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A novel molecular pathway for Snail-dependent, SPARC-mediated invasion in Non Small Cell Lung Cancer pathogenesis

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A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Cellular and Molecular Pathology

by

Jeanette Lynn Grant

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ABSTRACT OF THE THESIS

A novel molecular pathway for Snail-dependent,
SPARC-mediated invasion in Non Small
Cell Lung Cancer pathogenesis

by

Jeanette Lynn Grant
Doctor of Philosophy in Cellular and Molecular Pathology
University of California, Los Angeles, 2013
Professor Steven M. Dubinett, Chair

In the U.S., lung cancer is the leading cause of cancer death, with a median survival of eight months following diagnosis and only 16% of patients surviving more than five years. The low survival rate is attributable, in part, to a lack of early detection, limiting the benefit of surgical resection as metastatic progression has already occurred. The early events of lung cancer pathogenesis have not yet been well defined, contributing to the paucity of early detection techniques clinically. For tumors to progress to metastatic disease, they must acquire characteristics that allow them to become migratory, degrade and invade their local basement membrane, and migrate to a new site before forming a micrometastasis. As part of this process, tumors undergo a series of events known as epithelial-to-mesenchymal transition (EMT), where they lose epithelial characteristics and become mesenchymal in phenotype and molecular profile.

Snail is a zinc-finger transcription factor that exerts global effects on epithelial cell gene expression profiles, resulting in regulation of EMT. Recent studies by our lab have shown that

Snail is upregulated in human non-small cell lung cancer (NSCLC) tissues, is associated with poor prognosis, and promotes NSCLC tumor progression *in vivo*. Furthermore, Snail overexpression in NSCLC is associated with differential gene expression related to diverse aspects of lung cancer progression, including angiogenesis. Herein we demonstrate that overexpression of Snail leads to upregulation of Secreted Protein, Acidic and Rich in Cysteine (SPARC). Immunoblot and qRT-PCR analysis of multiple NSCLC cell lines with and without Snail overexpression validated the relationship between Snail and SPARC in established cancers. Similar results were found in Snail-overexpressing Human Bronchial Epithelial Cells (HBECs), a model of early pathogenesis, as well as in human lung adenocarcinomas and squamous cell carcinomas immunostained for Snail and SPARC. Taken together, these data indicate that SPARC is upregulated by Snail at early and late points during lung carcinogenesis and may play a role in lung cancer initiation and progression.

In all cell lines evaluated, Snail overexpression leads to increased SPARC-dependent invasion *in vitro*. The promoter region of SPARC does not contain a binding site for Snail, indicating that Snail upregulates SPARC by an indirect mechanism. Bioinformatic analysis of array data revealed potential intermediaries in Snail-mediated upregulation of SPARC, including miR-29b and the TGF-β and MEK/ERK pathways. Both the TGF-β1 ligand and TGF-βR2 are upregulated following Snail overexpression. In addition, treatment of HBEC cell lines with TGF-β1 resulted in phosphorylation of ERK1/2 as well as upregulation of SPARC and Snail. Inhibition of TGF-β1 mRNA decreased the Snail-dependent activation of ERK1/2 and protein expression of SPARC. Inhibition of MEK phosphorylation by the chemical inhibitor U0126 in Snail-overexpressing cell lines leads to a loss of SPARC upregulation, indicating that the TGF-β-driven MEK/ERK pathway is necessary for Snail-dependent upregulation of SPARC. The

microRNA miR-29b is downregulated in Snail-overexpressing cell lines. As the 3'UTR of SPARC mRNA contains multiple consensus sequences for miR-29b, we have hypothesized that downregulation of miR-29b by Snail, downstream of ERK, allows for upregulation of SPARC. Transient overexpression of a miR-29b mimic in both the vector control and Snail-overexpressing cell lines inhibited SPARC expression, confirming the ability of miR-29b to regulate SPARC. In addition, miR-29b was upregulated following ERK inhibition, indicating a pathway by which Snail overexpression leads to activation of TGF-β and ERK signaling, resulting in downregulation of miR-29b and upregulation of SPARC.

Upregulation of SPARC is associated with metastatic potential of melanomas and gliomas as well as an invasive phenotype in breast, prostate, and colorectal carcinomas. Expression of SPARC in the tumor stroma of NSCLC is associated with poor patient prognosis, though its role in tumor progression, especially in relation to Snail expression, has not been evaluated. Our novel discovery of SPARC overexpression as an early event during lung carcinogenesis has potentially important implications, especially as related to the parallel model of cancer development and metastatic progression. Delineating pathways involved in Snail-dependent and SPARC-mediated parallel progression may yield new targets for lung cancer prevention and treatment.

The dissertation of Jeanette Lynn Grant is approved.

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2013

For Sr. Peg, Gma W, Aunt Sally, Tammie, and my family.

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LIST OF ABBREVIATIONS

ADC	Adenocarcinoma
ALK	Anaplastic lymphoma kinase
AML	Acute myeloid leukemia
BSA	Bovine serum albumin
CAGR	Cancer-associated genomic region
Cdk4	Cyclin-dependent kinase 4
COX-2	Cyclooxygenase-2
CSC	Cancer stem cell
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
ECM	Extracellular membrane
EGFR	Epidermal growth factor receptor
EMT	Epithelial-to-mesenchymal transition
ERK	Extracellular signal-related kinase
FBS	Fetal bovine serum
FGFR	Fibroblast growth factor receptor
GTPase	Guanosine triphosphatase
HBEC	Human bronchial epithelial cell
HGFR	Hepatocyte growth factor receptor
HSC	Hepatic stellate cell
hTERT	Human telomerase

IGFR	Insulin-like growth factor receptor
IL-1β	Interleukin-1 beta
ILK	Integrin-linked kinase
IPF	Idiopathic pulmonary fibrosis
FRA	Fragile site loci
JNK	C-Jun NH_2-terminal kinase
LCC	Large cell carcinoma
LOH	Loss of heterozygosity
MAP3K	Mitogen-activated protein kinase kinase kinase
MAPK	Mitogen-activated protein kinase
MEK/MKK	Mitogen-activated protein kinase kinase
MET	Mesenchymal-to-Epithelial Transition
miRNA	MicroRNA
MKP	MAP kinase phosphatase
MMP	Matrix metalloprotease
MPA	Mycophenolic acid
mRNA	Messenger RNA
MSC	Mesenchymal stem cell
NSCLC	Non Small Cell Lung Cancer
PBS	Phosphate buffered saline
PI3K	Phosphoinositol-3-kinase
qRT-PCR	Quantitative real-time–polymerase chain reaction
RNA	Ribonucleic acid

SCC	Squamous cell carcinoma
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SPARC	Secreted protein, acidic and rich in cysteine
TGF-β	Transforming growth factor Beta
TGFβR	Transforming growth factor Beta receptor
TIC	Tumor-initiating cell
TKI	Tyrosine kinase inhibitor
TME	Tumor microenvironment

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Grant JL, Walser TC, Hong LS, Minna JD, Shay JW, Fishbein MC, Dubinett SM. Snail mediates NSCLC invasion through indirect upregulation of SPARC. *In Submission.*
Heinrich EL, Walser TC, Krysan, K, Liclican EL, **Grant JL**, Rodriguez NL, Dubinett SM. The inflammatory tumor microenvironment, epithelial mesenchymal transition, and lung carcinogenesis. Cancer Microenvironment. EPub ahead of print, 16 September 2011.

Abstracts and Presentations:

Grant, JL, Walser TC, Yanagawa J, Lee G, Minna JD, Prasad S, Hazra S, Dubinett SM. *Lovastatin and low dose celecoxib decrease proliferation and inflammation in a cellular model of lung pre-malignancy.* AACR 101st Annual Meeting, 2010, Washington D.C. Poster Presentation.

Hazra S, Lee G, **Grant JL**, Walser TC, Prasad S, Minna, JD, and Dubinett SM. *Modulation of prostaglandin E2 by statins in human bronchial epithelial cells harboring K-ras mutation: the potential advantage of combination therapy.* AACR Frontiers in Cancer Prevention Research 2010, Philadelphia, PA. Poster Presentation.

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Grant JL, Walser TC, Hong LS, Minna JD, Shay JW, Fishbein MC, Dubinett SM. Snail mediates NSCLC invasion through indirect upregulation of SPARC. Ninth AACR-Japanese Cancer Association Joint Conference: Breakthroughs in Basic and Translational Cancer Research, February 2013, Maui, HI. Poster Presentation.

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A Review of the Literature:

Inflammation in the Pulmonary Microenvironment and Cancer Progression

Lung Cancer

In the U.S., lung cancer is the leading cause of cancer death, with a median survival of eight months following diagnosis and only 16% of patients surviving more than five years [1]. The low survival rate is attributable, in part, to a lack of early detection, limiting the benefit of surgical resection of the primary tumor as metastatic progression has already occurred. The two main types of lung cancer are Small Cell Lung Cancer and Non Small Cell Lung Cancer (NSCLC), which are classified based on histological evaluation. The majority of lung cancers are classified as NSCLC and can be further classified as the subtypes of Adenocarcinoma (ADC), Squamous Cell Carcinoma (SCC), and Large Cell Carcinoma (LCC) [2]. The majority of patients diagnosed with lung cancer have a history of chronic obstructive pulmonary disease (COPD) and/or tobacco use, both of which are associated with inflammation [3]. COPD includes the diseases chronic bronchitis and emphysema and is characterized by luminal airway narrowing and destruction of lung parenchyma. Chronic bronchitis is characterized by chronic irritation and inflammation of the airways, leading to thickening of the airway lining and increased mucus production. Emphysema is characterized by the damage or destruction of the air sacs in the lungs, preventing proper gas exchange [4].

While knowledge of the molecular mechanisms of lung cancer behavior has increased and new therapeutic agents have been introduced, lung cancer survival rates have remained relatively unaltered over the last 30 years. This plateau may be due to a lack of understanding of the factors promoting lung cancer development and metastatic progression. In recent years, focus has changed from histological classification to classification by driver mutations such as those in the KRAS, epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase (ALK) genes [5]. These mutations may play a role in both tumorigenesis and therapeutic resistance.

Driver mutations occur in genes that encode signaling proteins crucial for cellular proliferation and survival. Mutant oncogenes drive both tumor formation and maintenance and this phenomenon is also termed oncogene addiction [6].

Epidermal growth factor receptor (EGFR) is a transmembrane tyrosine kinase receptor that initiates a signaling cascade following ligand binding. Overexpression has been observed in several cancers, including NSCLC, and is the target of two tyrosine kinase inhibitors (TKIs), gefitinib and erlotinib [5]. Activating mutations in the EGFR gene have been detected in approximately 50% of Asian patients and 10% of non-Asians, making it an effective target for therapy. However, the exon 20 T790M mutation is associated with acquired resistance to TKI therapy [5,7]. EGFR activation may also be caused by increased copy number [5]. Given that EGFR is expressed on the cell surface of a substantial percentage of NSCLCs, it has also been suggested that EGFR inhibition could also be used in lung cancer prevention [7]. Inhibition of EGFR may result in activation of alternate signaling pathways. The most frequently encountered alterations include KRAS mutations, MET amplification, ALK gene fusion, PIK3CA mutations, BRAF mutations, and IGF1R overexpression [5].

Activating mutations in the KRAS gene lead to constitutive Ras signaling, are found in approximately 25% of NSCLCs, are associated with poor prognosis, and are associated with EGFR TKI resistance [8]. The gene MET encodes the receptor tyrosine kinase hepatocyte growth factor receptor (HGFR). Amplification of this gene has been associated with secondary resistance to EGFR TKIs and has been reported in about 20% of tumors from patients with acquired resistance [9]. In adenocarcinomas, MET amplification is independent of KRAS mutation or EGFR amplification. Amplification of MET results in constitutively active HGFR, which has been shown to initiate tumorigenesis [6]. ALK is a receptor tyrosine kinase not

normally expressed in the lung. Its fusion with an upstream partner, EML4, results from diverse small inversions within the short arm of chromosome 2 [6]. This fusion results in protein oligomerization and constitutive kinase activation and ultimately cellular proliferation. EML4-ALK fusions are detected in approximately 7% of all NSCLCs, though frequency is increased in adenocarcinomas, young adult patients, and never- or light-smokers [5,9]. A small molecule tyrosine kinase inhibitor of ALK, crizotinib, has proven effective in NSCLC patients with EML4-ALK fusions [10]. PIK3CA is a gene encoding the main catalytic subunit of PI3K proteins, which are key mediators between growth factor receptors and intracellular downstream signaling pathways [6]. Mutations in PIK3CA occur in about 2% of NSCLC cases and lead to a gain of enzymatic function, allowing activation of the protein kinase B signaling pathway in the absence of growth factors. B-RAF is one of three members of the RAF kinase family that links RAS GTPases to downstream proteins of the MAPK family, controlling cell proliferation [6]. Mutations in BRAF are found in 1-3% of NSCLC tumors and are associated with increased kinase activity due to constitutive activation of MAPK2 and MAPK3. Identification of additional driver mutations is an active area of study and could yield additional targets for chemoprevention and therapy as well as provide additional clues into the molecular pathogenesis of NSCLC.

Inflammation in the Pulmonary Microenvironment

Inflammatory mediators, including TGF-β, COX2, and IL-1β are overexpressed in the lungs of smokers and patients with COPD and idiopathic pulmonary fibrosis (IPF); these patients have a heightened risk of developing lung cancer [11-13]. Smoking increases lung concentrations of TGF-β and EGF and is thought to contribute to formation of both COPD and IPF and subsequently lung cancer [7]. EGF potentiates TGF-β1-mediated COX-2 induction at both the mRNA and protein levels in HBECs in an EGFR- and ERK-dependent manner [7]. The

CXC chemokine interleukin-8 (IL-8) is an important proinflammatory mediator that promotes cell proliferation and angiogenesis in NSCLC and is associated with poor prognosis. IL-8 is a transcriptional target of RAS signaling and is therefore upregulated in the setting of oncogenic KRAS. Activating EGFR mutations in NSCLC also induce expression of IL-8 [8]. Overexpression of inflammatory mediator responsive receptors frequently occurs in NSCLC. One example is insulin-like growth factor 1 receptor (IGFR1R), a mediator of cellular proliferation [10]. Another is fibroblast growth factor receptor 1 (FGFR1), which is frequently amplified in the lungs of smokers and is correlated with poor prognosis. Given the interactions between the inflammatory microenvironment, oncogenic mutations, and tumor initiation, chronic inflammation is now considered a risk factor for the development of lung cancer and was recently described as one of the hallmarks of cancer development, with estimates of 15% of all cancer deaths being inflammation-related [13,14]. While some correlations have been made between the two disease states, the molecular mechanisms underlying the association between inflammation and lung cancer initiation and progression remain largely undefined.

The inflammatory tumor microenvironment (TME) is comprised of a number of different cell types and secreted molecules including cancer-associated fibroblasts, inflammatory cytokines, immune cells including macrophages and lymphocytes, and matricellular proteins [15]. Two pathways have been postulated for the origin of the inflammatory TME, the intrinsic and extrinsic pathways. The intrinsic pathway supposes that genetic alterations within the neoplastic cells lead to increased production of inflammatory mediators. The extrinsic pathway suggests that the inflammatory environment is present first and creates an accommodating niche for tumor development. Given what is currently being discovered about the ability of cytokines induced by inflammation in the lungs of smokers or those with COPD to cause transformation of

normal bronchial epithelial cells, the extrinsic pathway does seem to play a role [13]. The intrinsic pathway has also been indicated as being operative in human bronchial epithelial cells.

Understanding the molecular profile of the TME may enhance the diagnostic and therapeutic processes. Research into the gene expression profiles of the non-tumoral bronchial epithelium has revealed patterns that correlate with clinical endpoints including diagnosis, progression, and survival [15,16]. Use of non-steroidal anti-inflammatory drugs, including COX-2 inhibitors, has been linked to decreased incidence and delayed progression of a number of cancers, including lung [13,17,18]. The TME may also extend beyond the immediate tissue surrounding the tumor. Several studies have described a field of injury characterized by molecular and cellular changes in all airway epithelial cells exposed to cigarette smoke, termed a "field effect" [19].

Epithelial-to-Mesenchymal Transition

For tumors to progress from *in situ* to metastatic disease, they must acquire certain characteristics that allow them to become migratory, degrade and invade their local basement membrane, and migrate to either a proximal or distal site before forming a micrometastasis. As part of this process, tumors undergo a series of events known as epithelial-to-mesenchymal transition (EMT), wherein they lose epithelial characteristics and become more mesenchymal in phenotype and molecular profile.

