LINE-1 COUPLES EPITHELIAL-MESENCHYMAL TRANSITION PROGRAMMING WITH THE ACQUISITION OF ONCOGENIC PHENOTYPES IN HUMAN BRONCHIAL EPITHELIAL CELLS

by

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As members of the Master's Committee, we certify that we have read the thesis prepared by *Ivan Alspuro*, titled *LINE-1 couples Epithelial-Mesenchymal Transition programming with the acquisition of oncogenic phenotypes in human and bronchial epithelial cells* and recommend that it be accepted as fulfilling the thesis requirement for the Master's Degree.

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Final approval and acceptance of this thesis is contingent upon the candidate's submission of the final copies of the thesis to the Graduate College.

We hereby certify that we have read this thesis prepared under our direction and recommend that it be accepted as fulfilling the Master's requirement.

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ABSTRACT

Lung cancer has the highest cancer-related mortality in the United States; there is an imminent need to identify novel molecular targets that can help improve the precision of current therapies. Recent studies have implicated the long interspersed nuclear element-1 (L1) in the onset and progression of lung cancers, but the molecular bases of this response remain largely unknown. L1 is an abundant and highly polymorphic genetic element that mobilizes via retrotransposition using L1-encoded ORF1p and ORF2p proteins. L1 expression is tightly regulated in somatic tissues and reactivated by tobacco carcinogens, such as benzo(a)pyrene (BaP). Its mobilization to new sites throughout the genome serves to globally regulate genetic structure and function and has been associated with epithelial-to-mesenchymal transition (EMT), a key event in malignant transformation which has been associated with chemoresistance and invasive phenotypes leading to metastasis. Therefore, to assess whether L1 induces EMT, chemoresistance, and invasive phenotypes involved in lung cancer progression and metastatic conversion, non-malignant, human bronchial epithelial BEAS-2B cells were stably transfected with vectors that constitutively expressed wildtype L1; a mutant counterpart that lacked reverse transcriptase activity, and was therefore unable to retrotranspose (mutant L1); or empty vector as a control. Immunoblotting showed that cells expressing L1 and mutant L1 proteins exhibited increased expression of mesenchymal markers coupled with decreased expression of epithelial markers compared to control cells. These changes in markers were seen in the cell lines transfected with both L1 and mutant L1, which is incapable of retrotransposition. Remarkably, blockade of L1 production with siRNA in cells constitutively expressing L1 and mutant L1 resulted in regression to a more epithelial phenotype. Furthermore, our data showed that cells

transfected with L1 have increased survival rates when exposed to tyrosine kinase inhibitor sunitinib-malate. Finally, *in-vivo* experiments in which nu/nu mice were injected with cell lines constitutively expressing either L1 or mutant L1 resulted in tumor formation in both populations of mice, not seen in control mice. Based on these findings, we conclude that: 1) L1 alters non-malignant bronchial epithelial cell plasticity to induce EMT phenotypes via retrotransposition-independent mechanisms, 2) L1-mediated changes are partially reversible, 3) L1-mediated EMT may contribute to lung cancer progression by promotion of chemoresistance, and 4) tumorigenesis induced by L1 is mediated by both retrotransposition-competent and retrotransposition-deficient L1. These findings suggest that the retrotransposition-independent pathway of L1 is sufficient for cellular transformation, and that retrotransposition events are not necessary for L1-induced cellular transformation in the BEAS-2B cell line.

INTRODUCTION

Significance

Non–small cell lung cancers (NSCLC) are the most frequent forms of lung cancer, the leading cause of cancer-related mortality, and highest cancer-related economic burden in the United States (US) and worldwide[1]. NSCLC primarily consists of squamous cell lung cancer and lung adenocarcinoma, which account for up to 30 and 40 percent of NSCLC, respectively. Lung cancer is strongly correlated to the prevalence of tobacco smoking, as evidenced by the high rates of adenocarcinoma seen in Kentucky, the state with the highest rates of smoking in the country[2]. Conversely, Utah with the lowest rates of smoking, has the lowest rates of NSCLC[2]. Although cigarette smoking has peaked in the US due to aggressive public health education campaigns, NSCLC is most frequently diagnosed in former smokers[2]. This means that even if smoking rates continue to decline in the US, diagnosis of NSCLCs may continue to rise for years to come. Furthermore, secondhand smoke, alcohol, air pollution, lack of physical activity and occupational exposures to silica or asbestos have been linked to the development of NSCLC[2]. Currently, there is great interest to increase the effectiveness of lung cancer diagnostics.

NSCLC are heterogeneous in nature. Current chemotherapeutic treatments have relatively low response rates in patients with advanced disease and surgery remains the major treatment for NSCLC, if detected early[3]. Therefore, novel molecular targets must be identified to increase the efficacy of current therapeutic options and decrease unwanted toxicities. Epigenetic factors, such as global DNA hypermethylation and hypomethylation can be used as prognostic determinants in patients with NSCLC[4]. DNA hypermethylation leads to transcriptional silencing of genes, and hypermethylation in the promoter regions of *APC, CDH13* and *RASSF1* may serve as indicators of early recurrence of lung cancer[4]. Less focus has been placed on global DNA hypomethylation.

However, an increasing number of studies links hypomethylation of Long Interspersed Nuclear Element-1 (L1) with poor prognosis of NSCLC patients[4-6]. Given this correlation, we sought to determine whether ectopic expression of L1 proteins in bronchial epithelial induced a more mesenchymal phenotype, induced chemoresistance, and induced a tumorigenic phenotype *in vivo*.

L1 Molecular Genetics and Physiology

L1 is an autonomous and highly mutagenic genetic element that mobilizes throughout the genome via retrotransposition[7,8]. L1 reactivation in somatic cells elicits a variety of genetic alterations including aberrant splicing, exon skipping, gene fusions, and genome rearrangements that can change gene expression and promote genomic instability[5]. These genetic alterations may be critical to neoplastic transformation and cancer progression[9,10]. In humans, L1 copies constitute 17-20% of the genome, though only ~100 L1s per individual remain retrotransposition competent due to truncations, inversions, or point mutations within the L1-encoded proteins in the inactive copies[5]. A functional L1 element in humans is ~6 kb in length and consists of a 5′ UTR (untranslated region), two open reading frames, and a 3′ UTR terminating in the poly (A) tail[11,12]. L1 encodes two proteins – ORF1p and ORF2p. ORF1p is a 40 kDa protein with nucleic acid binding activity, while ORF2p is a 150 kDa protein with endonuclease and reverse transcriptase activities[5]. A full L1 retrotransposition cycle begins with transcription of the gene itself. L1 mRNA is then transported to the cytosol, where ORF1p and ORF2p are produced independently via translation of L1 mRNA. In the cytosol, ORF1p and ORF2p preferentially bind to their own coding mRNA, which results in the formation of a ribonucleoprotein particle (RNP). It is unknown how the RNP is transported to the nucleus, but studies have shown that retrotransposition occurs in the absence of cell division, which means that nuclear transport does

not require the nuclear capsule to be dissolved[7]. Once in the nucleus, ORF2p breaks one strand in the genomic DNA. This break exposes a 3'-OH group, which is used as a primer for reverse transcription using L1 mRNA as a template. The process by which complementary DNA (cDNA) completes the insertion is also currently unknown. The completion of the cycle may result in the insertion of a full, retrotransposition-competent L1 or truncated L1 fragment in genomic DNA[5,7]. Figure 1 shows a visual representation of a full L1 retrotransposition cycle, used with permission from Copyright Clearance Center.

