissue specific genes in several organs (69). C/EBPα has been shown to inhibit proliferation and cause cell cycle arrest through protein-protein interactions with several proteins involved in cell-cycle regulation including p21 and Rb/E2F1 protein complex (70-74). On the other hand, C/EBPβ has been found to promote proliferation, and its levels are increased in number of tumors (75). Further, C/EBPγ has been shown to be responsible for regulating transcription of key antioxidant and DNA repair genes in nonbronchogenic individuals (76). Based on this knowledge, we hypothesized that RA may inhibit cell proliferation and induce cell differentiation through C/EBPs transcription factors, and that these in turn may regulate genes involved in controlling cell proliferation, differentiation and apoptosis.

In the current study, we evaluated the effect of RA on the transcript abundance levels of C/EBPs transcription factors in cultured BECs. We found downregulation of C/EBPα,
C/EBPβ and C/EBPγ transcription factors in response to RA treatment. These results suggest that C/EBPs may not be involved RA induced differentiation of BECs in culture. To verify successful RA treatment of our BECs, we measured gene expression levels of a previously identified RA-regulated gene, tissue transglutaminase 2 (TGM2) (77). RA-regulated genes have functional retinoid response elements (RARE) in their promoters that allow retinoic acid receptor to enhance transcription. Functional RAREs are found in the promoter region of TGM2 (78). TGM2 has roles in the regulation of proliferation, differentiation, apoptosis and other biological function (79). Our results showed that TGM2 was upregulated by RA treatment of BECs after 24 hours. To investigate whether RA treatment of BECs enhanced differentiation of BECs in culture, we measured the transcript abundance levels of two differentiation genes MUC1 and FOXA1. MUC1 has been shown to be expressed in respiratory epithelium and is considered to be a marker for respiratory epithelium differentiation (80). In our study, we observed that MUC1 transcript abundance levels in cultured BECs were upregulated by RA treatment. FOXA1 transcription factor is another differentiation marker in which we found induction in the transcript abundance level in response to RA treatment of BECs. FOXA1 transcription factor has been shown to be expressed in the pulmonary epithelium and regulate cell differentiation and lung development and studies on FoxA1–/– mice showed some delay in lung epithelial maturation during embryogenesis (81).

The small, proline-rich (SPR1) gene is known to be a biomarker associated with squamous cell differentiation of airway epithelium (82). Spr1 is absent in normal mucociliary epithelium of the respiratory tract and epithelia that undergo squamous differentiation express SPR1. In our study we showed that all-trans RA treatment of
BECs prevented squamous differentiation and enhanced normal differentiation through downregulation of Spr1 transcript abundance levels in RA treated BECs compared to untreated controls.

BCL-2 is an anti-apoptotic gene that is associated with regulation of apoptosis (83). Due to the important role of Bcl-2 in apoptosis regulation, we sought to test whether all-trans RA treatment can inhibit spontaneous apoptosis of BECs in culture. In our study, we observed that all-trans RA treatment induced Bcl-2 transcript abundance levels in RA treated BECs compared with untreated controls. This is consistent with recent reports that RA can activate the survival pathway through the nuclear receptor PPARβ/δ in normal mammary tissues that express low level of CRABP-II and they have shown that in human breast adenocarcinoma cell line (MCF-7) that has increased FABP5/CRABPII ratio, RA is converted from a proapoptotic to an anti-apoptotic agent (84).

Materials and Methods

Cell lines and culture conditions

The H661 large cell carcinoma cell line (American Type Culture Collection, VA, USA) was cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (Clonetics, San Diego). Cells were grown to confluence in a T-75 culture flask. Cells were washed in PBS buffer, trypsin dissociated, resuspended in medium, and transferred into 6-well plastic plates at a concentration of 100,000 cells/ml. Cells were cultured in the presence or absence of 30 nM RA (Sigma, Inc.) for 24, h, 48 h and 72 h and then sent for flow cytometric analysis. Primary human bronchial epithelial cells were suspended in a serum-free hormone-supplemented medium (BEBM, Clonetics, Cambrex Bioscience)
supplemented with growth factors and cytokines, necessary for cell growth and plated into T-75 tissue culture flasks coated with a layer of collagen and fibronectin, and incubated until confluency (7-10 days). The confluent primary cultures then were passaged into a Transwell (Corning Coster, NY) chamber (25 mm) at 10,000-20,000 cells/cm² in serum-free medium with the addition of 30nM concentration of All-trans-RA. After 24 hours, the cells were lysed and RNA was extracted for StaRT-PCR transcript abundance measurement of selected genes. Cultures used in the study were incubated for 14 days after the initial plating.