EMT in Human Development

During the embryonic development process, cells undergo several rounds of EMT and the converse process mesenchymal-to-epithelial transition (MET) in order to migrate and differentiate into the specialized cell types and complex structures that form adult tissues and organs. These processes are highly regulated and have been classified into three distinct

sequences- primary, secondary, and tertiary EMT [20]. Primary EMTs center on the gastrulation process and includes formation of the mesoderm and primitive streak and neural crest determination. The transcription factors Snail and Twist, inhibitors of E-cadherin and evolutionarily conserved initiators of EMT, are key mediators of the gene regulatory network responsible for this process in vertebrates [21]. Expression of Snail is induced by transforming growth factor β (TGF-β) superfamily members and its expression is maintained by additional growth factor signaling [22].

Following the migration and primary differentiation events of gastrulation and neural crest formation, secondary EMT events generate the cell types required for formation of multiple organs. Mesenchymal cells in the neural crest differentiate into determined cell fates, migrate together, and undergo MET to form transient epithelial structures that will later form adult neural tissues, the musculoskeletal system, and the urogenital system, among others. The condensed epithelial structures undergo another round of EMT to differentiate further and migrate into more complex structures [20]. Tertiary EMT is primarily responsible for heart formation. Primary EMT specifies formation of cardiac mesodermal cells that organize into a two-layer epithelium via MET. These two layers fold around the primitive foregut in a secondary EMT followed by the endocardial tube formation surrounded by myocardial epithelium formed by a second MET. Finally, endothelial cells undergo another EMT to form the endocardial cushion [23].

As the phenotypic switching and cell migration required during development are not necessary in adult tissues, the EMT program is dormant in adult tissues, with a few exceptions. In response to epithelial tissue injury, keratinocytes along the edge of a wound undergo an EMT-like process during which they acquire an intermediate phenotype known as the “metastable” state. In this state they keep loose contact with neighboring cells while moving as a group to

close the wounded area [24]. When EMT is induced in chronically injured epithelium, such as in liver cirrhosis, fibrosis can develop over time. Fibrotic tissue forms from the conversion of epithelial cells to myofibroblasts that accumulate and secrete organ-damaging amounts of collagen [25]. The same molecules involved in developmental EMT have been implicated in fibrosis, including TGF-β and Snail [26-28].

EMT in Cancer Progression

While the EMT process and its associated transcription factors (especially the Snail family described below) are active in the embryonic development process, their activation in adult tissues is associated with malignant transformation. The transcription factors associated with EMT have been linked to a number of malignant phenotypes including transformation, migration, invasion, and apoptosis resistance [21,29,30]. The invasive front of numerous carcinomas, including breast, colon, and cervical, have a strong EMT profile. As the invasive front receives signals from both the tumor and the stroma, it is likely that the conversion is a result of the interplay between these two tissues. As the subset of cells that undergo EMT are likely to be responsible for metastatic progression, it is not surprising that an EMT molecular profile is correlated with poor clinical outcome and disease relapse in patients with breast, colorectal, and ovarian carcinomas [20].

Tumor Initiating Cells

Studies of neoplastic tissues have revealed a small subpopulation of cells within the tumor capable of self-renewal. These cells have been termed tumor-initiating cells (TICs) or cancer stem cells (CSCs) and are thought to not only give rise to the initial neoplasia but also the heterogeneous lineages of cells that comprise the total tumor [31,32]. Additionally, due to their self-renewal capabilities, these cells are thought to be responsible for relapse following

chemotherapy or radiation treatment [33]. These properties have recently lead TICs to become a critical target for new therapeutics. The origins of the TIC have not been identified and may be tumor-specific, but they have been hypothesized to arise from either a mature tissue stem cell that has had a malignant transformation or from a more differentiated cell whose "stemness" program has been re-initiated [33]. While the overall complement of TIC markers likely differs between tumor types, the $CD44^{high}/CD24^{low}$ antigenic phenotype has been correlated with a TIC-enriched subpopulation in breast cancer and other carcinomas [34,35].

In order for disseminated cells to give rise to macrometastases they must be capable of self-renewal, suggesting that they arise from the TIC subpopulation. As metastatic cells have undergone EMT, it would follow that the EMT process generates stem-like TICs. In a report from 2009, Mani and colleagues demonstrated that induction of EMT through ectopic expression of Snail family members or exposure to the inflammatory cytokine TGF-β1 in normal mammary epithelial cells gave rise to a stem-like ($CD44^{high}/CD24^{low}$) phenotype [35]. Examination of these stem-like cells confirmed their mesenchymal phenotype and tumorigenic potential. Additionally, murine mammary stem cells expressed markers associated with the EMT phenotype. Another inflammatory cytokine, IL-1, secreted by carcinoma cells is able to induce PGE_2 production in recruited mesenchymal stem cells (MSCs) in the surrounding tumor stroma. A resulting autocrine/paracrine signaling cascade between MSCs and carcinoma cells leads to increased cytokine production in MSCs and ultimately induction of the EMT program in the carcinoma cells. This EMT program includes increased Snail expression, invasion, and tumor initiation, as well as an enriched TIC population [36].

As potential drivers of both oncogenesis and metastatic progression, the TIC population is an obvious target for therapeutic intervention. A high-throughput screen of 16,000 small

molecules identified salinomycin as a selective inhibitor of breast cancer stem cells [37]. The stem cell population was created through induction of EMT and analysis of the drug in a xenograft model resulted in decreased primary mammary tumor burden as well as decreased metastases. The tumor cells remaining after salinomycin treatment had a distinctly differentiated and epithelial phenotype when compared to standard chemotherapy, suggesting preferential targeting of cells that had undergone, or were capable of undergoing, EMT. Importantly, salinomycin-treated cells no longer expressed stem cell markers and were unable to form tumorspheres. Salinomycin has subsequently been shown to be efficacious in cancer stem cell populations of many tumor types and is in early clinical evaluation [38-40].

Parallel Progression and Metastasis

The classical model of tumor progression proposes that a small population of cells within the invasive edge of an established tumor acquire characteristics necessary for EMT. This model assumes that a large tumor is established before metastases are able to form. Recent findings have shown that many patients present with a number of micrometastases before the primary tumor has become completely established. According to a new proposed model of parallel tumor progression, metastatic dissemination can occur throughout the course of primary tumor development and potentially occurs as an early event in the pathogenesis of the disease. These metastases arise from a subpopulation of tumor-initiating cells present at tumor initiation that express proteins, such as Snail, that induce EMT and confer the stem cell with migratory and invasive capacity [41].

The Transcriptional Regulators of EMT

The transcriptional regulators of EMT were first classified together by their ability to inhibit transcription of the epithelial adhesion molecule, E-cadherin. They can be further divided

into two groups based on their repression mechanism, direct or indirect. The direct repression group binds to and represses the activity of the E-cadherin promoter and includes Snail, Slug, Zeb1/2, E47, and KLF8. Twist, Goosecoid, E2.2, and FoxC2 repress E-cadherin transcription through an indirect mechanism [20]. The direct repressors bind to E-box consensus sequences in the promoter regions of target genes with the aid of chromatin modifying proteins such as HDAC1 and HDAC2 and other transcription modifying proteins [42]. Due to their profound effects on cellular phenotype, the expression of Snail family members is tightly regulated at multiple levels. Posttranslational modifications that control either nuclear localization or degradation include phosphorylation/dephosphorylation by PAK, GSK3β, and SCP [21,43].

The Role of Snail in EMT

The zinc-finger transcription factor *Snai1* (called Snail) has been shown to be upregulated following exposure to the inflammatory mediators COX-2 [44] and TGF-β [45]. Snail exerts global effects on epithelial cell gene expression profiles, resulting in regulation of EMT [46,47]. Snail plays a pivotal role in inducing EMT in a number of solid tumors, including breast and colorectal cancers [48-50]. In addition to transcriptional repression of E-cadherin, a critical aspect of EMT is loss of cell polarity. Through repressing transcription of apicobasal polarity molecule *Crumbs3* and inhibition of formation of the Par and Crumbs complex at cell junctions, Snail alters epithelial cell polarity [51]. The transition to a mesenchymal phenotype allows cells to become motile, but in order to leave their primary location, metastatic cells must also be able to degrade their basement membrane. Snail increases expression of matrix metalloproteases (MMPs), a class of protease enzymes that degrade extracellular matrix (ECM) proteins [52-54]. Some MMPs are able to prolong the EMT signaling process through a positive feedback loop that stimulates expression of Snail or other EMT drivers [55]. Recent studies suggest that Snail

may play a broader role in carcinogenesis. Yanagawa *et al* showed that Snail is upregulated in human NSCLC tissues, is associated with poor prognosis, and promotes NSCLC tumor progression *in vivo*. Furthermore, Snail overexpression in NSCLC is associated with differential gene expression related to diverse aspects of lung cancer progression, including angiogenesis [56]. Identification of the mechanisms by which the inflammation-induced transcriptional repressor Snail contributes to lung cancer initiation and parallel progression would be a step forward in targeting and impeding inflammation-induced lung cancer development.

The Role of Snail in Tumor Initiation

Given that Snail is upregulated by COX-2, TGF-β, and other cytokines secreted in the inflammatory TME, the role of Snail in tumor initiation has recently been investigated. Snail is expressed in a stem cell-like subpopulation within immortalized human mammary epithelial cells that are capable of transformation [35]. A subset of A549 lung cancer cells expressing Snail and its family members Zeb1 and Zeb2 have been shown to form dispersed villous colonies in an anchorage independent growth assay. In addition, this subset formed subcutaneous tumors more efficiently in a xenograft model than did the non-Snail expressing subset or total A549s, suggesting a role for Snail in the tumor-initiating cell population [57]. In a malignant human keratinocyte *in vitro* system, inhibition of Snail signaling depleted keratinocyte stem cell markers CD34 and K5 [58]. Given the relationship between the EMT program and the conversion to a tumor-initiating cell phenotype, further investigation into the role of Snail in early events and metastatic progression is needed.

The Role of Invasion in Metastatic Progression

In order for tumor cells to metastasize beyond their primary location, they must be able to degrade and invade into their tumor stroma and basement membrane and intravasate into either

blood vessels or lymph vessels. The acidity of tumors, a result of hypoxia, has been shown to mediate remodeling of the stroma and permit invasion [59]. Additionally, a number of molecular changes have been shown to enable tumor cells to become invasive, many of which are correlated with EMT. Inflammatory mediators associated with EMT, including TGF-β, COX-2, and IL-1β, lead to increased invasion and metastasis in melanoma, glioma, and NSCLC [13]. Tumor-derived TGF-β transdifferentiates fibroblasts at the invasive front of tumors into myofibroblasts, which share characteristics of mesenchymal cells and smooth muscle cells [60]. The presence of myofibroblasts has been correlated with invasion and progression in breast, colon, and lung cancers [61]. In reaction to signals received from the tumor cells, the fibroblasts in the tumor stroma also secrete ECM remodeling factors to enhance degradation, including collagen type I & IV, MMPs, and secreted protein, acidic and rich in cysteine (SPARC) [60-62].

SPARC

SPARC, also known as osteonectin, is an extracellular matrix glycoprotein first identified as a major noncollagenous component of bovine bone [63]. SPARC, as with all SPARC family members, possesses a characteristic conserved C-terminal extracellular calcium-binding domain and it binds both fibrillar collagen and basal lamina collagen IV with a long helical structure at the N-terminus [64,65]. Its expression modulates reversible interactions between cells and the extracellular matrix though it does not directly support cell attachment or play a structural role in the ECM [66]. When added to epithelial cells, SPARC induces focal adhesion disassembly, cell rounding, and an intermediate state of cell adhesion, indicative of a role for SPARC in ECM organization [67]. Indeed, SPARC-null (-/-) mice have altered ECM production and assembly with diminished and less mature collagen production than wild-type mice [68]. SPARC binds to integrin-linked kinase (ILK), a serine/threonine kinase that binds to the intracellular domain of

β1 integrin and controls the intracellular signaling cascades that influence cellular contractile elements, and is required for fibronectin-induced ILK activation [69].

The Role of SPARC in Wound Healing and Tumor Progression

Given its role in remodeling the ECM through its interaction with collagen and other matricellular proteins, it is unsurprising that SPARC plays a role in regulating the healing of wounded dermal tissues [66,70]. SPARC is expressed by macrophages and immunoreactive fibroblasts at the edge of wounds and stimulates angiogenesis at the wound site [70,71]. Given the importance of SPARC in the inflammatory response to injury and collagen deposition, it is unsurprising that it has been found to play a role in tissue fibrosis. For example, SPARC has been found to be upregulated in fibrotic tissue in cirrhotic livers, specifically secreted by hepatic stellate cells (HSC) in these tissues. In an *in vivo* model of cirrhotic liver fibrosis, SPARC knockdown in HSCs decreased fibrosis through increased fibronectin adhesion and decrease chemokine-mediated migration [72]. TGF-β and SPARC expression in HSCs cooperates in an autocrine-feedback loop, with SPARC knockdown reducing TGF-β1 gene expression and secretion and TGF-β1 treatment increasing SPARC gene expression.

Upregulation of SPARC is associated with metastatic potential of melanomas and gliomas, as well as an invasive phenotype in breast, prostate, and colorectal carcinomas [73]. SPARC is regulated by Integrin β4 to promote invasion [74]. Additionally, SPARC has been shown to regulate the activity of MMPs, which, as mentioned above (See The Role of Snail in EMT), are enzymes that play a major role in ECM proteolysis, a requirement for cancer cell invasion into the basement membrane and stroma [64]. Inhibition of SPARC expression in metastatic melanoma cell lines decreased their *in vitro* invasion through downregulation of MMP2 and MMP9. In this same model, the cell lines were unable to form tumors *in vivo*

following SPARC inhibition, suggesting a role for SPARC not only in tumor invasion but in tumorigenesis as well [75]. In leukemia cells, increased levels of intracellular SPARC were correlated with increased cellular survival and chemoresistance, further indicating a role for SPARC in the TIC/EMT phenotype [76]. Expression of SPARC in the tumor stroma of NSCLC is associated with poor patient prognosis [77], though its role in lung tumor progression, especially in relation to Snail expression, has not been evaluated.

SPARC-Targeted Therapies

Albumin-bonded paclitaxel (nab-paclitaxel), an injectable form of the mitotic inhibitor drug paclitaxel, is known to accumulate preferentially in tissues expressing SPARC, due to SPARC's albumin-binding properties [78]. A Phase II study of nab-palitaxel in advanced NSCLC demonstrated decreased toxicity and increased response compared to traditional paclitaxel treatment [79]. In this study, tumoral SPARC expression was not evaluated. In a retrospective study of monotherapy nab-paclitaxel in head and neck cancer patients, response to treatment correlated positively with SPARC expression due to the SPARC-albumin interaction [80]. Additional studies in pancreatic, adrenocortical, and advanced breast cancers confirm the enhanced efficacy of nab-paclitaxel therapy in SPARC-expressing tumors [81-83]. Following correlation of SPARC expression levels with bone-metastatic potential of prostate cancers, Thomas and colleagues developed a nanoparticle targeted to SPARC. This nanoparticle is designed to be used as a molecular imaging agent for prostate cancer patient prognostic stratification and could potentially be used for specific delivery of cytotoxic agents to SPARC-expressing cancer cells in multiple tumor types [84].

Transforming Growth Factor-β and Cancer

Transforming Growth Factor-β (TGF-β) is one of a number of polypeptide growth factor family members that activate downstream signaling cascades in response to activation. The three TGF-β isoforms (TGF-β1, TGF-β2, and TGF-β3) are structurally and functionally similar and are secreted as latent precursor molecules [85]. Activation of the latent molecules by interaction with integrins, proteolytic cleavage, or changes in environmental pH results in cleavage of the propeptide from the noncovalently bound latent TGF-β-binding protein [86]. Once activated, TGF-β binds to its constitutively active transmembrane serine/threonine kinase receptor, TGFβRII, which in turn recruits and phosphorylates TGFβRI. Phosphorylation of TGFβRI is required for activation of downstream signaling cascades, which can include the canonical Smad pathway, as well as the non-canonical mitogen-activated protein (MAP) kinase pathways, phosphoinositol-3-kinase (PI3K)/AKT pathway, and PP2A pathway [87].