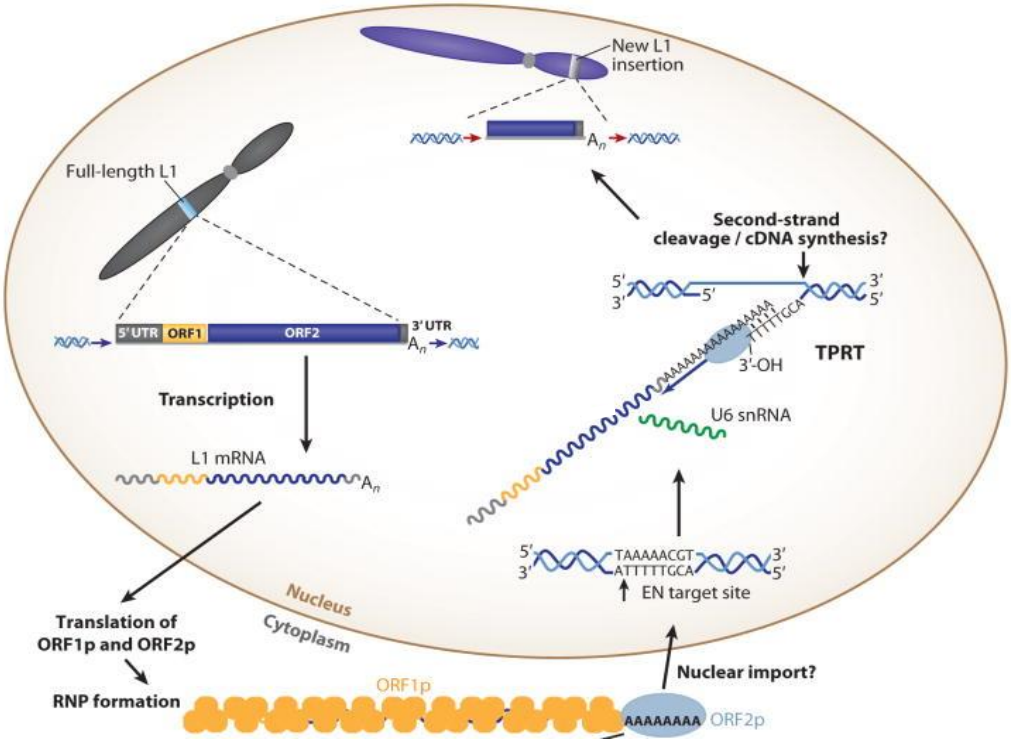

Figure 1[5]: A full cycle of L1 begins with transcription of the L1 gene. L1 mRNA is then exported into the cytoplasm where ORF1p and ORF2p are translated. ORF1p and ORF2p preferentially bind to L1 mRNA and form a ribonucleoprotein particle (RNP). The RNP is then imported back into the nucleus, wherein ORF2p binds a target site for its endonuclease component at a different site, breaks one strand of DNA, uses L1 mRNA as a template for retrotransposition and finally incorporates a new L1 copy into a different genomic location[72].

L1 is silenced epigenetically in somatic tissues[18]. Epigenetic mechanisms influence how biological complexes access the genome in a cell-specific, heritable manner, without changes in DNA sequence. The epigenetic L1 regulatory network includes histone modifications, nucleosome positioning, small non-coding RNAs, and DNA methylation. When L1 elements are methylated, few or no L1 proteins are expressed[20]. CpG methylation inhibits L1 promoter activity via recruitment of histone deacetylases, which create transcriptionally inactive chromatin structures. Methyl-CPG-binding protein 2 (MeCP2) is especially important for L1 silencing. MeCP2 is a member of the methyl-CpG-binding domain (MBD) protein family, which binds to methylated L1 5' UTR to inhibit transcription. In addition to DNA methylation, RNA editases have also been implicated in regulating the expression of L1[20]. During the process of RNA editing, nucleotide sequences in an RNA molecule are modified. RNA-dependent deaminases are responsible for changing cytidine into uridine, or adenosine into inosine. Specifically, apolipoprotein B mRNA-editing enzyme complex (APOBEC) 3A inhibits L1 retrotransposition by deaminating exposed single-stranded DNA that is created as a result of *de novo* L1 integration events[20]. APOBEC3A deaminates cytosine to uracil, which is removed from DNA by uracil DNA glycosylase and apurinic/apyrimidinic endonuclease, leading to degradation of L1 retrotransposition intermediates[73]. Other L1 regulators include Moloney leukemia virus 10 (MOV10), an RNA helicase that inhibits retrotransposition by associating with the L1 RNP particle[20].

L1 and Lung Carcinogenesis

L1 is reactivated in several types of cancer, including NSCLC[14,19]. The genome of lung cancer cells is one of the most frequently affected by L1 insertions. The reactivation of L1 is associated with insertional mutagenesis into different locations and global changes in gene

expression[5,14,20]. Studies of a panel of different human lung neoplasms revealed that more than 50% of malignant epithelial lung tumors express ORF1p; this expression was not detected in non-neoplastic somatic tissues[14].

L1 is reactivated by the lung carcinogen benzo(a)pyrene (BaP), a polycyclic aromatic hydrocarbon (PAH)[21] and a strong carcinogen present in tobacco smoke[28,29] and in various other forms of environmental pollution[21,28,30,31]. L1 reactivation by BaP is effected through the aryl hydrocarbon receptor (AHR) and subsequent deregulation of epigenetic control[22,23]. Exposure to environmental carcinogens such as BaP leads to epigenetic alterations that represent a fast-adaptive response of the cellular machinery for survival[32]. Indeed, exposure to cigarette smoke and/or environmental carcinogens is (are) the major culprit(s) in lung carcinogenesis[33-35]. PAHs are found in diesel exhaust, air-borne fine particulate matter (PM2.5), and second-hand tobacco smoke[36,37]. Carcinogenic PAHs exert direct biological effects by binding and activating the transcription factor AHR. AHR biology has been extensively characterized and shown to not only regulate the expression of PAH metabolic enzymes, but also to regulate differentiation programs in all cell types examined[38,39].

The carcinogenic activity of PAHs has mainly been attributed to the formation of diol epoxides and quinones that give rise to DNA adducts and induce oxidative DNA lesions, processes which are highly mutagenic[40,41]. However, a long series of studies completed in the Ramos laboratory have shown that BaP and related PAHs also activate L1 via epigenetic mechanisms, and this response is linked directly to AHR signaling[23,42-44]. L1 reactivation by BaP is directly linked to AHR activity. BaP diffuses across plasma membranes and binds to AHR present in the cytosol. Ligand-AHR complexes then translocate into the nucleus, where they interact with the nuclear aryl hydrocarbon receptor nuclear translocator (ARNT) protein.

Thereafter, the ligand-AHR-ARNT complex binds to dioxin responsive elements (DRE) on DNA to regulate the transcription of target genes[60]. AHR protein is present in the macromolecular complex that assembles on the promoter of both human and murine L1[57]; genetic silencing of AHR precludes transcriptional activation of L1 by PAHs[43,61].

BaP regulates L1 expression via epigenetic modification in the L1 promoter that involves: 1) enrichment of transcriptionally active chromatin markers histone H3 trimethylated at lysine 4 (H3K4Me3) and histone H3 acetylated at lysine 9 (H3K9Ac), and 2) reduction of the association of DNA methyltransferase-1 (DNMT1) to the promoter[23]. These modifications are tightly controlled by E2 transcription factors (E2F) and retinoblastoma protein (pRB)[56]. pRB is recruited to promoters by E2Fs, which in turn recruit corepressors that can remodel neighboring regions to silence transcription. L1 silencing by pRB is carried out via the NuRD multiprotein complex repressor[57]. NuRD has unique ATPase and histone deacetylase (HDAC1 and HDAC2) dual enzymatic activity among its six core subunits[58,59]. Histone deacetylation by HDACs causes compaction of chromatin structure and repression of transcriptional activity.

L1 reactivation is highly mutagenic by promoting somatic mutations and genomic rearrangements, suggesting that disruption of L1 biology contributes significantly to lung carcinogenesis induced by PAHs. However, beyond the association of L1 hypomethylation with poor prognosis of lung adenocarcinoma[15-17], the specific molecular mechanisms disrupted by environmental carcinogens that are responsible for aberrant expression of L1 in lung epithelial cells remain to be fully elucidated.

The Potential Role of L1 in EMT

Epithelial-to-mesenchymal transition (EMT) is the process by which epithelial cells lose their epithelial phenotype, characterized by epithelial cell-cell junctions and apical-basal polarity,

and acquire a mesenchymal-like phenotype[71]. EMT occurs normally during embryogenesis and wound healing, however it also plays a critical role in tumor metastasis and chemoresistance[24-26,71]. More specifically, EMT leads to dissolution of cell junctions, which is marked by decreased levels of claudin1, occludin, E-cadherin, connexin and ZO1 from cell-cell contacts[25]. The reduction of these epithelial markers inhibits new production of epithelial cell-cell junction complexes. The decrease in E-cadherin is offset by an increase in N-cadherin production. N-cadherin expression alters cell adhesion, and cells undergoing EMT no longer associate with neighboring epithelial cells. Instead, these cells acquire affinity for other mesenchymal cells via N-cadherin-N-cadherin associations[25]. These changes facilitate migration and invasion, enhance cell motility, and increase production of matrix metalloproteinases[25].