**RNA extraction and Reverse Transcription**

For cultured or primary cells, total RNA was extracted by phenol/chloroform methods using TRI-Reagent (Molecular Research Center Inc., Cincinnati, OH). The aqueous phase from each lysate (containing total RNA) was precipitated in isopropanol, washed in ethanol, and resuspended in 30µl of RNase-free water. The RNA then was subjected to DNase I (Ambion, Inc.) for 30 minutes at 37°C in order to remove any genomic contamination. Approximately 1µg of denatured RNA was combined with 500µg /ml Oligo dT primer (Promega, Inc), 10mM dNTPs, 25 units/µl RNasin (Promega, Inc), and 200 units/µl M-MLV-Reverse Transcriptase (Invitrogen, Inc) and incubated for 1 hour at 37°C. Reaction then stopped by incubating at 94°C for five minutes.

**Standardized RT-PCR (StaRT-PCR)**

StaRT PCR was used for all transcript abundance measurements as previously described (85-86). Briefly, each gene is co-amplified with an internal standard within a standardized mixture of internal standard (SMIS) at a known concentration ranging from
10E-12 M to 10E-17. Each internal standard is 10-20% shorter in length than the target gene PCR product. Internal standards for genes, FOXA1, Bcl-2, TGM2 and MUC1 were commercially prepared (Gene Express, Inc.) while internal standards for CEBPα, CEBPβ and CEBPγ were prepared in this laboratory. For each PCR reaction, the amount of cDNA in balanced with β-actin internal standard was used. The appropriate dilution of cDNA was determined by calibrating the native β-actin in 1µl of cDNA to (600,000 molecules) β-actin. Calibrated cDNA samples were used in all PCR reactions. Prior to amplification, equal volumes of cDNA and internal standard were combined into a master mix along with the appropriate volume of RNase free H₂O, 30 mM MgCl₂, 2 mM dNTPs, and a minimum of 0.1 unit of taq polymerase (Promega, Inc.). All PCR reactions were performed in a rapidcycler (Idaho Technologies, Inc.). After PCR, 1µl of the PCR product was electrophoresed and quantified on an Agilent 2100 bioanalyzer using DNA chips with DNA 1000 kit reagent according to manufacturer’s recommended protocol (Agilent Technologies, Palo Alto, CA), and then each gene was quantified based on the ratio of the endogenous gene product (NT, native templet) to its respective internal standard (IS) within the SMIS. Sequence information for primers that have been used for TA measurement in all cultured and primary samples is provided in Table(2).

**Flow Cytometry**

To determine DNA content and cell cycle phase, (D2) bronchial epithelial cells were incubated for 24, 48 and 72 hours with 30 nM RA. Adherent and suspended cells were collected as described above. Cells were fixed in 100% ethanol for 20 minutes and
washed with PBS before staining with 50 µg/ml propidium iodide (PI). DNA content was analyzed on FACSCalibur Flow Cytometer (Beckman-Coulter Corp., Miami, FL).

**Statistical analysis**

An unpaired student’s T-test was used to determined if each gene was significantly affected by RA treatment (p-value less than 0.05 was considered statistically significant). All statistical tests were done using SPSS 11.5.1 for Machintosh (SPSS, Chicago, IL). Creation of graphs were accomplished using Excel 2000 (Microsoft Corp., Redmond, WA).

**Conclusions**

Our results indicate that RA exerts two effects on culture bronchial epithelial cells, a differentiating effect through downregulation of Spr-1 and C/EBPβ and upregulation of FOXA1 and MUC1 and a protective effect from apoptosis through upregulation of Bcl-2 antiapoptotic gene.