The p38 MAPK signal transduction pathway

One of the signaling cascades induced by TGF-β is known as the p38 group of MAP kinases. Members of this group are known to be involved in inflammation as well as cell death, growth, and differentiation [88]. The p38α protein (or p38) was the first member isolated as it was rapidly phosphorylated in response to LPS stimulation [89]. Like the other MAP kinases, p38 contains dual phosphorylation sites in a regulatory loop between two of its kinase subdomains [90]. There are three p38 homologues that are ubiquitously, though differentially expressed, in mammalian tissues. The p38δ isoform (also known as SAPK4) is predominantly expressed in the lung, as well as in the kidney, testes, pancreas, and small intestine [91]. The p38 group of kinases is activated by any of the MAP kinase kinases (MKKs), though typically MKK3 or MKK6, which are in turn activated by any of the MKK kinases (MAP3Ks). Due to the commonality of activators between the MAP kinases, the different MAPK groups are often

coactivated. However, specific activation of a MAP kinase group (e.g. p38 over JNK) can be achieved by different MAP3Ks [92,93]. Likewise, selective deactivation of a MAP kinase group can be achieved by dephosphorylation by a MAP kinase phosphatase (MKP), of which several have been identified [94,95].

The JNK MAPK signal transduction pathway

The c-Jun NH_2-terminal kinase (JNK) pathway is another of the TGF-β-induced MAP kinase signaling group members. Like p38, JNK is activated by MKKs, typically MKK4 or MMK7 [96]. The most well studied target of JNK signaling is the transcription factor Activator Protein-1 (AP-1), the activation of which is mediated by the phosphorylation of c-Jun and related molecules [97]. JNK signaling can be selectively regulated by MAP kinase phosphatases, including the dual specificity (p38 and JNK) phosphatase MKP7 [98]. A small molecule inhibitor of JNK activity, SP600125, has been developed and is currently being evaluated in cellular models of various epithelial cancers [99,100]

The MEK/ERK signal transduction pathway

The ERK pathway is another of the MAP kinase signaling group members that can be activated by TGF-β or other cell-surface receptors. Following signaling from the receptor, one of the Ras family small guanosine triphosphatases (GTPases) is recruited to the receptor by adaptor proteins. Its activation stimulates the first kinase of the phosphorylation cascade, the MAP3K Raf family members, followed by the second kinase MKK, or MEK, family. MEK1 and MEK2 are dual-specific and phosphorylate both threonine and tyrosine residues in their MAPK targets, extracellular signal-regulated kinase (ERK) 1 and ERK2. Activation of ERK1/2 activates their catalytic activity and results in their nuclear translocalization and ultimately changes target gene expression [101]. ERK targets a number of genes responsible for cellular activities as diverse as

proliferation, differentiation, survival, and cellular metabolism. The biochemical activity of ERK occurs both through kinase-dependent and kinase-independent pathways [102]. In the nucleus, ERK regulates gene expression by a number of interactions. One such interaction is the activation of the poly (ADP-ribose) polymerase PARP-1 and its substrate Elk-1, a transcription factor [102].

Given its role in processes necessary for tumor cell propagation and survival, it is unsurprising that the ERK pathway is activated in many cancers. Normal lung tissue stains negatively for ERK phosphorylation while 34% of lung cancer specimens have activated ERK signaling, with activation correlating positively with disease progression and metastasis [103]. While EGFR mutant lung cancers are highly responsive to the EGFR TKI class of drugs, all patients eventually acquire resistance and develop recurrence [104,105]. One of the mechanisms for resistance is activation of the ERK1/2 pathway either through amplification of the *MAPK1* gene encoding ERK2 or downregulation of negative regulators of ERK signaling DUSP5, SPRY4, and SPRED2 [106].

Given the role for ERK signaling in tumorigenesis, progression, and chemoresistance, an effort has been made to target ERK in patients. A number of MEK inhibitors have been developed for the clinic, including the MEK 1 and 2-specific small molecule selumetinib. Selumetinib has been shown to be efficacious in KRAS-mutant NSCLC [107].

TGF-β and EMT

TGF-β1 has been shown to induce EMT in a variety of epithelial tumor types, including alveolar carcinomas [108]. This EMT induction has been shown to be mediated by transcription factors including the Snail family of transcriptional repressors [109]. Snail and Slug are integral in TGF-β induced EMT in *in vitro* models of lung fibrosis and were detected in the affected

tissues of patients with Idiopathic Pulmonary Fibrosis (IPF), a common precursor to lung cancer [110]. MEK/ERK activation by TGF-β1 increases collagen deposition in a Smad2/3-dependent and JNK-independent manner [111,112]. Activated Notch signaling was shown to be directly responsible for the upregulation of Snail by TGF-β in an *in vitro* lung cancer model [113] and the high-mobility group protein HMGA2 has been shown to control the expression of Snail during TGFβ-induced EMT [114]. TGF-β signaling also works to control the hierarchy of transcriptional repressors during EMT. The Snail and Slug gene promoters contain responsive elements to TGF-β targets AP1, AP4, Smad, and LEF [21]. Snail is frequently expressed at the initiation of EMT, while Slug is subsequently induced to maintain the mesenchymal state, indicating cooperation between TGF-β and Snail family members for both initiation and maintenance of EMT. Clinical studies targeting various TGF-β pathway molecules are underway, including an autologous tumor cell vaccine carrying a TGF-β2 antisense transgene [115].

MicroRNAs and Cancer

MicroRNAs (miRNAs) are small (18-25 nt) RNA sequences that, when incorporated into an miRNA-protein complex, bind the 3' UTR of target mRNA which contain at least partial homology with the miRNA "seed" sequence [116]. Binding of an miRNA to its target leads to inhibition of target translation or destabilization of the target transcript leading to degradation [117]. Recent bioinformatic analysis suggests that 30-60% of the human genome may be regulated by miRNAs [117]. Deregulation of miRNA genes in cancer cells could alter the expression levels of their downstream targets, including oncogenes and tumor suppressor genes. Gain or loss of miRNAs can contribute at many points of the life span of a cancer cell including during tumor initiation, development, and dedifferentiation [118]. Unsurprisingly, miRNAs are

differentially expressed in human cancers versus normal tissues and this differential expression varies among tumor subtypes [119]. *In vitro* manipulation of miRNAs potentially involved in cancer has revealed their functions. For example, downregulation of the miRNA Let-7 in human lung carcinomas occurs frequently and *in vitro* overexpression of Let-7 in the human lung cancer cell line A549 induced cell growth inhibition [120]. The development of microarrays containing all known and predicted human miRNAs has allowed comparison of the entire miRNAome of malignant and normal tissues [121].

In silico analysis of the genome predicts that 44% of known cancer-related genes could be targeted by miRNAs differentially expressed in cancers. *In vitro* analysis of this data set confirms 75% of these interactions, including those with important cancer genes such as *RB1* and *FGFBR2* [119]. Further evidence linking aberrant miRNA expression with cancer was found when analyzing the genes encoded in fragile regions of the chromosomes. Fragile sites (FRAs) are specific loci in the genome that form gaps and constrictions on metaphase chromosomes that have been exposed to partial replicative stress [122]. Common fragile sites are suspected to be part of the normal chromosomal structure while other fragile sites are rare and only occur in a subset of the population. Both common and rare fragile sites co-localize with sites of deletion, amplification, translocation, and loss of heterozygosity (LOH) in human tumors and are especially susceptible to integration of viral DNA [122]. One such fragile site is the chromosome 13q14 region. This region is deleted in more than half of B-cell chronic lymphocytic leukemias and frequently in other cancers [123]. This region does not contain any known tumor suppressor genes, however, it contains the genes encoding miR-15a and miR-16a, known to play a role in degradation of CDK family members. Carlo Croce and colleagues further investigated this link between miRNAs and FRAs using a bioinformatic approach. In total, 186 miR genes were

mapped and their nonrandom chromosomal distribution allowed them to be spatially grouped into 36 clusters. The mapped genes were compared to the location of FRAs and 19% of the miRs were located in or adjacent to FRAs, a rate of occurrence 9.12 times higher than in non-fragile sites [124]. Correlation between miRNA gene location and cancer-associated genomic regions (CAGRs) in a variety of tumor types was significantly higher (52.5%). CAGRs are regions of amplification, LOH, and breakpoints common in cancer cells. High frequency of LOH at 17p13.3 is found in solid tumors with a low frequency of *TP53* mutations, indicating that these tumors have an alternative method of tumor suppression, possibly related to an miRNA in 17p13.3 [124,125].

Another microRNA, miR-29b has been found to target apoptosis, cell cycle, and proliferation pathways in acute myeloid leukemia (AML) [126]. Mir-29b is downregulated in primary AML samples and its overexpression induced apoptosis and inhibited expression of tumorigenic proteins in AML cell lines. In a panel of breast cancer cell lines, miR-29b expression was identified as inversely correlating with invasiveness [127]. The miR-29b target genes (verified by luciferase activity in the 3'UTR) responsible for invasion included C1QTNF6, COL4A2, and SPARC [128].

MicroRNAs have been found to play a role in the EMT process. For example, the miR-200 family of microRNAs is capable of inhibiting EMT by downregulating the expression of Zeb and Slug, and is itself downregulated by Slug [129-131]. MiR-200 is frequently downregulated in the lungs of patients with idiopathic pulmonary fibrosis, and forced overexpression of miR-200 family members in lung adenocarcinoma (ADC) cell lines inhibits their ability to undergo EMT, migrate, and invade, suggesting a role for microRNAs in the pathogenesis of lung cancer [130,132,133].

MicroRNA expression patterns have been used to characterize metastatic disease as well as predict progression and clinical outcome [134,135]. For example, Carlo Croce and colleagues used miRNA array technology in 2005 to identify an miRNA signature of eleven miRNAs significantly altered in breast cancer. Differential expression of miRNAs was also noted between biopathologic subtypes, allowing for further classification of breast cancer based on their miRNA signature [136].

Restoration of tumor-suppressor miRNA expression in patients has been successfully evaluated clinically. Intratumoral injection of miR-29 miRNA mimics in xenograft models of human liver cancer, rhabdomyosarcoma, and AML resulted in tumor regression [126,137,138], though this method has not been evaluated in human patients. Use of adenovirus-associated vectors to efficiently transduce tumors intravenously has been proposed as an improved delivery mechanism that will allow miRNA mimic use in the clinic [139].

THE ROLE OF SPARC IN SNAIL-OVEREXPRESSING NSCLC

Introduction

The molecular mechanisms underlying the association between inflammation and lung cancer initiation and progression remain largely undefined. Identification of the mechanisms by which the inflammation-induced transcriptional repressor Snail contributes to lung cancer initiation and parallel progression would be a step forward in targeting and impeding inflammation-induced lung cancer development. Expression of SPARC in the tumor stroma of NSCLC is associated with poor patient prognosis [77], though its role in lung tumor progression, especially in relation to Snail expression, has not been evaluated. To understand the molecular changes that occur in NSCLC initiation and development, Snail was overexpressed in cellular models of premalignant and established lung cancers. SPARC is upregulated by Snail in both models and both proteins are co-expressed in NSCLC tissues. Snail overexpression leads to increased invasion in both models in a SPARC-dependent manner, suggesting a role for Snail and SPARC in metastatic progression.

Materials & Methods

Human Cell Lines and Reagents

To facilitate the study of normal lung differentiation and pathology, we use a system for immortalizing human bronchial epithelial cells (HBECs) in the absence of viral oncoproteins via ectopic expression of human telomerase (hTERT) and cyclin-dependent kinase 4 (Cdk4) under control of puromycin and geneticin respectively [140]. Over 50 such HBEC cell lines established from proximal airway epithelial cells derived from unique patients are currently available; the cell lines designated HBEC2, HBEC3, and HBEC7 were used in these studies [141]. The HBEC3 cell line was transfected with shRNA targeting the tumor suppressor P53 under control of zeocin and a plasmid overexpressing the oncogenic protein KRAS with an activating mutation

under control of blasticidin. Based on extensive molecular characterization and their ability to differentiate into each of the major cell types of the normal pseudostratified columnar bronchial epithelium in the 3D organotypic model, HBECs are known to have bronchoalveolar stem-like characteristics that allow modeling of the pulmonary airways and their associated malignant transformation [140,142-144]. Utilization of this unique cell-based resource to model lung carcinogenesis allows us to systematically test the functional importance of Snail and SPARC *in vitro* and *in vivo*, affording us a more comprehensive understanding of the pathogenesis of NSCLC and new opportunities for early clinical intervention.

Lung cancer cell lines A549, H1437 and H292 were obtained from American Type Culture Collection (ATCC) (Rockville, MD). HBEC cell lines were a kind gift from Dr. John D. Minna at the University of Texas, Southwestern. All cell lines were routinely tested for the presence of *Mycoplasma* using the MycoAlert *Mycoplasma* Detection Kit (Lonza, Walkerville, CA). Cell lines were authenticated in the UCLA Genotyping and Sequencing Core utilizing Promega's (Madison, WI) DNA IQ System and Powerplex 1.2 system according to the manufacturer's instructions. All cells were utilized within 10 passages of genotyping. Lung cancer cell lines were grown in RPMI 1640 (Mediatech Inc, Herndon, VA) supplemented with 10% fetal bovine serum (Gemini Biological Products, Calabasas, CA), 1% penicillin/streptomycin (Gibco, Gibbstown, NJ) and 2mM glutamine (Gibco). HBEC cell lines were grown in Keratinocyte Serum-Free Media (Gibco) supplemented with 30ug/mL Bovine Pituitary Extract and 0.2ng/mL recombinant Epidermal Growth Factor 1-53 (Gibco).

Stable Overexpression of Snail

Cells were stably transduced as follows: wild-type Snail cDNA pcDNA3 (a gift from Dr. E. Fearon, University of Michigan, Ann Arbor, MI) was excised from the plasmid with HindIII

and EcoRV and subcloned into the retroviral vector pLHCX (Clontech), which includes a drug resistance (Hygromycin B) marker. All constructs were verified by restriction endonuclease digestion. For virus production, 70% confluent 293T cells were transfected with pLHCX-Snail or pLHCX (vector alone). Tumor cells were then transduced with high-titer supernatants producing either Snail or pLHCX virus. Following transduction, the tumor cells were selected with Hygromycin B (Invitrogen). Cells were verified by genotyping and tested for Mycoplasma as above.

Immunoblotting

Cells were washed with PBS and whole-cell lysates were collected over ice using lysis buffer prepared according to standard methods [145]. Protein concentrations were measured with a BCA protein assay reagent (Pierce, Rockford, IL). Proteins were resolved by 10% SDS-PAGE and analyzed by western blot using polyvinylidene difluoride membranes (Millipore, Bedford, CA) according to standard methods. Membranes were blocked with 5% nonfat dry milk or 5% BSA in TBS plus 0.05% Tween 20. The membranes were probed overnight at 4^{o}C with anti-E-cadherin antibody (BD Biosciences PharMingen/Transduction Laboratories), anti-Snail, and anti-SPARC (all from Cell Signaling). Secondary antibodies, goat anti-mouse (Bio-Rad, Hercules, CA) and goat anti-rabbit (Santa Cruz), were incubated at room temperature for 60 minutes. Membranes were developed using Supersignal Chemiluminescence System (Pierce) or Western Lightning Plus-ECL (Perkin-Elmer, Waltham, MA) and exposed to X-ray film (Life Sciences Products, Inc, Frederick, CO). Equal loading of samples was confirmed by probing the membranes with alpha-tubulin (Cell Signaling).

Modified Boyden Chamber Invasion Assay

Cells were serum-starved and plated at a density of $2x10^4$ (cancer cells) or $1x10^4$ (HBEC) cells per well in Corning HTS Transwell-96 Permeable Support Plate (Sigma-Aldrich). Prior to plating, transwells were coated with Type I Rat Tail Collagen (BD Biosciences) to create a "membrane" for the cells to degrade and invade through. Cells were allowed to invade for 48 hours into a lower chamber containing media with 20% FBS (cancer cells) or 2% FBS (HBEC) as a chemoattractant. The upper chamber was aspirated and washed with PBS to remove noninvasive cells. The lower chamber was washed with PBS and invasive cells were released from the underside of the top chamber with Cell Dissociation Solution (Trevigen, Gaithersburg, MD). Calcein AM (Invitrogen) was used to stain viable cells in the lower chamber only and fluorescence was quantified and compared to a control plate from Day 1 containing the total number of cells plated.

Immunohistochemistry

Sections were obtained from human lung cancer specimens archived in the University of California at Los Angeles Lung Cancer Specialized Programs of Research Excellence tissue bank (IRB#10-001096). Antigen retrieval was accomplished with sodium citrate 10 mmol/L (pH 6.0). Serial sections were blocked with 10% normal goat serum and then probed with an antibody against Snail (ab85931, Abcam) or SPARC (AON-5031, Heamatologic Technologies) using a working dilution of 1:500 for tissue staining. Primary antibodies were incubated overnight at 4°C. After incubation with secondary antibody (Vector Laboratories), staining was developed using DAB Substrate kit for Peroxidase (SK-4100, Vector Laboratories). Snail and SPARC expression was evaluated by a pathologist (MCF) specializing in cardiopulmonary disease. Evaluation of tumors was based on staining intensity and correlation of staining between serial sections. Photomicrographs were obtained using an Olympus BX50 microscope, with Plan

APO objective lenses. An Olympus DP11 camera and Olympus Camedia software were used to produce the images.