Emerging evidence reveals that EMT is not a binary construct under which cells either display a full epithelial or mesenchymal phenotype. Rather, EMT is a spectrum – at one end is a complete epithelial phenotype while a complete mesenchymal code is found at the other[71]. In most pathological states, epithelial cells acquire mesenchymal phenotypes and remain within this spectrum; that is, they retain both epithelial and mesenchymal markers and characteristics[71]. Furthermore, directional movement along this spectrum may be reversible in certain cases. Mesenchymal-to-epithelial transition (MET) also occurs, as seen during renal morphogenesis where cells of mesodermal origin differentiate into epithelial phenotypes[71]. For this reason, new, broader criteria have been introduced which define EMT and MET as “changes in cellular properties together with a set of molecular markers”[71]. Furthermore, the concept of epithelial-mesenchymal plasticity is being invoked to describe cells that are able to undergo EMT or MET[71].

BaP is known to induce EMT in lung cells following activation of AHR[45,46]. AHR can regulate TGF-β1 signaling through deregulation of TGF-β1 secretion, modulation of TGF-β1 expression or downregulation of latent TGF beta binding protein (LTBP-1) expression[62-64]. TGF-β1 is a pleotropic cytokine whose effects are context-dependent; it is known to be a potent immunosuppressive and pro-inflammatory cytokine[65] that plays a dual role in carcinogenesis, having the ability to inhibit proliferation of cancer cells or activate migration and invasiveness through EMT[66] or neoangiogenesis[67]. TGF-β1 signaling is mediated through a receptor complex that includes type-I and type-II membrane receptors with serine/ threonine kinase activity. Type-II receptors phosphorylate type-I components. Type-1 receptors transduce the signal from the cell membrane to the nucleus canonically through a complex of SMAD proteins. Alternatively, the non-canonical TGF-β1 signaling pathway operates through Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinases/mitogen-activated protein kinases (ERK/MAPK)[68]. Importantly, depending on the cellular context, TGF-β1 signaling has been strongly implicated in the regulation of epigenetic mechanisms[75]. Activated SMAD proteins closely interact with chromatin to remodel chromatin structure and/or access cryptic loci secluded by repressive histone marks. SMAD proteins recruit histone acetyl transferases (HATs) p300 and CBP to stimulate transcription, leading to acetylation of Lys9, Lys14, Lys18 and Lys23 on histone H3 [62,63].

TGF-β1-mediated SMAD protein activation can induce expression of SNAIL. SNAIL is a master regulator of EMT during development, fibrosis, and tumorigenesis. SNAIL works with the TGF-β-activated SMAD3/SMAD4 complex to repress E-cadherin and occludin expression[25]. Figure 2 shows two pathways by which TGF-β1 induces EMT, used with permission from Copyright Clearance Center.

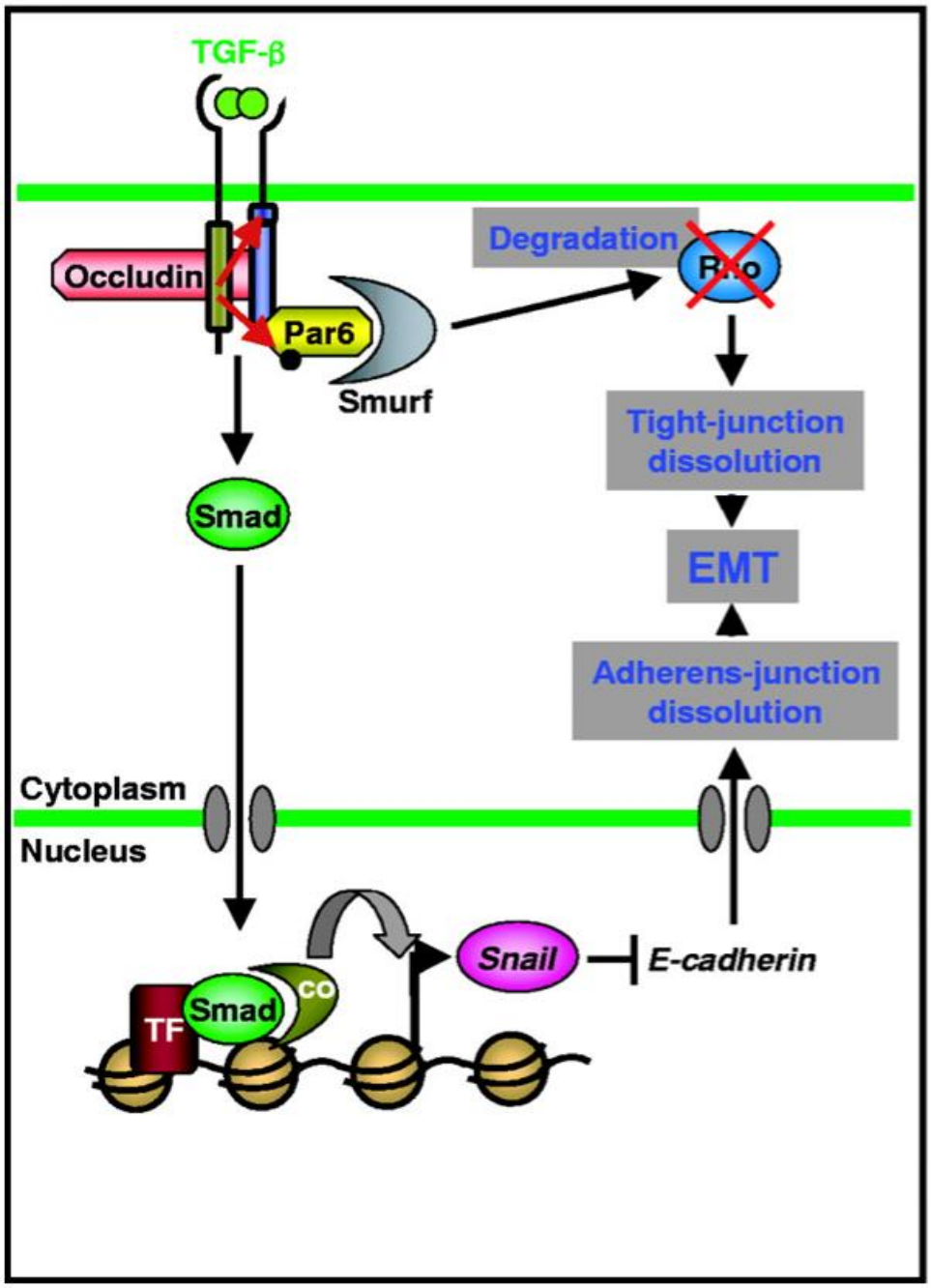

Figure 2: Regulation of the actin cytoskeleton and the EMT response. TGF-β induces Smads, which regulate genes such as that encoding Snail, the transcriptional repressor of E-cadherin gene expression that leads to the dissolution of adherens junctions. Alternatively, the receptors constitutively associate with occludin and the polarity protein Par6. Upon ligand stimulation, the type II receptor phosphorylates Par6 directly. This then recruits the ubiquitin ligase Smurf1, which ubiquitylates and degrades RhoA, thus leading to dissolution of tight junctions. The combined outcome of the two pathways cooperatively promotes EMT[74].