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Figures

Fig.1. All-trans RA up-regulates TGM2

Expression of known retinoid-regulated gene by standardized quantitative reverse transcription-PCR (StaRT-PCR). BECs were treated with 30 nM All-trans RA for 24 hours. mRNA expression levels were normalized to β-actin; All-trans RA was determined to significantly up-regulate TGM2 mRNA as determined by unpaired student’s t-test of unequal variances. Each column and error bar represents mean and standard deviations of at least triplicate measurements.
Fig. 2a. All-trans RA down-regulates C/EBPA

Expression of C/EBPα mRNA by StaRT-PCR. BECs were treated with 30 nM All-trans RA for 24 hours. mRNA expression levels were normalized to β-actin; All-trans RA down-regulates C/EBPα mRNA in all individuals but it did not reach the statistical significance as determined by unpaired student’s t-test of unequal variances. Each column and error bar represent mean and standard deviation of at least triplicate measurements.
Fig. 2b. All-\textit{trans} RA down-regulates C/EBP\(\beta\)

Expression of C/EBP\(\beta\) mRNA by StaRT-PCR. BECs were treated with 30 nM All-\textit{trans} RA for 24 hours. mRNA expression levels were normalized with \(\beta\)-actin; All-\textit{trans} RA significantly down-regulates C/EBP\(\beta\) mRNA on 17684X1 BECs as determined by unpaired student’s t-test of unequal variances. Each column and error bar represent mean and standard deviation of at least triplicate measurements.
Fig. 2c. All-trans RA down-regulates C/EBPγ

Expression of C/EBPγ mRNA by StaRT-PCR. BECs were treated with 30 nM All-trans RA for 24 hours. mRNA expression levels were normalized with β-actin; All-trans RA significantly down-regulates C/EBPγ mRNA on 17684X2 BECs as determined by unpaired student’s t-test of unequal variances. Each column and error bar represent mean and standard deviation of at least triplicate measurements.
Fig. 3a. All-trans RA up-regulates MUC1

Expression of MUC1 mRNA by StaRT-PCR. BECs were treated with 30 nM All-trans RA for 24 hours. mRNA expression levels were normalized with β-actin; All-trans RA up-regulates MUC1 mRNA on 17684X1 BECs significantly as determined by unpaired student’s t-test of unequal variances. Each column and error bar represent mean and standard deviation of at least triplicate measurements.
Fig. 3b. All-trans RA up-regulates FOXA1

Expression of FOXA1 mRNA by StaRT-PCR. BECs were treated with 30 nM All-trans RA for 24 hours. mRNA expression levels were normalized with β-actin; All-trans RA up-regulates FOXA1 mRNA on all individual and it was significant on 17684X1 BECs as determined by unpaired student’s t-test of unequal variances. Each column and error bar represent mean and standard deviation of at least triplicate measurements.
Fig. 4. All-trans RA up-regulates Spr-1

Expression of Spr-1 mRNA by StaRT-PCR. BECs were treated with 30 nM All-trans RA for 24 hours. mRNA expression levels were normalized with β-actin; All-trans RA significantly down-regulates Spr-1 mRNA on 17684X2 BECs as determined by unpaired student’s t-test of unequal variances. Each column and error bar represent mean and standard deviation of at least triplicate measurements.
Fig. 5. All-trans RA up-regulates Bcl-2

Expression of Bcl-2 mRNA by StaRT-PCR. BECs were treated with 30 nM All-trans RA for 24 hours. mRNA expression levels were normalized with β-actin; All-trans RA significantly up-regulates Bcl-2 mRNA on 17684X2 BECs as determined by unpaired student’s t-test of unequal variances. Each column and error bar represent mean and standard deviation of at least triplicate measurements.
Fig. 6a: Lung section comparing genes expression from normal and squamous metaplasia

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<th>Gene Expression Associated with Different Airway Epithelial Histologies in Organ Donor 012401</th>
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<td>520,000</td>
<td>230,000</td>
</tr>
<tr>
<td>ARNT</td>
<td>15,000</td>
<td>5,200</td>
</tr>
<tr>
<td>CYP1A1</td>
<td>NQ</td>
<td>LOW</td>
</tr>
</tbody>
</table>
Fig. 6b: Lung section comparing genes expression from normal and squamous metaplasia

Variation in Gene Expression Associated with Different Airway Epithelial Histologies in Organ Donor 012401