Total RNA Preparation, cDNA Synthesis and Real-Time PCR

RNA was isolated using the miRNeasy Mini kit (Qiagen, Valencia, CA) and cDNA was prepared using the High Capacity RNA-to-cDNA Kit (Applied Biosystems) according to the manufacturers' protocols. Transcript levels were measured by quantitative real-time–polymerase chain reaction (qRT-PCR) using the TaqMan Probe-based Gene Expression system (Applied Biosystems) in a MyiQ Cycler (Bio-Rad) following the manufacturer's protocol. Amplification was carried out for 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C. Samples were run in triplicate and their relative expression was determined by normalizing the expression of each target to GUSB. These were then compared with the normalized expression in a reference sample using the efficiency corrected Pfaffl method to calculate a fold-change value [146].

Stable Inhibition of SPARC by Short-Hairpin RNA

SPARC shRNA plasmids on the pLKO.1-Puro vector backbone and relevant controls were obtained from Sigma-Aldrich. Cancer cell lines were stably transduced using the same viral transduction method as above with one of five SPARC shRNA sequences (designated –shSPARC1, -shSPARC2, -shSPARC3), a nonsilencing sequence (-shNS), or the pLKO.1 vector backbone (-shV). The cell lines were selected with optimized concentrations of puromycin (EMD Chemicals) and verified by genotyping and tested for Mycoplasma as above. For the HBEC cell lines, the puromycin selection marker was replaced with the IMPDH gene, encoding resistance to mycophenolic acid (MPA). After viral transduction, the cells were selected with optimized concentrations of MPA.

Results

Snail overexpression drives upregulation of SPARC in cellular models of premalignant and established NSCLC

Recently, we have shown that forced overexpression of Snail in NSCLC cell lines leads to global expression changes, including increased angiogenesis and invasion. Microarray and bioinformatic analysis, using Ingenuity Systems Pathway Analysis (IPA) software, indicated that a panel of Snail-overexpressing NSCLC cell lines have significantly increased expression of Secreted Protein, Acidic and Rich in Cysteine (SPARC) [56]. Immunoblot and qRT-PCR analysis of multiple NSCLC cell lines with and without Snail overexpression confirms the relationship between Snail and SPARC in established cancer cell lines **(Fig 2.1, 2.2)**. As we are interested in the mechanism of NSCLC pathogenesis, we introduced the same Snail overexpression plasmid into HBECs to model early pathogenesis. Immunoblot and qRT-PCR analysis of these cell lines shows that Snail overexpression leads to SPARC expression in this model as well **(Fig 2.3, 2.4)**. Taken together, these data indicate that SPARC is upregulated by Snail at early and late points during lung carcinogenesis and may play a role in lung cancer initiation and progression.

Snail overexpression is correlated with SPARC overexpression in human NSCLC tissues

To confirm that the relationship between Snail and SPARC is physiologically relevant to human tumors, we stained serial sections of 10 human adenocarcinomas and 9 human squamous cell carcinomas for Snail and SPARC protein expression. Cells within both adenocarcinomas and squamous cell carcinomas with nuclear Snail staining also have cytoplasmic SPARC staining (**Fig 2.5**). These results establish an *in situ* correlation between Snail and SPARC expression.

Snail overexpression leads to increased invasion in cellular models of premalignant NSCLC

Because SPARC is known to be associated with invasive cancers and is correlated with poor patient prognosis in NSCLC, we evaluated the HBEC cell lines in an *in vitro* invasion assay. Prior to plating, transwells were coated with Type I Rat Tail Collagen to create a "membrane" for the cells to degrade and invade through. In all HBEC cell lines, Snail overexpression lead to increased invasion **(Fig 2.6)**.

Snail overexpression leads to increased invasion in cellular models of established NSCLC

Because SPARC is known to be associated with invasive cancers and is correlated with poor patient prognosis in NSCLC, we evaluated the NSCLC cell lines in an *in vitro* invasion assay. Prior to plating, transwells were coated with Type I Rat Tail Collagen to create a "membrane" for the cells to degrade and invade through. In all the NSCLC cell lines Snail overexpression lead to increased invasion **(Fig 2.7)**.

Snail-mediated invasion is SPARC-dependent in *in vitro* models of premalignant and established NSCLC

Having established that Snail overexpression leads to both increased SPARC expression and invasion, we hypothesized that the increased invasion is SPARC-mediated. The cancer cell lines A549 and H1437 and HBEC cell line HBEC3mutP53/KRAS with stable Snail overexpression (-S) or vector control (-V) were stably transfected with a plasmid containing an shRNA sequence specific to the 3'UTR of SPARC or non-silencing controls (-NS) **(Fig 2.8, 2.9,** and data not shown**)**. Utilizing the Modified Boyden Chamber Assay previously described, we plated the cells and allowed them to invade for 48 hours. Inhibition of SPARC by shRNA in the

NSCLC lines and HBEC lines prevented Snail-mediated invasion **(Fig 2.10, 2.11)**, indicating that SPARC is at least partially responsible for increased invasion downstream of Snail.

Figures

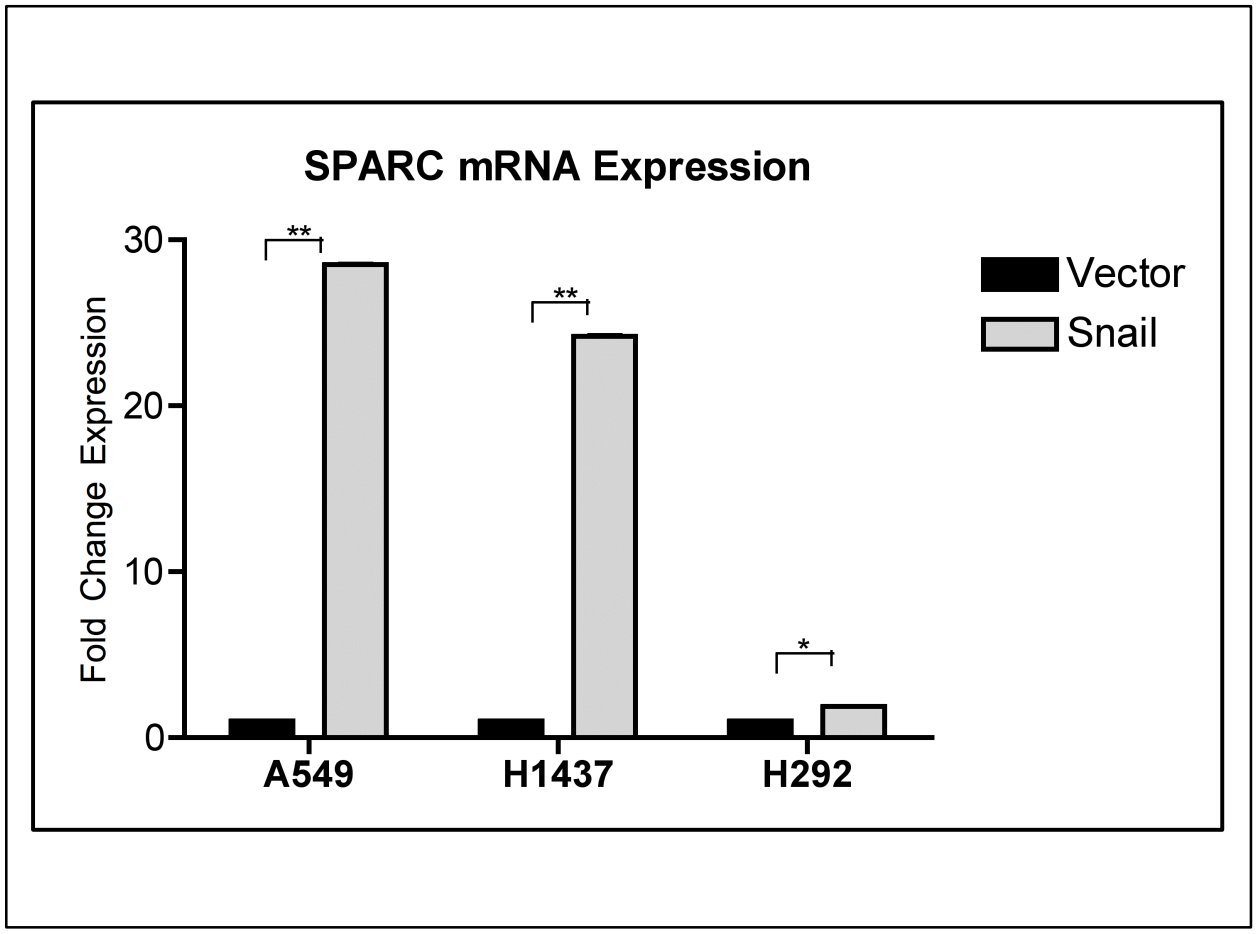

Figure 2.1. SPARC mRNA is upregulated by Snail in NSCLC cell lines. Total RNA was isolated from NSCLC cell lines H292, H1437, and A549 stably transfected with either a Vector control plasmid (V) or a Snail expression plasmid (S). Expression levels of SPARC were evaluated by qRT-PCR using TaqMan primers. mRNA levels were normalized to GUSB.

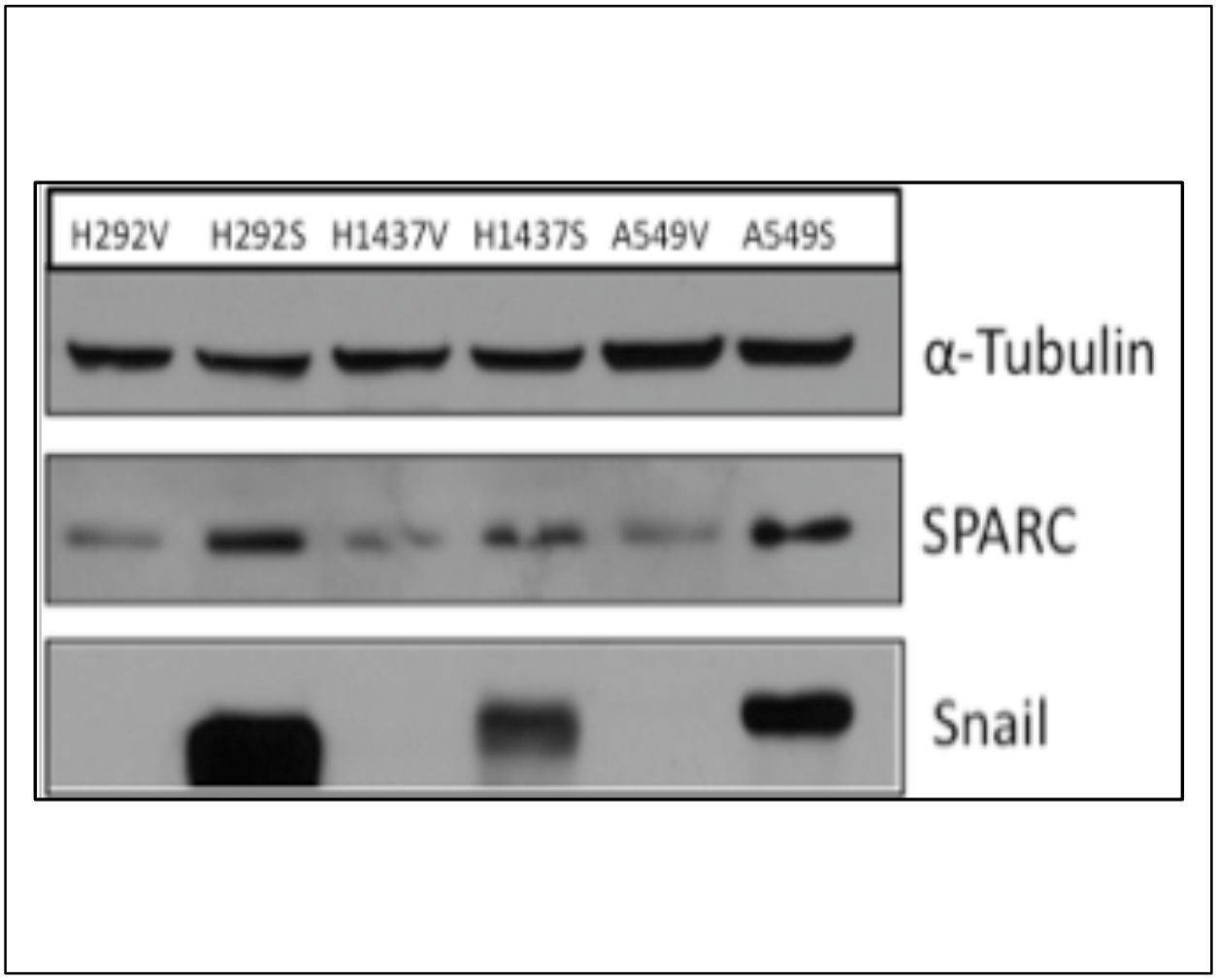

Figure 2.2. SPARC protein is upregulated by Snail in NSCLC cell lines. Levels of Snail and SPARC protein were evaluated by Western blotting in A549-V/S, H1437-V/S, and H292-V/S cell lines. Protein levels were normalized by α-Tubulin.

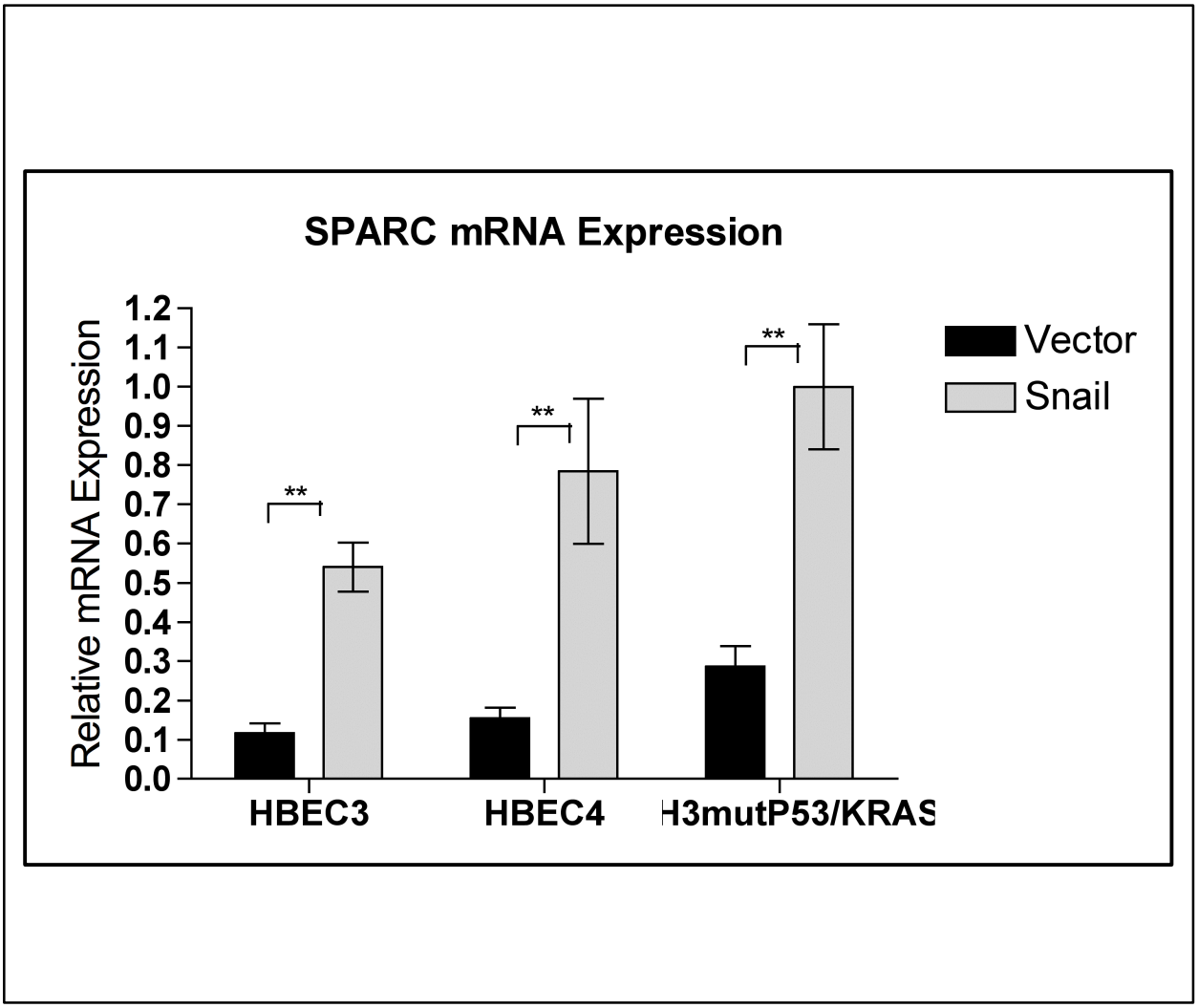

Figure 2.3. SPARC mRNA is upregulated by Snail in HBEC cell lines. Total RNA was isolated from HBEC cell lines HBEC2, HBEC4, and H3mutP53/KRAS stably transfected with either a Vector control plasmid (V) or a Snail expression plasmid (S). Expression levels of SPARC were evaluated by qRT-PCR using TaqMan primers. mRNA levels were normalized to GUSB.