A connection between L1 and EMT has also been established. Studies in the Ramos laboratory have shown that retrotransposition-independent mechanisms contribute to genetic reprogramming of somatic cells by L1[10]. Moreover, this research group has shown that HepG2 cells challenged with BaP increase expression of ORF1p and ORF2p. These results were confirmed to be due to AHR activation, as genetic knockdown using AHR-specific siRNAs blocked L1 mRNA and protein expression[61]. In the same series of experiments, BaP was found to increase TGF-β mRNA expression and TGF-β levels preceded induction of L1 proteins. The effects of BaP on downstream TGF-β targets were also tested, and it was found that BaP increased phosphorylation of SMAD2 and downregulated expression of E-cadherin and N-cadherin[61]. Next, it was determined that TGF-β induced the expression of ORF1 and ORF2 mRNA, thus establishing TGF-β as a regulator of L1 expression. To determine whether AHR signaling activated L1 via the TGF-β pathway, HepG2 cells were treated with BaP, and downstream targets of TGF-β such as TGF-β receptor 1 (TGFBR1), SMAD2, and SMAD3 were knocked down via siRNA transfection. Inhibition of TGFBR1, SMAD2, and SMAD3 resulted in decreased BaP-induced L1 mRNA expression, bringing to light at least one mechanism by which BaP induces L1 activation in HepG2 cells[61].

The studies above established a potential pathway through which L1 may mediate cancer progression in certain cell types. However, the role of L1 reactivation in lung neoplastic transformation and EMT has not been elucidated. EMT has a profound influence on NSCLC progression, metastasis, and drug resistance[25,47-50]. Evidence is presented here that L1 couples EMT programming with tumorigenesis in human bronchial epithelial cells (BEAS-2B). These findings provide important insights into the role of L1 in lung cancer and identify a TGFβ1-SNAIL-L1-EMT axis as a critical molecular effector pathway in L1-mediated carcinogenesis.

MATERIALS AND METHODS

Reagents

BaP was purchased from Ultra Scientific (Kingstown, RI). Recombinant human TGF-β1 was purchased from R&D Systems (Minneapolis, MN). Monoclonal anti-GAPDH, and horseradish peroxidase (HRP) linked anti-mouse IgG antibodies were from Santa Cruz Biotech (Dallas, TX). Rabbit anti-AhR (13790), anti-E-cadherin (24E10), anti-vimentin (D21H3), anti-N-cadherin (D4R1H), anti-ZO-1 (D7D12), anti-claudin1 (8685), anti-SNAIL1 (C15D3), anti-Akt (C67E7), anti-phospho-Akt (T308) (C31E5E), anti-ERK1/2 (137F5), anti-phopho-ERK1/2 and horseradish peroxidase (HRP) linked anti-rabbit IgG antibodies were from Cell Signaling Technology (Beverly, MA). DMSO was from American Type Culture Collection (ATCC). Small interfering RNA (siRNA) duplex sequences were chemically synthesized and annealed by Thermo Fisher Scientific. The sequences of siRNA duplexes were 5′-CAGUUACUGUGGAAGGAAUtt-3′(TGFBR1 siRNA, Silencer®Select ID#s229438), (5′-GCUUCUCUGAACAAACCAGtt-3′ (SMAD2 siRNA, Silencer®Pre-designed ID#115715). 5′-GGCCCAGUGCAUAUGCAAUtt-3′ (SMAD3 siRNA, Silencer®Pre-designed ID#107877), 5′-CAAUGGAAGAUGAAAUGAAtt-3′ (ORF1 siRNA #1, Silencer®Select- Custom ID# s501620), 5′-GGGAGGACAUUCAAACCAAtt-3 (ORF1 siRNA #2 Silencer®Select- Custom ID# s501621), 5′-GGUGUGACUAACUAUGCAAtt-3′ (SNAIL siRNA #1, Hs_SNAI1_1 Qiagen), and 5 GAAUGUCCCUGCUCCACAAtt-3′ (SNAIL siRNA #2, Hs_SNAI1_5 Qiagen). BLAST analysis showed no homology to any sequence in the Human Genome Database, other than the intended target. The scrambled siRNAs used were Silencer® Negative Control #1 siRNA (AM4635), Silencer® Select Negative Control #2 siRNA (4390846), and Qiagen negative control

(1022076). The siRNAs were transfected using Lipofectamine™ RNAiMAX (Thermo Fisher Scientific), according to the manufacturer's directions.

Polyclonal Anti-Human ORF1p Antibody

A custom made, validated polyclonal ORF1p antibody was obtained from New England Peptide LLC was diluted 1:1000 and used in all experiments.

Cell Culture and Treatments

The human bronchial epithelial cell line BEAS-2B and Non-Small Cell Lung Cancer (NSCLC) cell lines (NCI-H460, NCI-H520 and NCI-H1993) were purchased from the American Type Culture Collection (ATCC). Cell lines were confirmed to be free of mycoplasma contamination (MycoAlert; Lonza). BEAS-2B were grown in LHC-9 medium while NSCLC cell lines were grown in RPMI media containing 10% FBS, Thermo Fisher Scientific, Grand Island, NY) in a humidified incubator at 37°C and 5% CO2. RPMI and LHC-9 medium were supplemented with 62.5 µg/mL penicillin and 100 µg/mL streptomycin (Thermo Fisher Scientific). Verification of all cell lines was performed by short tandem repeat (STR) using reference databases from ATCC (Genetics Core, University of Arizona, AZ). Cells were plated one day before treatments and treated with desired concentrations of TGF-β1 (1–10 ng/mL) or BaP (0.1 µM-1 µM) as indicated in figure legends. For biochemical analyses, cells were lysed with buffer containing 150 mmol/L NaCl, 2 mmol/L EDTA, 50 mmol/L Tris-HCl, 0.25% deoxycholic acid, 1% IGEPAL CA-630 (pH 7.5), supplemented with protease and phosphatase inhibitor cocktails (EMD Millipore) for 5 min at 4°C, and then cleared by centrifugation at 16,000 × g for 10 minutes at 4°C. All protein concentrations were determined using the bicinchoninic acid assay (Thermo Fisher Scientific).

Immunoblotting

Total cell lysates were resolved by SDS-Tris PAGE and transferred onto polyvinylidene fluoride membranes (Thermo Fisher Scientific) in Tris-glycine buffer containing 20% methanol. Proteins were detected by immunoblotting. Where indicated, membranes were stripped of bound antibodies using 62.5 mmol/L Tris-HCl (pH 6.7), 100 mmol/L 2-mercaptoethanol, and 2% SDS for 30 minutes at 60°C and re-probed as detailed in figure legends.

Cell Proliferation Assays

Cell proliferation was examined directly counting cells or indirectly using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay[45]. The MTT assay monitors metabolic activity and is routinely used as an indirect measure of cell proliferation. Its utility was confirmed by cell counts and biochemical testing to rule out chemical interference or confounding by changes in cellular morphology. Briefly, 3,000 cells were seeded in quadruplicate into 96-well plates and allowed to adhere overnight. Cells were treated with different concentrations of sunitinib malate and incubated for different periods of time without changing the culture medium. The signal corresponding to medium with no cells was subtracted as background. Cell proliferation was determined by normalizing to the proliferation of untreated cells for each cell type.

Stable Cell Lines

Expression vectors pB015^{WT} (wild type L1), pB016^{MUT} (L1 mutant carrying a single point mutation (D702Y) in ORF2 that destroys RT activity) and pB001^{CTR} (empty vector) generation have been previously described[10]. BEAS-2B cells were transfected with each expression vector using lipofectamine. 1,500,000 cells were plated in 10 cm plates and placed in complete media without antibiotics. On day 2, cell media was replaced with 8 mL Opti-MEM 1 medium.

Lipofectamine 2000 was diluted in Opti-Mem 1 medium at a ratio of 20µl/1,000ul and incubated for 5 minutes at room temperature. DNA plasmids were diluted separately in Opti-Mem 1 medium at a ratio of 8 µl/1,000 µl. Diluted lipofectamine and DNA plasmids were mixed and incubated at room temperature for 20 minutes. 2,000 µl of the DNA/Lipofectamine mix were added to the wells containing the cells and 8 mL of Opti-MEM 1 medium. Cells were incubated under standard conditions for three days before selection with hygromycin until the appearance of clones. Single clones were expanded and screened for L1 expression. Clones that showed similar expression of L1 and mutant L1 and no overt differences in cell growth under basal conditions were chosen for subsequent analyses. Figure 3 shows a schematic diagram of the cassettes used for transfection.