HistoMorphology

Squamous metaplastic

Normal, ciliated, mucous secreting

StaRT-PCR Gene Expression Data

Higher in Normal

<table>
<thead>
<tr>
<th>Gene</th>
<th>Normal</th>
<th>Squamous</th>
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</thead>
<tbody>
<tr>
<td>HNF3A</td>
<td>&lt;600</td>
<td>2,900</td>
</tr>
<tr>
<td>MUC1</td>
<td>&lt;60</td>
<td>55,000</td>
</tr>
<tr>
<td>ICAM1</td>
<td>&lt;1000</td>
<td>5,900</td>
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</table>

Higher in Squamous

<table>
<thead>
<tr>
<th>Gene</th>
<th>Normal</th>
<th>Squamous</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPR1</td>
<td>&lt;100</td>
<td>360,000</td>
</tr>
<tr>
<td>KERATIN 5</td>
<td>150,000</td>
<td>&lt;100</td>
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</tbody>
</table>
### Tables

Table 1: Cell Cycle Distribution and apoptosis of BECs and H661 cell line in the presence or absence of 30 nM RA

<table>
<thead>
<tr>
<th></th>
<th>G0 &amp; G1 phases</th>
<th>S Phase</th>
<th>G2 &amp; M phases</th>
<th>Apoptosis</th>
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<tbody>
<tr>
<td>24 h CTR</td>
<td>D2 35%</td>
<td>22.6%</td>
<td>23.4%</td>
<td>7%</td>
</tr>
<tr>
<td>24 h + RA</td>
<td>D2 33.7%</td>
<td>21%</td>
<td>22%</td>
<td>8%</td>
</tr>
<tr>
<td>48 h CTR</td>
<td>D2 41.5%</td>
<td>19.9%</td>
<td>14.8%</td>
<td>10%</td>
</tr>
<tr>
<td>48 h + RA</td>
<td>D2 45%</td>
<td>21.8%</td>
<td>14.7%</td>
<td>6.7%</td>
</tr>
<tr>
<td>72 h CTR</td>
<td>D2 46.9%</td>
<td>18%</td>
<td>16.3%</td>
<td>3.6%</td>
</tr>
<tr>
<td>72 h + RA</td>
<td>D2 46%</td>
<td>18.5%</td>
<td>16.4%</td>
<td>3%</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>G0 &amp; G1 phases</th>
<th>S Phase</th>
<th>G2 &amp; M phases</th>
<th>Apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td>H661 49.60%</td>
<td>27.40%</td>
<td>8.20%</td>
<td>11.70%</td>
</tr>
<tr>
<td>24 h + RA</td>
<td>H661 49%</td>
<td>32.70%</td>
<td>6.13%</td>
<td>8.30%</td>
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<tr>
<td>48 h</td>
<td>H661 51.70%</td>
<td>22%</td>
<td>5%</td>
<td>18.70%</td>
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<tr>
<td>48 h + RA</td>
<td>H661 54.60%</td>
<td>22.60%</td>
<td>5.40%</td>
<td>15%</td>
</tr>
<tr>
<td>72 h</td>
<td>H661 63.60%</td>
<td>24.80%</td>
<td>6%</td>
<td>3.10%</td>
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<tr>
<td>72 h + RA</td>
<td>H661 60.80%</td>
<td>28%</td>
<td>6%</td>
<td>3.3%</td>
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</table>