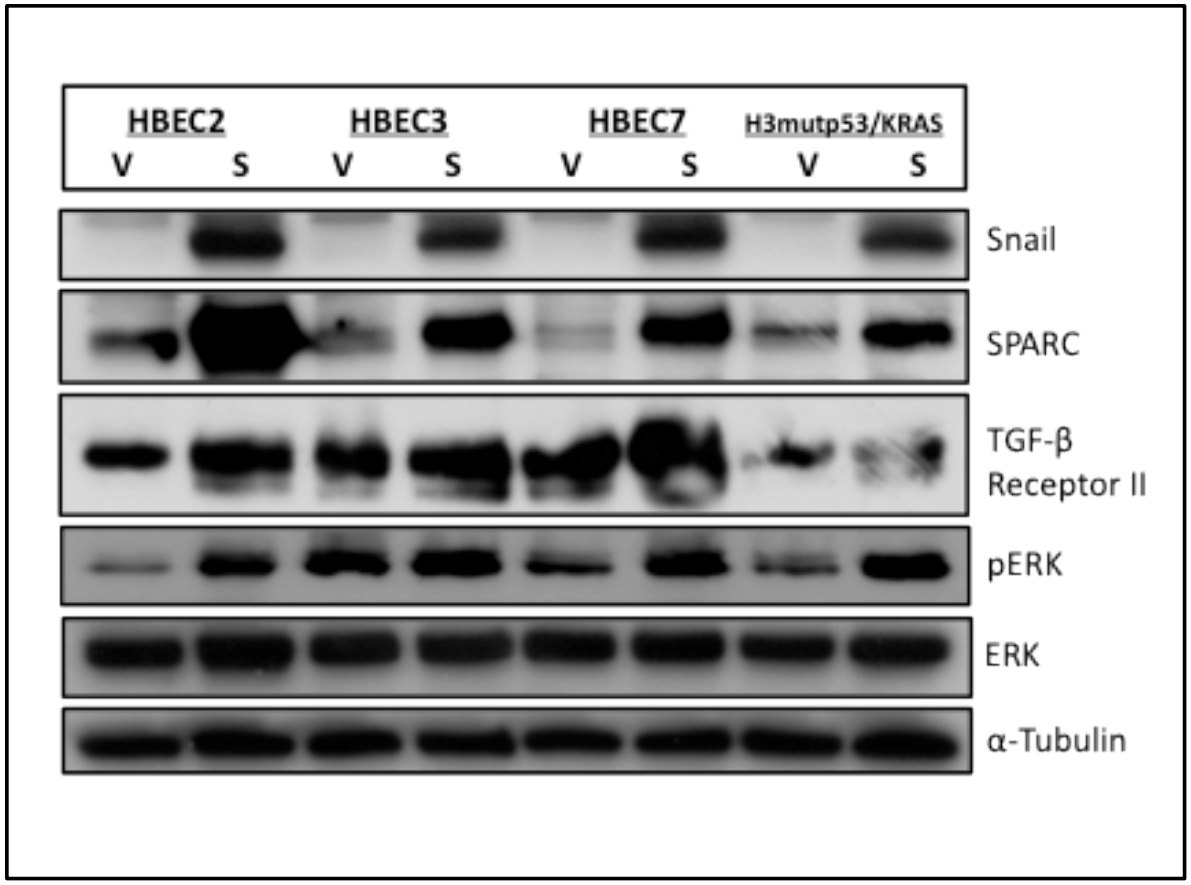

Figure 2.4. SPARC protein is upregulated by Snail in HBEC cell lines. Levels of Snail, SPARC, TGFβR2, pERK1/2, and tERK1/2 protein were evaluated by Western blotting in HBEC2-V/S, HBEC3-V/S, HBEC7-V/S, and H3mutp53/KRAS-V/S cell lines. Protein levels were normalized by α-Tubulin.

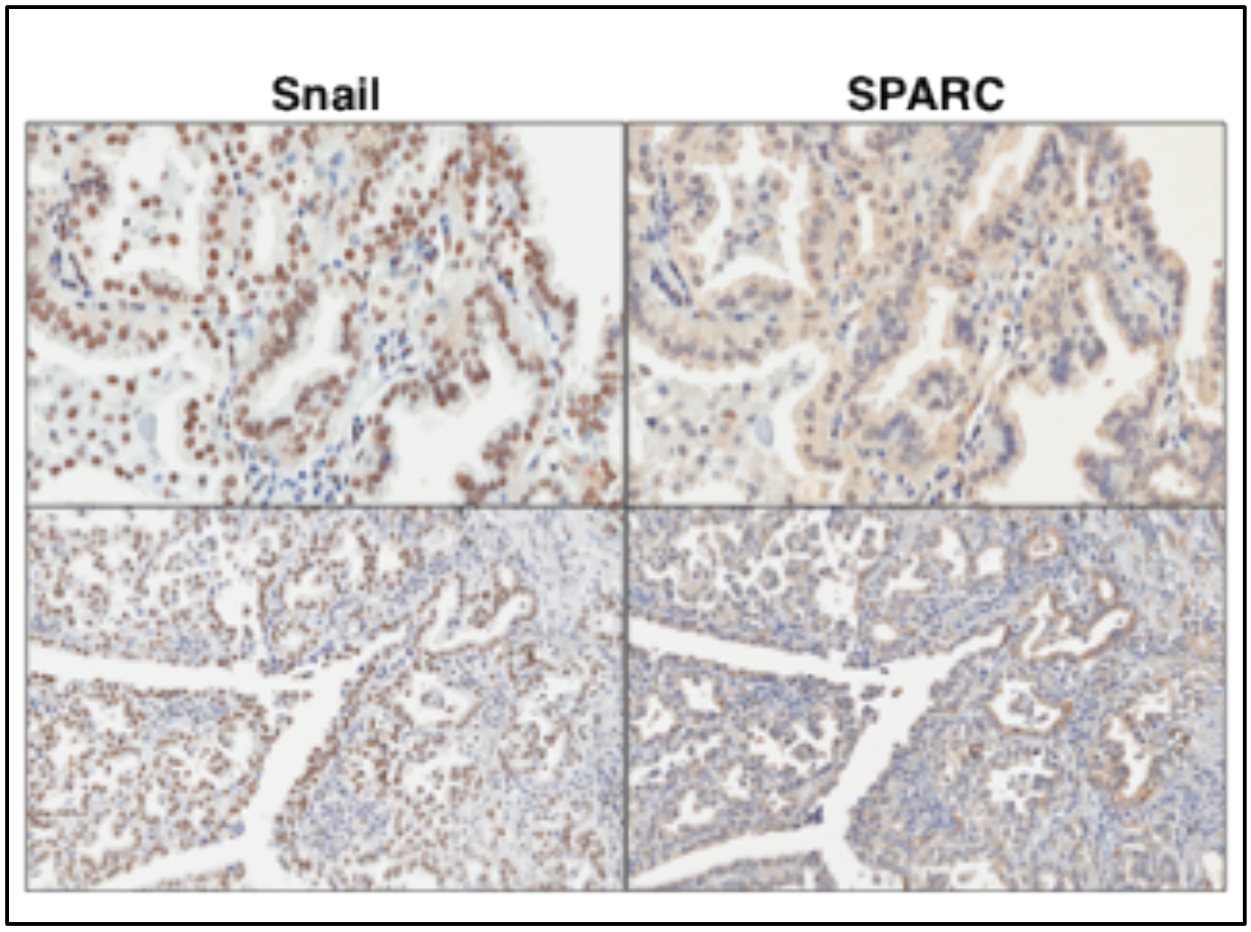

Figure 2.5. Snail and SPARC colocalize *in situ.* Serial sections of NSCLC tumors were immunostained for Snail (left) and SPARC (right). Sections were scanned using the Aperio ScanScope XT System and visualized with the Aperio ImageScope viewing software. Sections were reviewed by a pathologist (MCF) and scored based on Snail or SPARC staining intensities. 20X fields from infiltrating adenocarcinoma (ADC) and squamous cell carcinoma (SCC), showed correlating areas with both nuclear Snail and cytoplasmic SPARC staining. Representative sections are shown here.

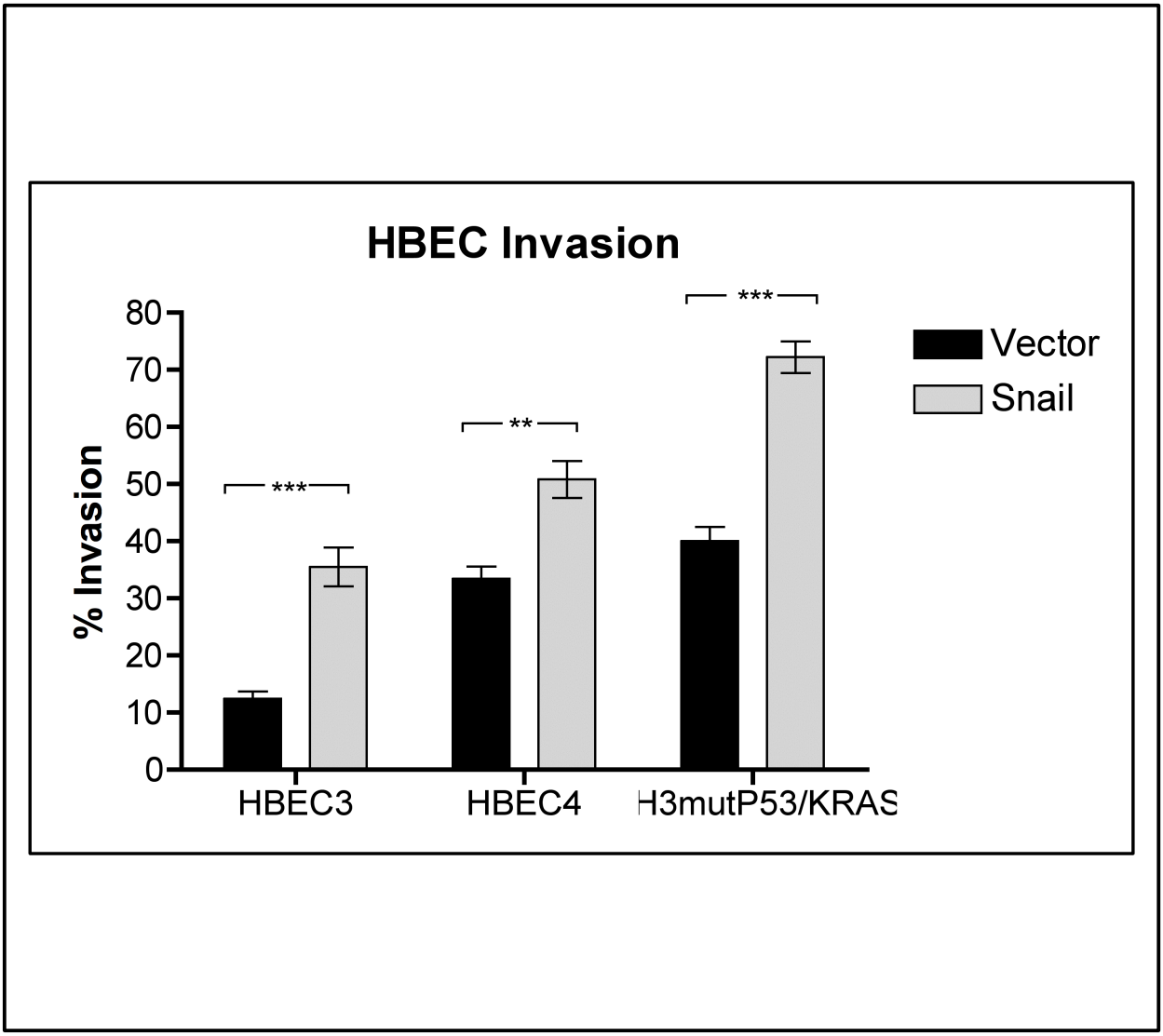

Figure 2.6. Snail overexpression leads to increased invasion in HBEC cell lines. The invasive capacity of the HBEC cell lines HBEC3, HBEC4, H3mutP53/KRAS (H3mut) with and without Snail overexpression were evaluated in a modified Boyden Chamber assay for invasion through a collagen matrix over 48 hrs.

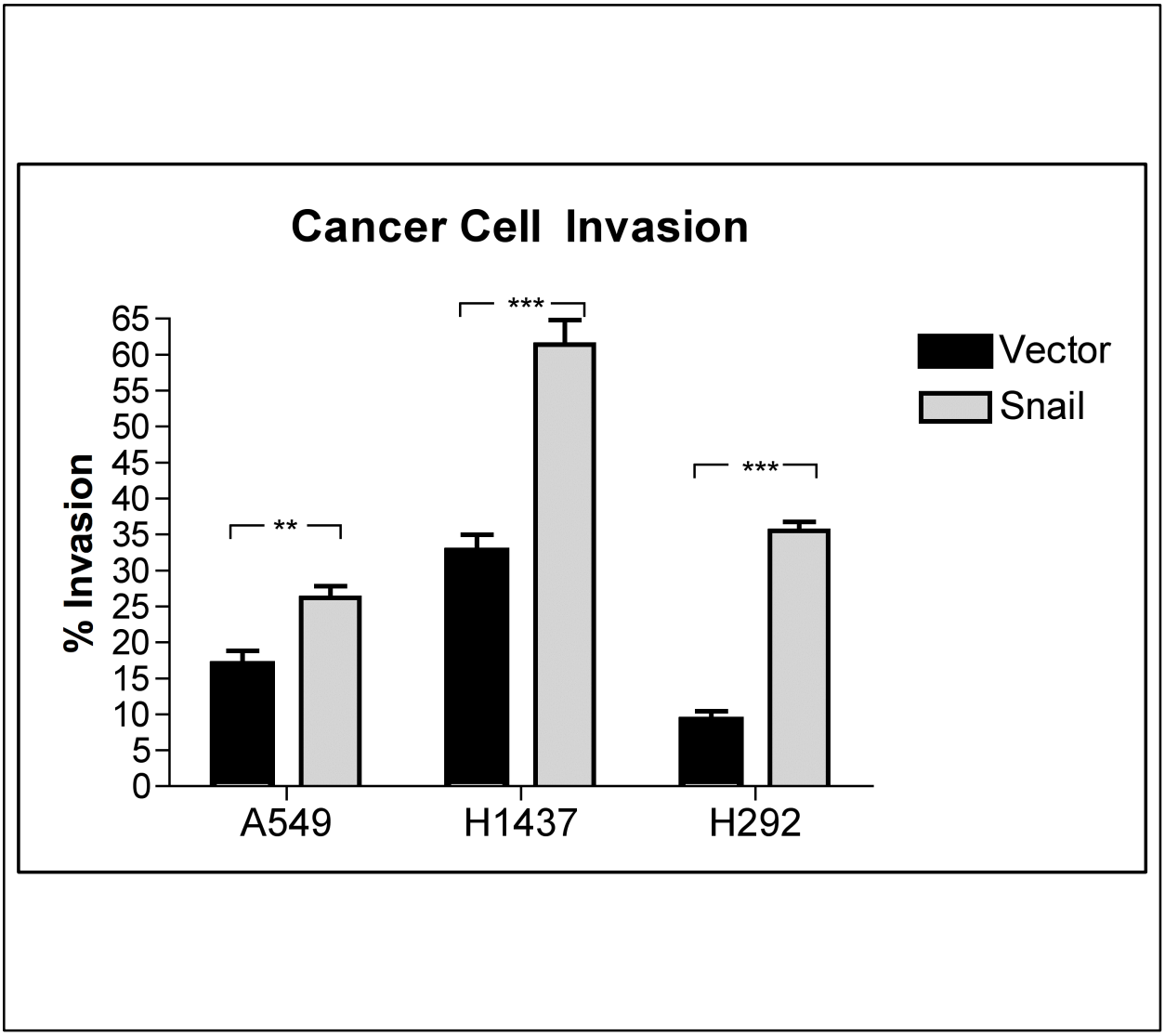

Figure 2.7. Snail overexpression leads to increased invasion in NSCLC cell lines. The invasive capacity of the NSCLC cell lines A549, H1437, and H292 with and without Snail overexpression were evaluated in a modified Boyden Chamber assay for invasion through a collagen matrix over 48 hrs.