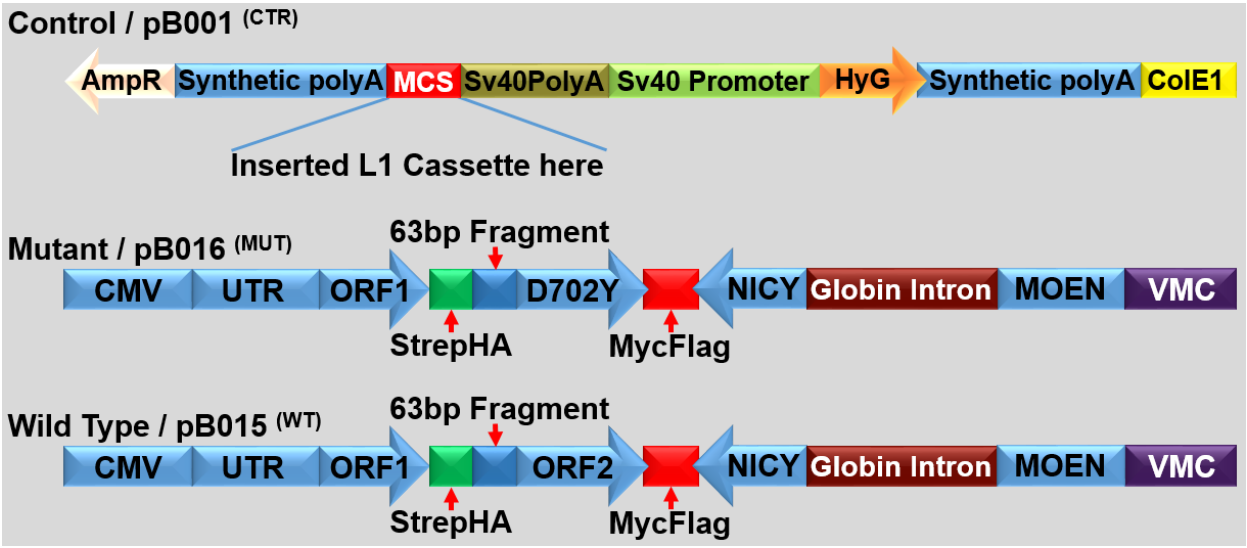

Figure 3: Schematic diagram of cassettes used for transfection. Control/pB001 was the backbone that contained a multiple cloning site into which the L1 cassettes were inserted. The L1 cassettes contained a CMV promoter to allow for continuous L1 protein expression. ORF1 was tagged with StrepHA. ORF2 was tagged with Myc and Flag. The ORF2 component of the Mutant/pB016 cassette had an aspartate-to-tyrosine substitution that disabled endonuclease activity. Both cassettes contained a neomycin resistance gene in antisense direction to assay for retrotransposition.

In vivo Studies

The Institutional Animal Care and Use Committee (IACUC) at the University of Arizona approved all experimental procedures involving animals. Healthy male, weanling nude mice (Nu/Nu) were purchased from Charles River Laboratories Inc. After acclimation for a week in the animal facility, mice were injected subcutaneously with a single-cell suspension consisting of 10^7 BEAS-2B cells expressing empty vector, L1 or mutant L1 (five mice per group) in 200 μL of Matrigel into each flank. Tumor volume and body weight were recorded every 2 or 3 days for 65 days.

Statistical Analysis

Experimental replicates were independent and performed on separate days. Comparisons were done between treated and control groups by ANOVA analysis as specified in figure legends.

RESULTS

Stable Transfection of BEAS-2B Cells with L1 Vectors

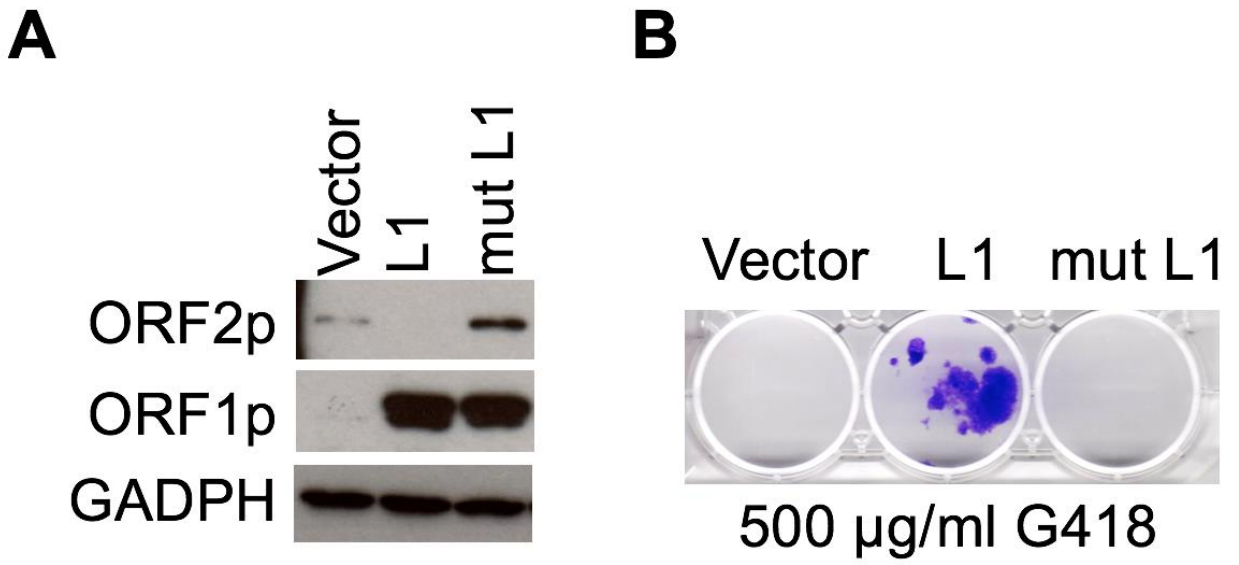

Figure 4: L1 Protein Expression and Retrotransposition Assay. In Figure **4A** BEAS-2B cells transfected with vector, wildtype L1 and mutant L1 were lysed, clarified by centrifugation, and analyzed by immunoblotting for L1 proteins ORF1p and ORF2p. In Figure **4B**, stably transfected BEAS-2B cells were treated with 500 µg/mL of geneticin for 15 days. Cells were washed, fixed and stained with 0.5% crystal violet.

The aim of this research was to determine the capacity of L1 to induce oncogenic transformation in non-malignant human bronchial epithelial cells. Therefore, we established BEAS-2B cell lines that constitutively overexpressed wildtype or mutant L1 proteins. This was achieved via stable transfection of the cells with the empty vector, wildtype L1, or mutant L1 cassettes. Figure 4A shows that the cells transfected with wildtype L1, and mutant L1 express similar levels of ORF1p, as expected. However, the mutant L1 strain expressed higher levels of ORF2p than the L1 wildtype strain. Our research team believes this may be due to the toxic effects of wildtype ORF2p, which may suppress ORF2p expression. Additionally, detection of ORF2p is a major challenge, and has been notoriously difficult to detect *in vitro* and *in vivo*[77,78].

ORF1p, on the other hand has been readily detected[78]. It is for this reason that ORF1p, rather than ORF2p, is used primarily as a measure of L1 expression. ORF2p levels are reported here for the sake of completion and to show that we, too, found ORF2p levels labile and inconsistent. In Figure 4B, the only cell line that was able to withstand geneticin treatment was the one expressing wildtype L1. Resistance to geneticin is conferred only to cell lines in which L1 could undergo a full cycle of retrotransposition. This is because the neomycin resistance cassette present in the antisense position must revert to the sense position, a process that requires retrotransposition. The neomycin gene can then be expressed in the appropriate sense position. Thus, although ORF2p does not appear to be present in L1, it must be there at a functional level because it is required for retrotransposition, which is documentable in these cells.