Values represent the mean percentage of cells in the indicated phase from three independent experiments ± SD.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession #</th>
<th>Primer</th>
<th>Sequence</th>
<th>Position</th>
<th>Prod.</th>
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<tr>
<td>ACTB</td>
<td>X00351</td>
<td>Forward</td>
<td>5' ATC CTC ACC CTG AAG TAC CC 3'</td>
<td>231</td>
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<td></td>
<td></td>
<td>Reverse</td>
<td>5' CCA TCT CTT GCT CGA AGT CG 3'</td>
<td>704</td>
<td>493</td>
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<tr>
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<td></td>
<td>CT</td>
<td>5' CCA TCT CTT GCT CGA AGT CCG CCA GCC AGG TCC AGA CGC A 3'</td>
<td>568</td>
<td>377</td>
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<tr>
<td>Bcl-2</td>
<td>M14745</td>
<td>Forward</td>
<td>5' TTT TAG GAG ACC GAA GTC CG 3'</td>
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<tr>
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<td>5' AGC CAA CTG GCC ATG TGC TAC CTC TGT TCC TCT CTA C 3'</td>
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<td>CEBPA</td>
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<td>350</td>
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<tr>
<td></td>
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<td>5' GAT CCC AGA AAA TAG CCT CCA ATG AAC ATT CAA GCC ACA AGC TC 3'</td>
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<td>282</td>
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<tr>
<td>HNF3A</td>
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<td>Forward</td>
<td>5' GCT CTA CGT TGC CCG CCA GCC TG 3'</td>
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</tr>
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<td>Reverse</td>
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<tr>
<td>MUC1</td>
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<td></td>
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<td>5' ACA ATT GAC TCT GGC CTT CCA CGA TCT CAG ACG TCA GCG T 3'</td>
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<td>Spr-1</td>
<td>M84757</td>
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<tr>
<td></td>
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<td>Reverse</td>
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<td>TGM-2</td>
<td>M55153</td>
<td>Forward</td>
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<td>397</td>
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<tr>
<td></td>
<td></td>
<td>CT</td>
<td>5' GAA AGG CTC CAG GTG TAG AGC ACG TAC CCG CAT GGC CAT C 3'</td>
<td>1551</td>
<td>223</td>
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</table>
Table 3: Transcript abundance levels after RA treatment

<table>
<thead>
<tr>
<th>Gene</th>
<th>17684X1</th>
<th>17684X2</th>
<th>D2</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>pvalue</td>
</tr>
<tr>
<td>30 nM RA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bcl-2</td>
<td></td>
<td>1.00E+01</td>
<td>8.8E+01</td>
</tr>
<tr>
<td>C/EBPα</td>
<td>1.77E+03</td>
<td>9.23E+01</td>
<td>0.077</td>
</tr>
<tr>
<td>C/EBPβ</td>
<td>1.10E+03</td>
<td>1.82E+02</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>C/EBPγ</td>
<td></td>
<td>1.95E+02</td>
<td>7.50E+01</td>
</tr>
<tr>
<td>FOXA1</td>
<td>5.90E+01</td>
<td>2.56E+03</td>
<td>0.008</td>
</tr>
<tr>
<td>MUC-1</td>
<td>5.28E+02</td>
<td>4.52E+03</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Spr-1</td>
<td>3.00E+05</td>
<td>2.93E+04</td>
<td>0.034</td>
</tr>
<tr>
<td>TGM-2</td>
<td>2.00E+01</td>
<td>3.73E+03</td>
<td>0.0031</td>
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</table>

BECs were treated with 30 nM RA for 24 hours. Transcript abundance was measured by StaRT-PCR and mean values are shown in units of molecules/10E6 β-actin molecules. Statistical significance was determined by an Unpaired Student’s T-test. P values were obtained from genes that had detectable transcript abundance and at least three or more independent measurements from treated and untreated samples. p values less than 0.05 are considered significant.
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Abstract

The goal of our studies was to elucidate mechanisms that control and modulate normal bronchial epithelial cell (BECs) proliferation and differentiation in respiratory epithelium. We have evaluated the effects of All-trans retinoic acid (RA) treatment of BECs in culture. BECs cultured in serum free media were exposed to 30 nM RA, and gene expression analysis was performed with a standardized quantitative reverse transcription polymerase chain reaction (StaRT-PCR) after 24 hours exposure. Treatment of BECs with RA enhances differentiation. This enhancement was correlated with a decrease in the transcript abundance levels of squamous differentiation marker, small, proline-rich 1 gene (Spr1) and the leucine zipper family transcription factors CCAAT/enhancer binding proteins, C/EBPα, C/EBPβ and C/EBPγ, and an increase in the levels of retinoic acid modulated gene tissue transglutaminase 2 (TGM2), anti-apoptotic protein B-cell leukemia/lymphoma 2 (Bcl-2) and differentiation genes, forkhead box A1 (foxA1) and cell surface associated gene (MUC1). Our results indicate that RA induced BEC differentiation independent of the cell cycle arrest and apoptosis pathway.