Figure 2.8. SPARC shRNA in H3mutP53/KRAS cell lines. SPARC shRNA sequences (sh1, sh2) were stably transfected into H3mutP53/KRAS vector control (V) and Snail-overexpressing (S) cell lines along with a nonsilencing (NS) shRNA control. Expression of SPARC in all stable lines was examined by Western blot. Protein levels were normalized to α-Tubulin.

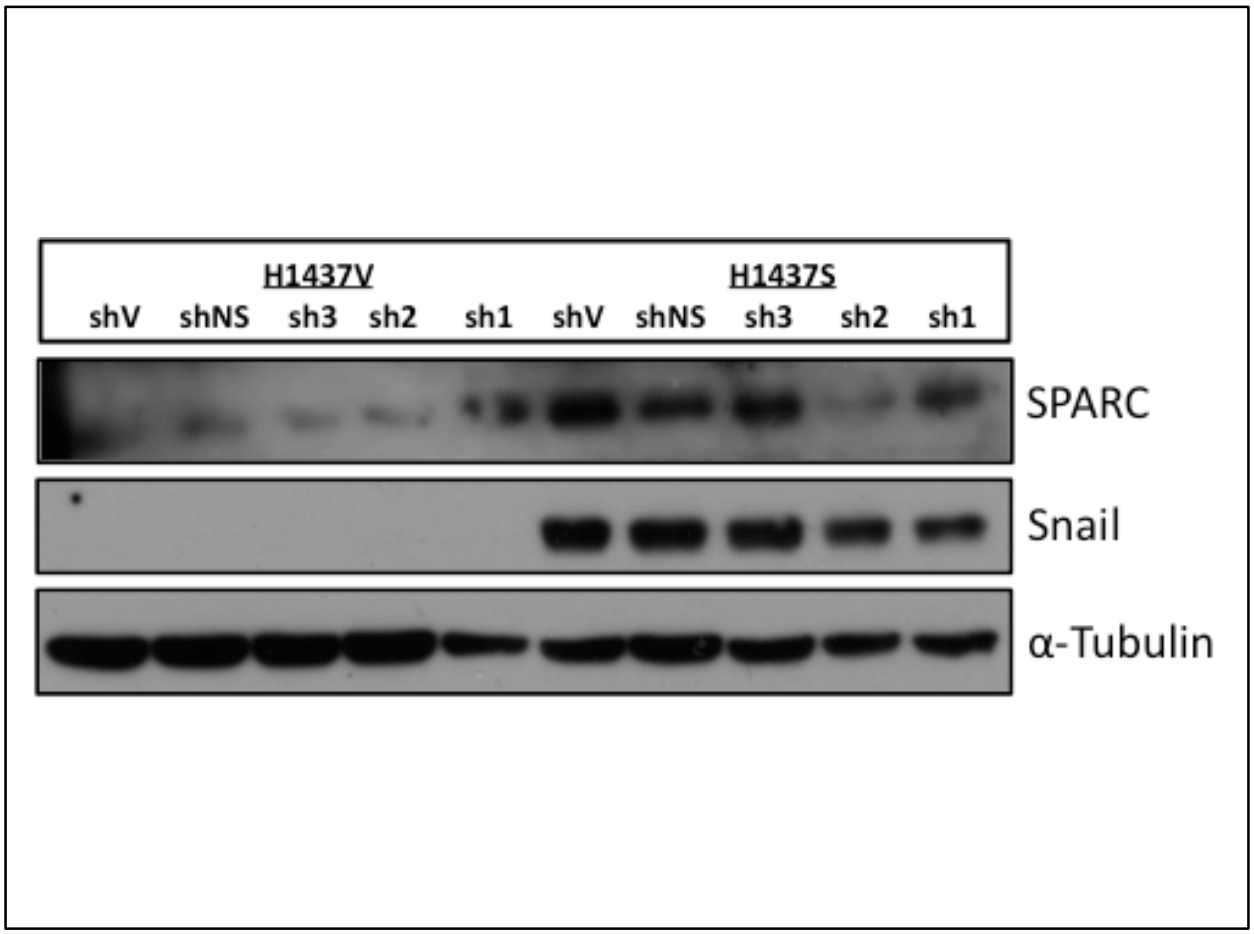

Figure 2.9. SPARC shRNA in H1437 cell lines. SPARC shRNA sequences (sh1, sh2, sh3) were stably transfected into H1437 vector control (V) and Snail-overexpressing (S) cell lines along with a nonsilencing (NS) shRNA control. Expression of SPARC in all stable lines was examined by Western blot. Protein levels were normalized to α-Tubulin.

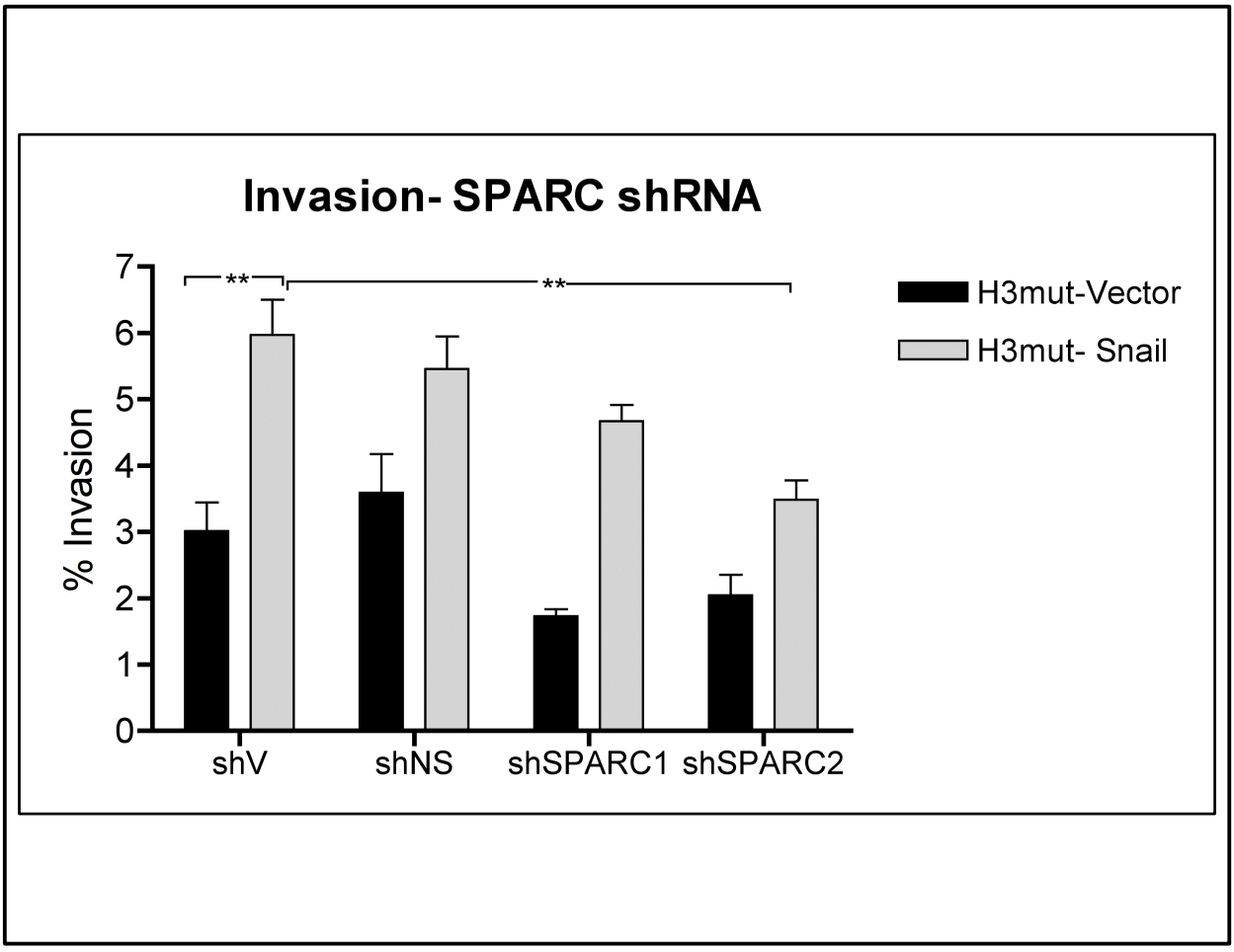

Figure 2.10. Snail-dependent invasion in HBEC cell lines is SPARC-mediated. The HBEC cell lines from Figure 2.8 were evaluated in a modified Boyden Chamber assay for invasion through a collagen matrix over 48 hrs as in Figure 2.6 . (** = $p<0.01$, *** = $p<0.0001$)

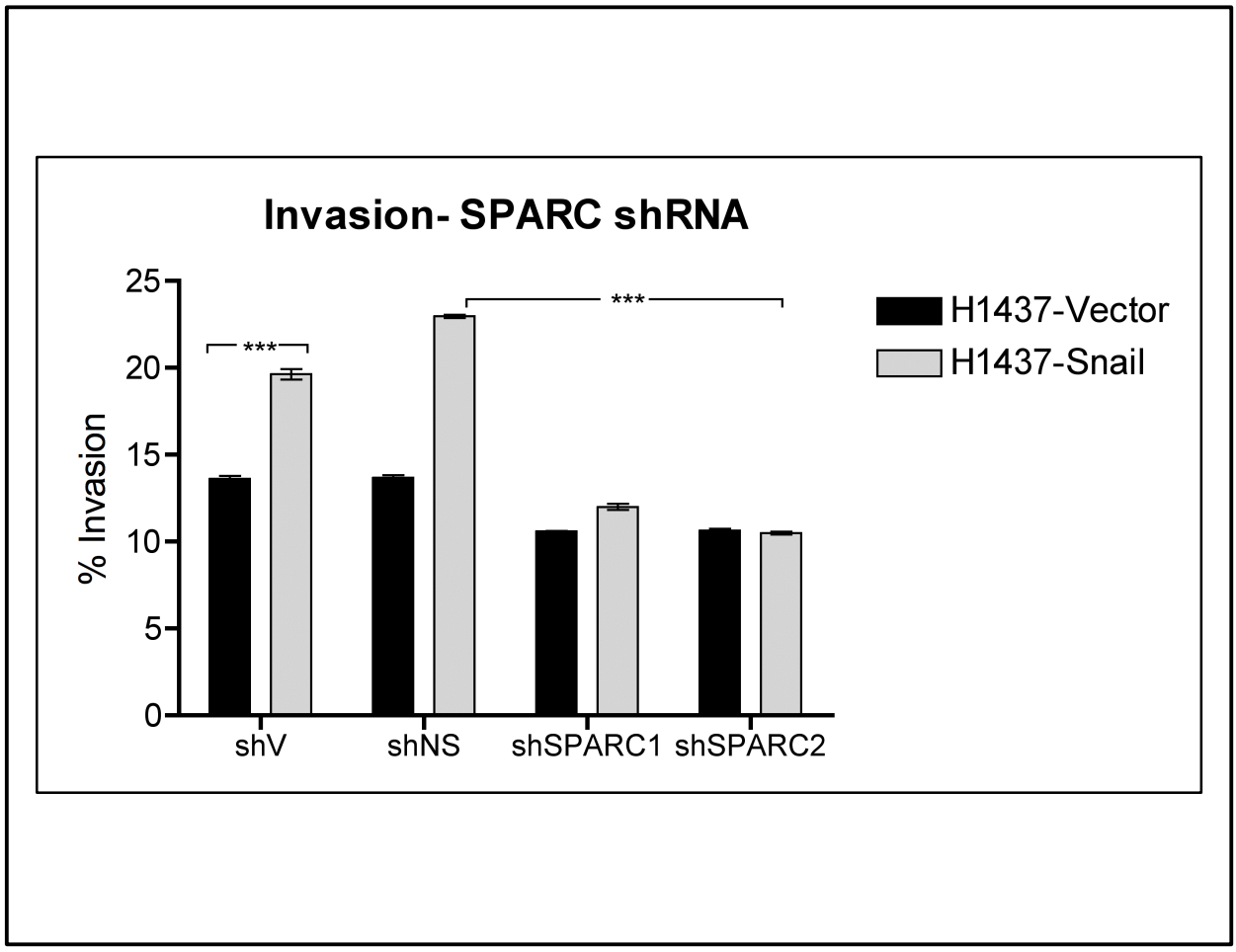

Figure 2.11. Snail-dependent invasion in NSCLC cell lines is SPARC-mediated. The NSCLC cell lines from Figure 2.9 were evaluated in a modified Boyden Chamber assay for invasion through a collagen matrix over 48 hrs as in Figure 2.6 . (** = $p<0.01$, *** = $p<0.0001$)

Discussion

For tumors to progress from *in situ* to metastatic disease, they must acquire certain characteristics that allow them to become migratory, degrade and invade their local basement membrane, and migrate to either a proximal or distal site before forming a micrometastasis. As part of this process, tumors undergo a series of events known as epithelial-to-mesenchymal transition (EMT), where they temporarily lose epithelial characteristics and become more mesenchymal in phenotype and molecular profile. The classical model of tumor progression proposes that a small population of cells within the invasive edge of an established tumor acquire characteristics necessary for EMT. Recent findings have shown that many patients present with a number of micrometastases before the primary tumor has become completely established, supporting a recently proposed model of parallel tumor progression in which metastatic dissemination occurs throughout the course of primary tumor development and potentially occurs as an early event in the pathogenesis of the disease [41]. These metastases arise from a subpopulation of stem cell-like cells present at tumor initiation, which express proteins that induce EMT and confer the stem cell with migratory and invasive capacity. This is the first report to show that Snail upregulates SPARC in models of both early and established NSCLC. The necessity of SPARC for Snail-mediated invasion in both models suggests a role for both Snail and SPARC in both the NSCLC stem-cell population and in parallel progression.

As mentioned in Section I, the relatively low five-year survival rate (16%) of patients with lung cancer is attributed, in part, to a lack of early detection. A recent national screening trial of low-dose computed tomography (CT) resulted in a decrease in mortality due to better detection of lung nodules that would have previously been undiagnosed or misdiagnosed on a chest x-ray [147,148]. However, understanding the molecular mechanisms contributing to

tumorigenesis and parallel metastatic progression could lead to identification of early disease biomarkers and therapeutic targets, adding to the success of the CT study.

Multiple genetic mutations have been identified as driving oncogenesis and promoting survival in NSCLC, including those in the genes encoding P53, KRAS, and EGFR [5,6]. Our results here are independent of mutation status, as the relationships between Snail, SPARC, and invasion were consistent among all cell lines tested. We showed here HBEC cell lines without known mutations, as well as a cell line in which P53 was silenced and KRAS was constitutively active. We also examined Snail-mediated SPARC expression in HBEC cell lines with single P53 or KRAS mutations as well as EGFR activating mutations and found the relationship to be the same in these lines. The three NSCLC cell lines that were evaluated, A549, H1437, and H292 had distinct mutation backgrounds. The cell line A549, derived from an adenocarcinoma, has mutations in KRAS, CDKN2A, and STK11. The cell line H1437, also derived from an adenocarcinoma has mutations in P53, CDKN2A, and STK11. The cell line H292, derived from a mucoepidermoid pulmonary carcinoma, contains only a CDKN2A mutation. Despite the differences in mutation status, they all responded to Snail overexpression with increased invasion, mediated by upregulation of SPARC. This mutation independence suggests that the mechanism of Snail-mediated, SPARC-dependent invasion may be occurring prior to or concurrent with tumorigenesis, supporting the role of Snail in the TIC population.

Research identifying novel driver mutations and therapies targeting them has been successful in NSCLC [5,6,9,10]. However, as has been the case with EGFR TKIs, almost all tumors initially responsive to treatment acquire resistance [149]. This may be due to activation of alternate signaling pathways, acquisition of additional oncogenic mutations from mutagens present in the tumor microenvironment, or due to the heterogeneity of the tumor makeup at the

time of initial treatment. The cancer stem cell model posits that the small subset of cells responsible for tumor initiation give rise to heterogeneous lineages of cells that comprise the bulk of the tumor [31,150]. Additionally, due to their self-renewal capabilities, these cells are thought to be responsible for relapse following treatment. Targeted treatment depletes those cells within a tumor expressing the target and those cells undergoing proliferation if targeted treatment is combined with classic chemotherapeutic agents [32]. If the tumor initiating cells are expressing EMT-associated factors such as Snail, as we posit here, that may be the mechanism by which they evade apoptosis. As chronic exposure to inflammatory cytokines such as TGF-β has been shown to induce EMT, chronic inflammation could lead to tumor initiation through upregulation of Snail and parallel progression through subsequent upregulation of SPARC.