L1 Induces EMT Phenotype via Retrotransposition-Independent Mechanisms

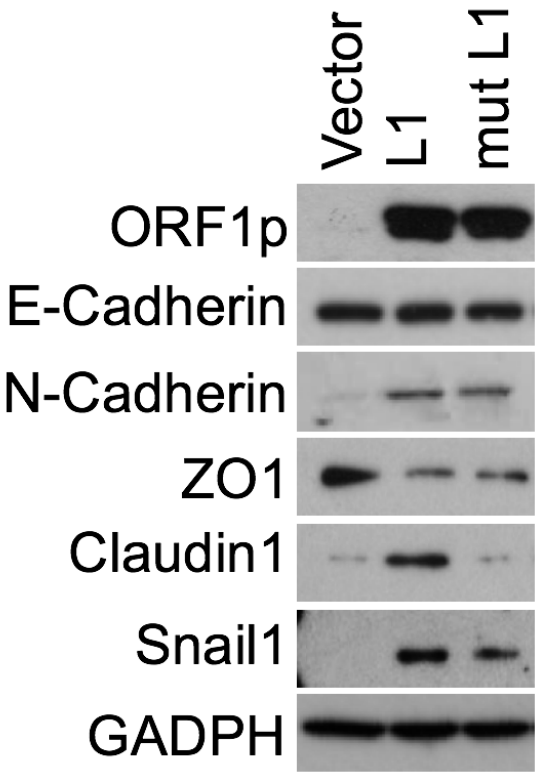

Figure 5: L1 and EMT Marker Expression. Stably transfected BEAS-2B cells with L1, mutant L1 or vector were analyzed by immunoblotting for L1 protein ORF1p; epithelial markers E-cadherin, ZO1, and claudin1; and mesenchymal markers N-cadherin and SNAIL.

Next, our lab sought to determine whether changes in L1 expression induce EMT in BEAS-2B cells. As expected, Figure 5 shows that the cell line expressing the vector showed high expression of epithelial markers E-cadherin and ZO1, minimal N-cadherin expression, and no SNAIL production. Both cell lines expressing either wildtype L1 or mutant L1 exhibited increased expression of the mesenchymal markers N-cadherin and SNAIL at comparable levels. Both cell lines exhibited decreased expression of ZO1. Interestingly, expression of E-cadherin was unaffected, while claudin1 was selectively induced by the cells expressing wildtype L1. Together, these changes indicate that expression of L1 may induce EMT programming in non-malignant bronchial epithelial cells. Furthermore, these findings suggest that EMT changes can be primarily mediated via retrotransposition-independent mechanisms, but that other changes such as elevated claudin1 expression may be retrotransposition-dependent.

EMT Phenotype Induced by L1 is Partially Reversible

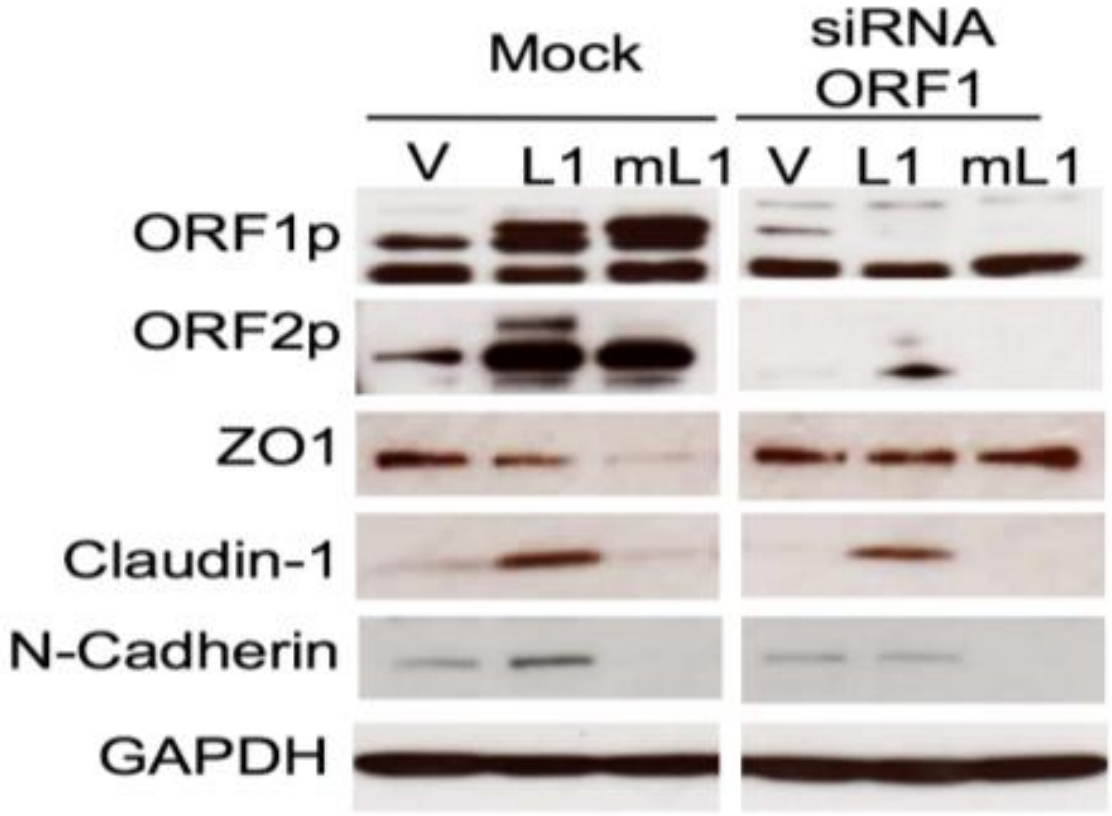

Figure 6: EMT Marker Expression after Silencing L1 with siRNA. Stably transfected BEAS-2B cell clones expressing L1 or mutant L1 were transiently transfected with mock or ORF1 siRNA and analyzed for immunoblotting for L1 proteins ORF1p and ORF2p; epithelial markers ZO1 and claudin1; and mesenchymal marker N-cadherin.

EMT is a dynamic process and cells may transition from an epithelial phenotype to a mesenchymal phenotype, and vice versa, depending on the cellular milieu. We sought to determine whether the EMT phenotype induced by L1 was reversible. Figure 6 shows that blockade of ORF1p expression with siRNA in cells constitutively expressing wildtype and mutant L1 successfully blocks expression of ORF1p and ORF2p. The reason we see double banding for ORF1p and ORF2P is because the exogenous L1 proteins are epitope-tagged with StrepHA and MycFlag, respectively. These tags cause an increase in molecular weight of the

exogenous proteins causing them to travel less their endogenous counterparts during electrophoresis, which results in two separate bands of ORF1p and ORF2p. Interestingly, silencing with ORF1 siRNA resulted in decreased expression of ORF2p. This is because LINE-1 mRNA is dicistronic[3-5], which means that two proteins are translated from the same mRNA strand. The siRNA targeted specifically for ORF1 mRNA therefore intrinsically targets ORF2 mRNA as well, since they are one and the same. The entire L1 mRNA is degraded and we see a decrease in both ORF1p and ORF2p.

Silencing ORF1p expression in cells transfected with either wildtype or mutant L1 reestablished expression of the epithelial marker ZO1. Similarly, silencing ORF1p expression in cells expressing wildtype L1 decreased expression of N-cadherin and claudin1. These results indicate that interference with L1 expression may be a strategy to regulate the invasive and metastatic potential of tumors.

Ectopic Expression of L1 Promotes Chemoresistance

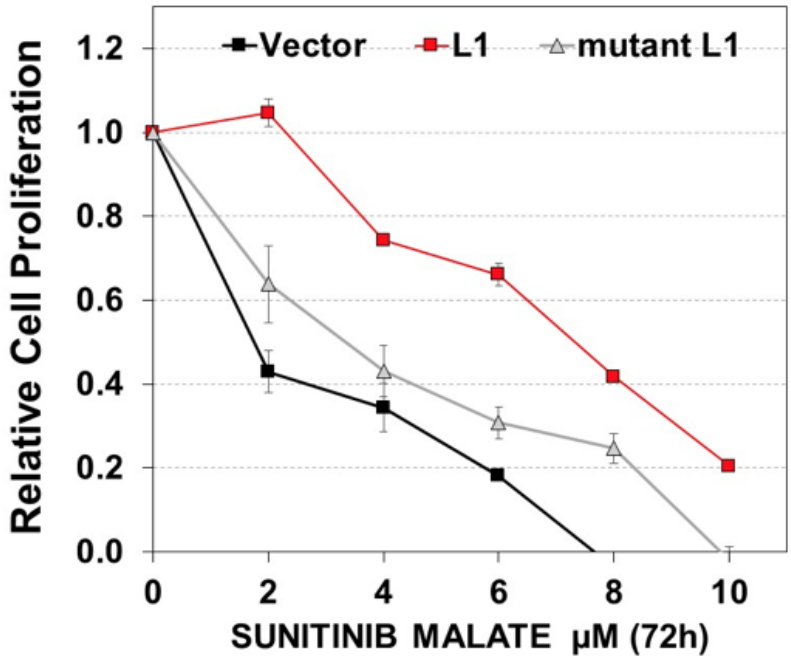

Figure 7: Proliferation Rates of Transfected Cells Exposed to Sunitinib. BEAS-2B cell clones constitutively expressing wildtype L1, mutant L1 or empty vector were treated with increasing concentrations of sunitinib malate. Cell proliferation was measured after three days of treatment by the MTT assay. Points represent the mean ± SE for individual samples from two to three independent experiments. Antiproliferative actions of sunitinib malate were significantly reduced in wildtype ($p<0.001$) in all concentrations tested and mutant L1 ($p<0.05$) in concentrations 2, 6, 8, and 10 μM compared to each other and vector cells.

In the past decade, chemotherapeutic drugs including monoclonal antibodies and tyrosine kinase inhibitors against epithelial growth factor receptor (EGFR) have been developed to treat NSCLC, however only a minority of patients with NSCLC respond to EGFR-directed therapy[47]. Additionally, studies have linked resistance to EGFR inhibitors such as gefitinib to EMT in NSCLC lines[47]. In fact, it has been shown that NSCLC cell lines that express genes associated with epithelial markers such as E-cadherin tend to be sensitive to gefitinib. On the other hand, resistant cell lines expressed high levels of the mesenchymal marker vimentin[47].

To determine whether L1-induced EMT promotes chemoresistance to tyrosine kinase inhibitor, BEAS-2B cell clones constitutively expressing wildtype L1, mutant L1 or vector were treated with sunitinib malate. Sunitinib malate is an inhibitor of the tyrosine activities of vascular endothelial growth factor receptor 2, platelet-derived growth factor receptor b, and c-kit. Figure 7 shows that clones constitutively expressing wildtype L1 were more resistant to the anti-proliferative effects of sunitinib. Furthermore, clones expressing the mutant L1 were also more resistant to sunitinib than those expressing the empty vector. These results indicate that high levels of chemoresistance of NSCLC may be due, at least partly, to L1 expression. Furthermore, the ability of L1 to undergo retrotransposition seems to confer higher levels of resistance.

Ectopic Expression of L1 Induces a Tumorigenic Phenotype

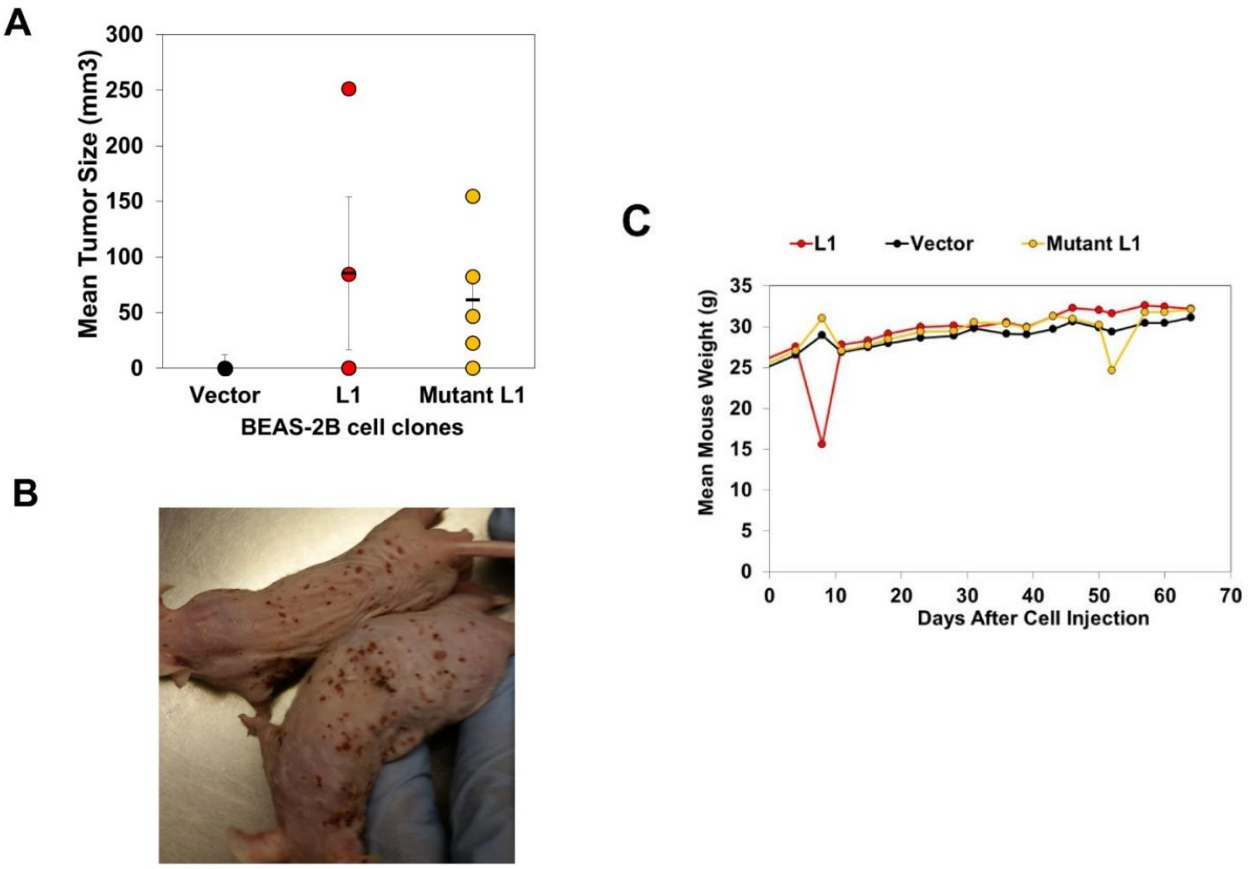

Figure 8: Tumor Size and Body Weight after Injection of Mice with Transfected Cells. BEAS-2B cell clones ($1x10^7$ cells) constitutively expressing wildtype, mutant L1, or empty vector were mixed with Matrigel and then injected subcutaneously into 5-week-old male Nu/Nu mice (n=4). **A)** Shows tumor sizes, 6 weeks post-implantation. **B)** Is a photograph of skin ulceration developed by mice injected with the mutant L1 cell line. **C)** Shows mean mouse weight according to cell line injected. It was not possible to do statistical analysis as mice injected with cells expressing vector and the tumors generated by the two other mice groups were too small.

Although we had obtained promising *in vitro* results, we wanted to test our working hypotheses *in vivo*. To this end, 5-week-old male Nu/Nu mice were injected with cell clones constitutively expressing the different L1 vectors. Remarkably, mice injected with cells expressing either wildtype or mutant L1 vectors developed tumors, with notable differences.

Tumors developed by mice injected with cells expressing wildtype L1 had the highest rate of growth, but growth eventually declined with subsequent tumor regression (data not shown). In contrast, tumors developed by mice injected with the cell line expressing mutant L1 had a slower rate of growth but continued to grow for the duration of the experiment, as shown in Figure 8A. Figure 8B shows skin ulceration developed in mice injected with cells expressing mutant L1; findings which warranted termination of the experiment. Figure 8C shows mean mouse weight according to cell line injected. These results indicate that expression of retrotransposition-deficient L1 is sufficient to promote an invasive tumorigenic phenotype in non-malignant bronchial epithelial cells. Further, these findings indicate that L1 retrotransposition is not required for induction of tumorigenic potential in BEAS-2B cells.

DISCUSSION

To date, little is known about the role of L1 proteins and their ability to drive lung neoplastic transformation and EMT phenotypes. Our current understanding of L1 biology includes: 1) identification of novel L1 insertions in several types of cancer, 2) study of mechanisms that control L1 retrotransposition during the later stages of the retrotransposition cycle, and 3) correlations of L1 methylation or ORF1p expression with cancer screening and prognosis. Many studies focus on epigenetic regulation of L1, but the complete pathway that controls this regulation has not yet been elucidated. Multiple studies point to hypomethylation of L1 genetic elements and L1 protein expression as potential prognostic determinants. Establishing a mechanism will allow targeted drug development for better treatment of NSCLC.