While we describe here an *in vitro* model of lung cancer invasion as well as an *in situ* correlation between Snail and SPARC expression, further investigation in an *in vivo* model is needed, considering the role the tumor microenvironment is known to play in progression [13]. Considering the role the tumor microenvironment is known to play in progression, further investigation in an *in vivo* model would give additional clues to the role of this pathway in parallel progression. For example, tumor-derived TGF-β transdifferentiates fibroblasts at the invasive front of tumors into myofibroblasts, which share characteristics of mesenchymal cells and smooth muscle cells [60]. The presence of myofibroblasts has been correlated with invasion and progression in breast, colon, and lung cancers [61]. In reaction to signals received from the tumor cells, the fibroblasts in the tumor stroma also secrete ECM remodeling factors to enhance degradation, including collagen type I & IV, MMPs, and SPARC [60-62]. Given the importance of SPARC in the inflammatory response to injury and collagen deposition, it is unsurprising that it has been found to play a role in tissue fibrosis. For example, SPARC has been found to be

upregulated in fibrotic tissue in cirrhotic livers, specifically secreted by hepatic stellate cells (HSC) in these tissues. In an *in vivo* model of cirrhotic liver fibrosis, SPARC knockdown in HSCs decreased fibrosis through increased fibronectin adhesion and decrease chemokine-mediated migration [72].

In addition, costaining of Snail and SPARC in premalignant tissues would yield further evidence of the role of Snail-mediated upregulation of SPARC in parallel progression. Additional experiments evaluating the role of SPARC in NSCLC tumor initiation could potentially yield interesting results, given the known role of Snail and EMT in the TIC population. Further refinement of the molecular phenotype of the Snail-expressing TIC population in NSCLC is currently being investigated in our lab. The role of SPARC in this specific subset of cells remains to be evaluated.

THE SNAIL-TO-SPARC PATHWAY INTERMEDIARIES

Introduction

Having established that Snail upregulates SPARC in models of premalignancy and established cancer and leads to an invasive phenotype, the next step was to establish a mechanism for this interaction. The promoter region of SPARC does not contain a binding site for Snail, indicating that Snail must upregulate SPARC by an indirect mechanism. The mechanism for Snail-induced SPARC expression was examined and key signaling pathways as well as a novel microRNA critical for this relationship were discovered.

Materials and Methods

Human Cell Lines and Reagents

To facilitate the study of normal lung differentiation and pathology, we use a system for immortalizing human bronchial epithelial cells (HBECs) in the absence of viral oncoproteins via ectopic expression of human telomerase (hTERT) and cyclin-dependent kinase 4 (Cdk4) under control of puromycin and geneticin respectively [140]. Over 50 such HBEC cell lines established from proximal airway epithelial cells derived from unique patients are currently available; the cell lines designated HBEC2, HBEC3, and HBEC7 were used in these studies [141]. The HBEC3 cell line was transfected with shRNA targeting the tumor suppressor P53 under control of zeocin and a plasmid overexpressing the oncogenic protein KRAS with an activating mutation under control of blasticidin. Based on extensive molecular characterization and their ability to differentiate into each of the major cell types of the normal pseudostratified columnar bronchial epithelium in the 3D organotypic model, HBECs are known to have bronchoalveolar stem-like characteristics that allow modeling of the pulmonary airways and their associated malignant transformation [140,142-144]. Utilization of this unique cell-based resource to model lung carcinogenesis allows us to systematically test the functional importance of Snail and SPARC *in*

vitro and *in vivo*, affording us a more comprehensive understanding of the pathogenesis of NSCLC and new opportunities for early clinical intervention.

Lung cancer cell lines A549, H1437 and H292 were obtained from American Type Culture Collection (ATCC) (Rockville, MD). HBEC cell lines were a kind gift from Dr. John D. Minna at the University of Texas, Southwestern. All cell lines were routinely tested for the presence of *Mycoplasma* using the MycoAlert *Mycoplasma* Detection Kit (Lonza, Walkerville, CA). Cell lines were authenticated in the UCLA Genotyping and Sequencing Core utilizing Promega's (Madison, WI) DNA IQ System and Powerplex 1.2 system according to the manufacturer's instructions. All cells were utilized within 10 passages of genotyping. Lung cancer cell lines were grown in RPMI 1640 (Mediatech Inc, Herndon, VA) supplemented with 10% fetal bovine serum (Gemini Biological Products, Calabasas, CA), 1% penicillin/streptomycin (Gibco, Gibbstown, NJ) and 2mM glutamine (Gibco). HBEC cell lines were grown in Keratinocyte Serum-Free Media (Gibco) supplemented with 30ug/mL Bovine Pituitary Extract and 0.2ng/mL recombinant Epidermal Growth Factor 1-53 (Gibco).

Stable Overexpression of Snail

Cells were stably transduced as follows: wild-type Snail cDNA pcDNA3 (a gift from Dr. E. Fearon, University of Michigan, Ann Arbor, MI) was excised from the plasmid with HindIII and EcoRV and subcloned into the retroviral vector pLHCX (Clontech), which includes a drug resistance (Hygromycin B) marker. All constructs were verified by restriction endonuclease digestion. For virus production, 70% confluent 293T cells were cotransfected with pLHCX-Snail or pLHCX (vector alone). Tumor cells were then transduced with high-titer supernatants producing either Snail or pLHCX virus. Following transduction, the tumor cells were selected with Hygromycin B (Invitrogen). Cells were verified by genotyping and tested for Mycoplasma

as above.

TGF-β ELISA

Secreted TGF-β1 levels were quantified using the eBioscience Human/Mouse TGF beta1 ELISA Ready-SET-Go! Kit. Cells were plated in 6-well plates at a density of $1.25x10^5$ cells per well. Once adherent, cells were washed and media replaced with 1ml serum-free media. Media supernatants and cell lysates were collected after 24 hours. Supernatant TGF-β levels were evaluated following the manufacturers' instructions and normalized to lysate protein values.

Inhibition of TGF-β with small-interfering RNA

Cells were plated in 6 well plates at $1.25x10^5$ cells per well and cultured for 24 hours in growth media. Complete media was replaced with serum-free media overnight prior to transfection with target siRNA (Integrated DNA Technologies) or Silencer® Negative Control #1 siRNA (Life Technologies). Transfections were carried out using the Lipofectamine RNAiMAX Transfection Reagent (Life Technologies) in serum-free media for 4 hours before replacement with fresh serum-free media and an additional 20-hour incubation.

Inhibition of ERK1/2 and p38 with a chemical inhibitor

Treatments were carried out in 6 well plates at a density of $1.25x10^5$ cells per well in serum-free medium, unless stated otherwise. The MEK/ERK inhibitor UO126 (Cell Signaling) was prepared in sterile DMSO at a concentration of 10mg/mL and cells were treated at a final concentration of 15ug/mL for 24 hours. The p38 inhibitor SB203580 (Cell Signaling) was prepared in sterile DMSO at a concentration of 10mg/mL and cells were treated at a final concentration of 15ug/mL for 24 hours.

Overexpression of microRNA-29b

Cells were plated in 6 well plates at $1.25x10^5$ cells per well and cultured for 24 hours in growth media. Complete media was replaced prior to transfection with the miRVana™ miR-29b mimic (Life Technologies) or miRVana™ miRNA mimic, Negative Control #1 (Life Technologies). Transfections were carried out using the Lipofectamine RNAiMAX Transfection Reagent (Life Technologies) in serum-free media for 4 hours before replacement with fresh media and an additional 20-hour incubation.

Immunoblotting

Cells were washed with PBS and whole-cell lysates were collected over ice using lysis buffer prepared according to standard methods [145]. Protein concentrations were measured with a BCA protein assay reagent (Pierce, Rockford, IL). Proteins were resolved by 10% SDS-PAGE and analyzed by western blot using polyvinylidene difluoride membranes (Millipore, Bedford, CA) according to standard methods. Membranes were blocked with 5% nonfat dry milk or 5% BSA in TBS plus 0.05% Tween 20. The membranes were probed overnight at 4^oC with anti-Snail, anti-SPARC, anti-TGFBR2, anti-ERK1/2, and anti-phospho-ERK1/2 antibodies (all from Cell Signaling). Secondary antibodies, goat anti-mouse (Bio-Rad, Hercules, CA) and goat anti-rabbit (Santa Cruz), were incubated at room temperature for 60 minutes. Membranes were developed using Western Lightning Plus-ECL (Perkin-Elmer, Waltham, MA) and exposed to X-ray film (Life Sciences Products, Inc, Frederick, CO). Equal loading of samples was confirmed by probing the membranes with alpha-tubulin (Cell Signaling).

Total RNA Preparation, cDNA Synthesis and Real-Time PCR

RNA was isolated using the miRNeasy Mini kit (Qiagen, Valencia, CA) and cDNA was prepared using the High Capacity RNA-to-cDNA Kit (Applied Biosystems) according to the manufacturers' protocols. Transcript levels were measured by quantitative real-time–polymerase

chain reaction (qRT-PCR) using the TaqMan Probe-based Gene Expression system (Applied Biosystems) in a MyiQ Cycler (Bio-Rad) following the manufacturer's protocol. Amplification was carried out for 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C. Samples were run in triplicate and their relative expression was determined by normalizing the expression of each target to GUSB (mRNA) or RNU6b (miRNA). These were then compared with the normalized expression in a reference sample using the efficiency corrected Pfaffl method to calculate a fold-change value [146].

Results

TGF-β1 is upregulated in response to Snail overexpression

The promoter region of SPARC does not contain a binding site for Snail, indicating that Snail must upregulate SPARC by an indirect mechanism. Combinatorial analysis using the Ingenuity Pathway Analysis (IPA) software revealed multiple potential intermediary molecules and signaling pathways that could be responsible for Snail-mediated upregulation of SPARC (**Fig 3.1)**. These intermediates were supported by the literature [72,151]. We evaluated the secreted protein levels of one potential candidate, the cytokine TGF-β1, utilizing ELISA. Expression levels were significantly increased ($p=<0.001$) in Snail-overexpressing HBEC cell lines compared to vector control **(Fig 3.2)**, indicating that TGF-β is upregulated by Snail and may be upstream of SPARC.

Treatment of parental HBEC cell lines with TGF-β1 results in upregulation of Snail, phosphorylation of ERK1/2, and upregulation of SPARC

Treatment of the parental HBEC cell lines with recombinant TGF-β1 resulted in increased expression of Snail, SPARC, and phosphorylation of ERK1/2 (**Fig 3.3),** suggesting an autocrine signaling mechanism for Snail and TGF-β1 expression. As the receptor for the TGF-β1

ligand was upregulated in response to Snail expression **(Fig 3.4)**, we hypothesized that increased TGF-β ligand binding increased production of receptor.

Inhibition of TGF-β1 by siRNA results in loss of Snail-driven SPARC expression

Furthermore, inhibition of TGF-β1 expression by siRNA abrogated Snail-mediated ERK1/2 phosphorylation and SPARC expression **(Fig 3.5, 3.6)**, suggesting that both TGF-β1 ligand and its receptor are necessary for Snail-mediated SPARC expression.

ERK1/2 is phosphorylated downstream of Snail and TGF-β overexpression

ERK1/2 was also indicated as a potential intermediate between Snail and SPARC **(Fig 3.1)** [76,152,153]. Immunoblot analysis of a panel of HBEC cell lines indicated that ERK has increased phosphorylation in Snail-overexpressing lines **(Fig 3.4)** and may be intermediate in the Snail-to-SPARC pathway. In addition, ERK1/2 was phosphorylated following TGF-β1 treatment, indicating that TGF-β is signaling through the ERK1/2 pathway **(Fig 3.3)**.

Chemical inhibition of ERK1/2 phosphorylation results in loss of Snail-driven SPARC expression and upregulation of miR-29b

Inhibition of ERK by the MEK1/2 phosphorylation inhibitor U0126 led to decreased mRNA and protein expression of SPARC in Snail-overexpressing HBEC cell lines (**Fig 3.7).**

MicroRNA-29b is downregulated in response to Snail overexpression

The microRNA array data analysis **(Fig 3.1)** indicated that microRNA-29b might be an intermediate regulator in the Snail to SPARC pathway. Analysis of the 3'UTR of SPARC mRNA by TargetScan software revealed a putative complementary sequence for miR-29b. We compared expression levels of miR-29b in three NSCLC cell lines and 4 HBEC cell lines with Snail overexpression (-S) to vector control (-V) lines by qRT-PCR using a TaqMan® microRNA primer assay. In both sets of cell lines, miR-29b expression levels were lower in Snail-

overexpressing lines versus vector control **(Fig 3.8)**, indicating that miR-29b is downregulated by Snail in NSCLC cell lines and may be upstream of SPARC.

Overexpression of a miR-29b mimic in Snail-overexpressing cell lines results in loss of SPARC expression

Overexpression of a miR-29b precursor led to downregulation of SPARC protein in a panel of Snail-overexpressing HBEC cell lines (**Fig 3.9).** In addition, miR-29b expression was significantly upregulated following MEK/ERK inhibition **(Fig 3.10)** and TGF=β inhibition **(Fig. 3.11)**, indicating a direct link between Snail, TGF-β, MEK/ERK, and miR-29b upstream of SPARC. Based on the results in this chapter, we propose a regulatory pathway wherein Snail upregulates TGF-β in an autocrine fashion, leading to activation of the MEK/ERK pathway, downregulation of miR-29b, and finally upregulation of SPARC (**Fig 3.14)**.

Alternate TGF-β signaling pathways are not activated downstream of Snail, with the exception of p38

As TGF-β1 signaling through its receptor is known to activate several signaling pathways in addition to MEK/ERK, we investigated if Snail-mediated TGF-β1 signaling activated these pathways in our system by immunoblotting for phosphoproteins. The p38 pathway is often coactivated with MEK/ERK and therefore, unsurprisingly is activated here **(Fig 3.12)**. Interestingly, the JNK and AKT signaling pathways were not activated downstream of TGF-β **(Fig 3.12)**, indicating that preferential signaling through p38 and MEK/ERK is occurring, possibly through upregulation of JNK and AKT inhibitor proteins. As Smad proteins are phosphorylated in multiple TGF-β pathways, investigating their contribution to this pathway would not be additive and was not pursued. To investigate the possibility of p38 also being intermediate between TGF-β and SPARC, downstream of Snail, p38 signaling was interrupted

by the chemical inhibitor SB203580 for 24 hours. Whole cell protein lysates were collected and probed for Snail and SPARC expression. Inhibition of p38 signaling did not effect SPARC expression, indicating that p38, though activated downstream of Snail, is not intermediate in the Snail-to-SPARC pathway **(Fig 3.13)**.

Figures

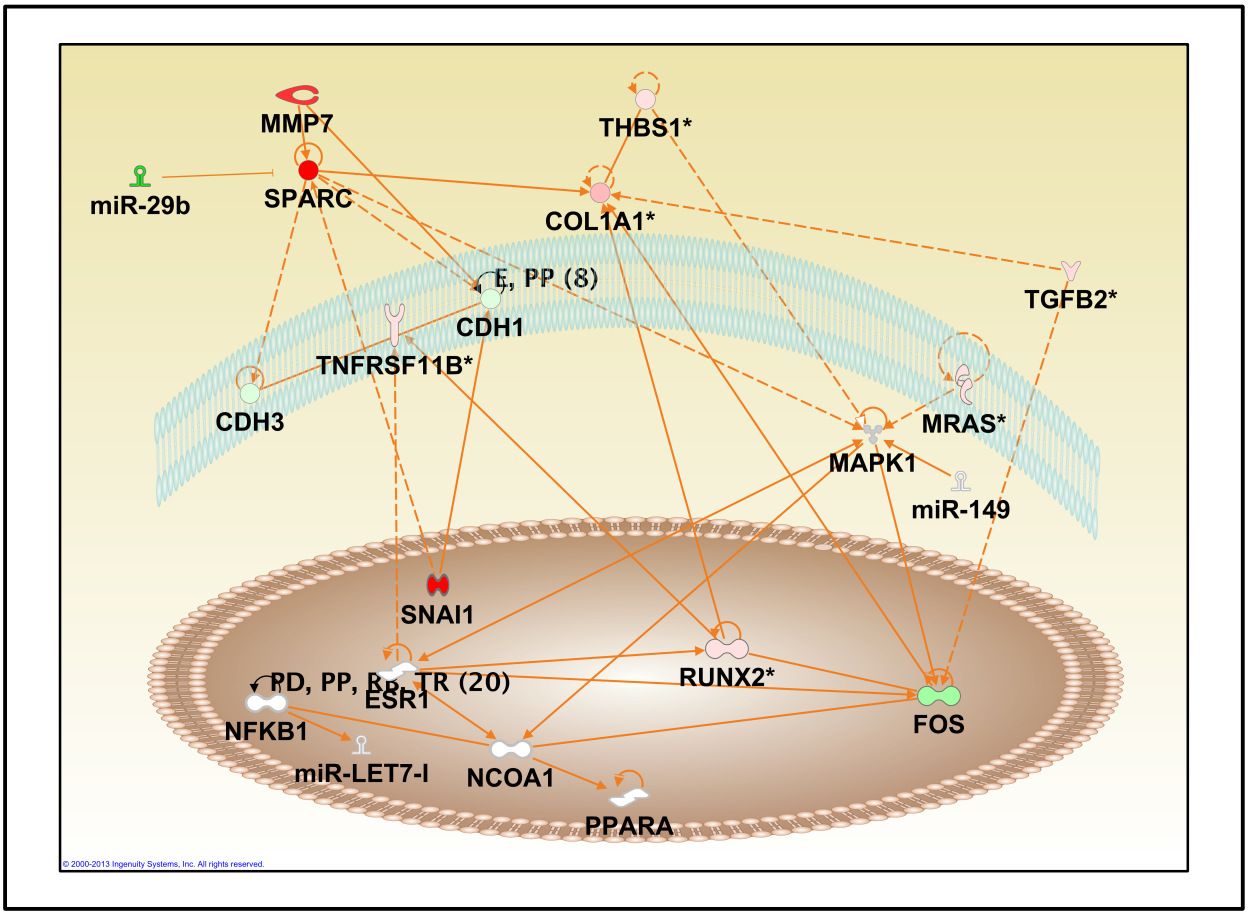

Figure 3.1. IPA Analysis revealed potential mechanism for Snail to SPARC pathway. a) Total RNA was isolated from panel of Snail-overexpressing NSCLC cell lines and vector controls and subjected to microRNA array. Array results were combined with mRNA array results published previously and analyzed by Ingenuity Pathway Analysis Software. A putative pathway for regulation of SPARC by Snail was produced.