L1 retrotransposition events have been considered the most important aspect of L1 biology by coupling neoplastic transformation and cancer progression[5]. However, recent evidence, including the work presented here, indicates that L1 retrotransposition-independent mechanisms modulate the expression of many genetic targets that regulate cancer progression including adhesion, inflammation and cellular metabolism[9,10]. Moreover, L1 induces EMT and disrupts chemokine expression in hepatic carcinoma cells via retrotransposition-independent mechanisms[10]. To date, little is known about the role of L1 proteins and their ability to drive lung neoplastic transformation and EMT phenotypes.

The data presented here demonstrate that ectopic expression of mutant L1, incapable of retrotransposition, in non-malignant HBECs induces an EMT-like phenotype characterized by induction of SNAIL and N-cadherin and downregulation of ZO1. Although ectopic expression of L1 or mutant L1 did not induce changes in E-cadherin expression, there could be several explanations for this. First, it is possible that L1 may not induce any changes in E-cadherin

expression as part of the mesenchymal transition it induces. Indeed, as noted above, there can be a spectrum between full polarization to a mesenchymal or epithelial phenotype. There could be, however, additional explanations for the lack of changes in E-cadherin expression. For example, the time required for L1 to affect E-cadherin levels may be longer than what was used in our experiments. It may be helpful to conduct similar experiments as those shown in Figure 5 with longer timelines to assess whether ectopic expression of L1 induces changes in E-cadherin expression. Another possibility is that L1 may not produce a quantitative effect on E-cadherin expression, but rather a qualitative one, such as spatial redistribution of E-cadherin within the cell membrane. It is important to note that the cellular migratory potential can be conferred by altering the process of junction formation and dissolution, and that a complete loss of junctions is not required[71]. This is evidenced by fibroblast growth factor-dependent pathways, which promote EMT and cell movement in chicken primitive streak without decreasing E-cadherin expression levels[76]. Part of the new criteria for EMT includes a change in a set of molecular markers, not only E-cadherin, which is what we see in our results.

A similar phenomenon occurred with claudin1 expression. As claudin1 is an epithelial marker, we would have expected L1 expression to cause a decrease in claudin1 expression. This was not the case. Instead, claudin1 levels increased only in the cell line expressing wildtype L1, per Figure 5. It may be possible that wildtype L1 induces a change in the E-cadherin-claudin1 ratio as part of the mesenchymal phenotype it induces. Changes in claudin1 are likely to be retrotransposition-dependent, as mutant L1 did not induce these changes.

Our data also suggest that L1-induced changes can be partially reversible, as evidenced by reestablishment of ZO1 expression after ORF1 blockade with siRNA in BEAS-2B cells that constitutively expressed L1 and mutant L1 (Figure 6). We found decreased N-cadherin

expression after ORF1 blockade with siRNA in BEAS-2B cells that constitutively expressed L1. The reversible changes seem to be those carried out by retrotransposition-independent mechanisms. Our results are limited because in previous experiments, we had seen that L1 and mutant L1 both induced N-cadherin expression (Figure 5). We were not able to replicate these findings in the reversal experiment. Although we were able to obtain reproducible results for each set of experiments independently (Figures 5 and 6), these two experiments had different outcomes. The major difference in terms of the technique in which these two sets of experiments were carried out was that the reversal experiments were carried out with older generations of cells, meaning they had been cloned multiple times before. This may have altered morphology and expression of certain cell-markers, such as N-Cadherin. Ideally, we would have conducted the experiments with a similar generation of cells. Meaning that cells that were immediately transfected, or even after cloning once or twice, would have been used for the experiments. We initially attempted to do this with both sets of experiments, but ran into difficulty transfecting a new batch of bronchial epithelial cells with the L1 constructs for the reversal experiment; additional studies are required.

Furthermore, we were able to show that ectopic expression of L1 or a mutant L1 enhances chemoresistance to tyrosine kinase inhibitors (Figure 7). Previous work conducted in head and neck squamous cell carcinoma cell lines have shown that EGFR expression is significantly lower in cell lines resistant to EGFR-targeted chemotherapy, which is associated with a concomitant increase in mesenchymal gene expression[47]. L1 expression may not only affect cell adhesion molecules but surface receptors as well. In this case, L1 may promote cellular survival via EGFR-independent mechanisms and may cause decreased expression of EGFR, leading to the inadequate response seen with current chemotherapeutic agents. Finally,

we showed that ectopic expression of L1 or a mutant L1 induces a tumorigenic phenotype in HBECs (Figure 8). These findings suggest that the retrotransposition-independent pathway is sufficient for cellular transformation, and that retrotransposition events are not necessary for L1-induced cellular transformation in the BEAS-2B cell line. Therefore, although we cannot establish a causal relationship between L1 expression and cellular transformation at this time, this work helps solidify L1 as a prognostic and diagnostic biomarker and provides a potential molecular target for precision-based interventions. Furthermore, we have identified additional players along the BaP-induced L1 reactivation pathway, including TGF-β1 and SNAIL that have been established as master regulators of EMT in a wide array of cell lines. Finally, these results have incredible implications for the treatment of NSCLC as they suggest that blocking the L1 pathway may decrease chemoresistance and metastatic patterns in patients with NSCLC.

MODEL

Dr. Ramos' laboratory has shown that challenging human bronchial epithelial cells with TGF-β1 induces ORF1 mRNA and ORF1p expression in a dose-dependent fashion[75]. Furthermore, we found that BaP induces TGF-β1, ORF1 and ORF2 mRNA expression; ORF1 and ORF2 mRNA expression is effectively silenced when cells are treated with TGF-β1 receptor inhibitor[75]. Additionally, we found that BaP-induced L1 mRNA expression was blocked after genetic knockdown with SMAD2 and SMAD3-specific siRNA, suggesting that BaP reactivates L1 via canonical TGF-β1 signaling[75]. Along these lines, genetic knockdown of SNAIL in cells challenged with TGF-β1 blocked induction of L1[75].

Based on the data presented in this paper, I propose that BaP induces L1 expression via the TGF-β1 canonical signal pathway during tumorigenesis. The whole pathway would go as follows: BaP found in cigarette smoke would lead to DNA damage via formation of reactive oxygen species and stimulate production of TGF-β1. TGF-β1 receptor activation would lead to activation of SMAD proteins which would recruit HATs to remodel chromatin and induce expression of SNAIL, a master regulator of EMT. SNAIL then would finally lead to expression of L1 proteins. In addition, we have evidence that suggests a synergistic relationship between SNAIL and L1 expression; SNAIL may initially activate L1, but L1 and SNAIL may actively recruit each other in a positive feedback loop to active a more complete EMT genetic program.

FUTURE STUDIES

Further work should be aimed at elucidating the complete mechanism by which BaP activates L1, and ultimately, a tumorigenic phenotype in bronchial epithelial cells with the hope of developing effective, non-surgical treatment of NSCLC. Additional studies are required to characterize epigenetic regulation of the L1 promoter effected through the BaP/AHR/TGF-β1/SMAD axis. For example, HBECs can be used as a model system with genetic approaches that determine promoter-specific changes in histone covalent modifications, DNA methylation, and recruitment of chromatin-modifying complexes following AHR activation by BaP via the TGF-β1/SMAD signaling pathway. Furthermore, we need to closely define molecular mechanisms that drive L1-induced EMT-like phenotype and enhanced survival (chemoresistance) of HBECs. Genetic and functional analyses in non-malignant and malignant HBEC lines (BEAS-2B, BZR, NL20, NCI-H1975, and NCI-H1993 cells) can be carried out to determine whether: 1) L1 regulates the expression of SNAIL by transcriptional or posttranslational mechanisms, and 2) L1 regulation of EMT phenotypes and enhanced survival during stress is regulated by SNAIL. Lastly, we hope to characterize spatiotemporal expression profiles of ORF1p, SNAIL, ZO1 and N-cadherin proteins in human lung tissue by immunohistochemistry.

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