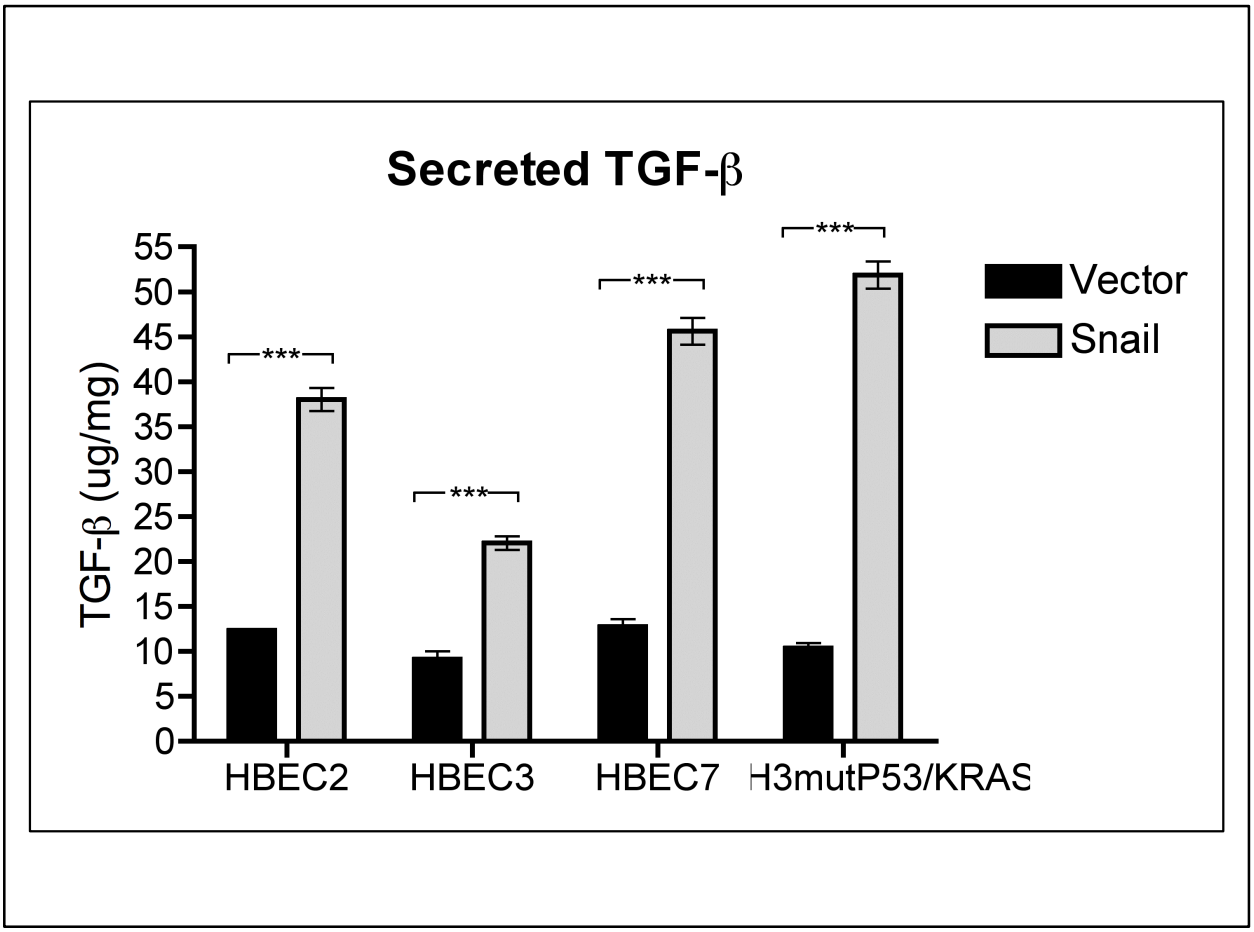

Figure 3.2. TGF-β1 is upregulated by Snail. The secreted protein levels of TGF-β1 were measured by ELISA from supernatants of Snail-overexpressing HBEC cell lines and compared to appropriate vector controls. (** = $p<0.01$, *** = $p<0.0001$)

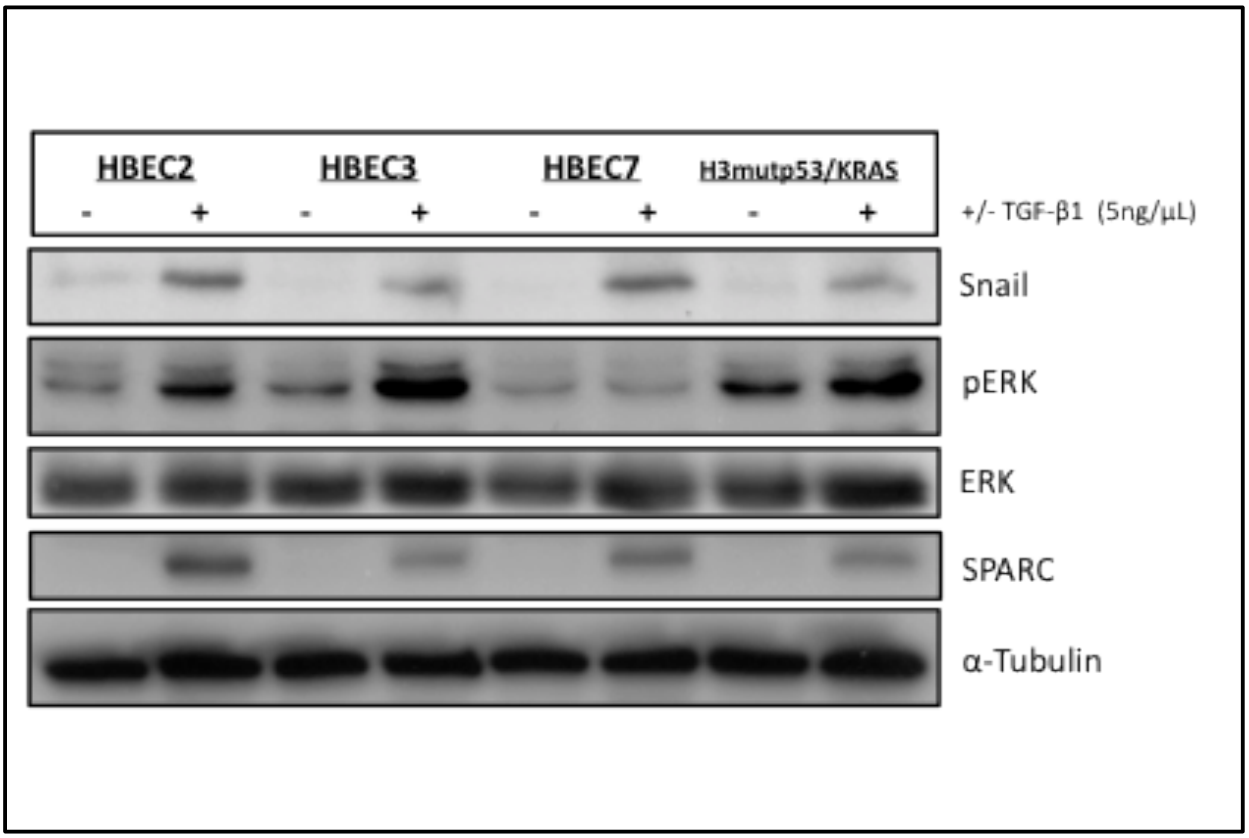

Figure 3.3. Treatment with TGF-β1 upregulates Snail and SPARC and activates ERK signaling. Parental HBEC cell lines were treated with 5ng/μL recombinant TGF-β1 for 24 hours in serum-free media. Lysates were collected and protein expression of Snail, pERK1/2, ERK1/2, and SPARC were measured by western blot.

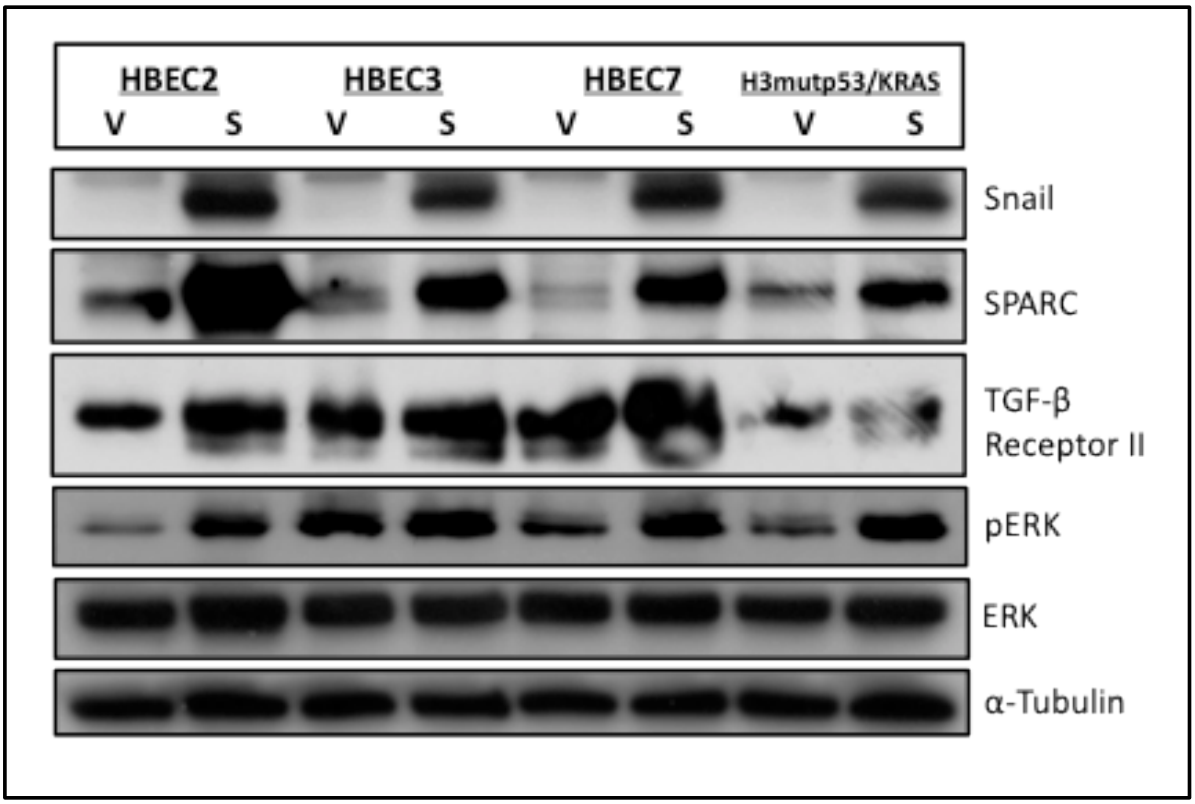

Figure 3.4. TGFβR2 is upregulated by Snail. Levels of Snail, SPARC, TGFβR2, pERK1/2, and tERK1/2 protein were evaluated by Western blotting in HBEC2-V/S, HBEC3-V/S, HBEC7-V/S, and H3mutP53/KRAS-V/S cell lines. Protein levels were normalized by α-Tubulin.

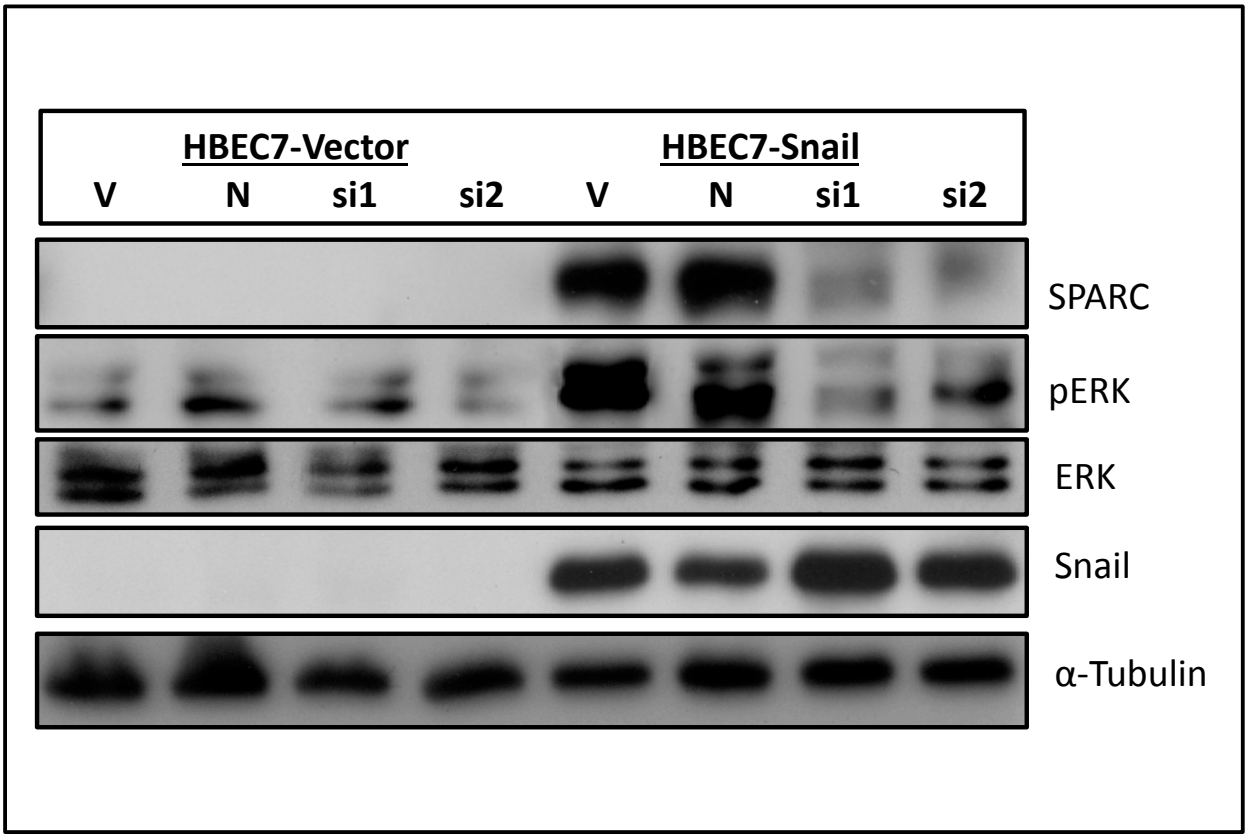

Figure 3.5. Inhibition of TGF-β1 expression downregulates ERK activity and SPARC expression. Snail-overexpressing HBEC cell lines and vector controls were treated with single siRNA sequences targeting TGF-β1 (1,2,3,4), a negative control siRNA (N), or untreated (C) for 24 hours in serum-free media. Lysates were collected and protein expression was measured as in (c).

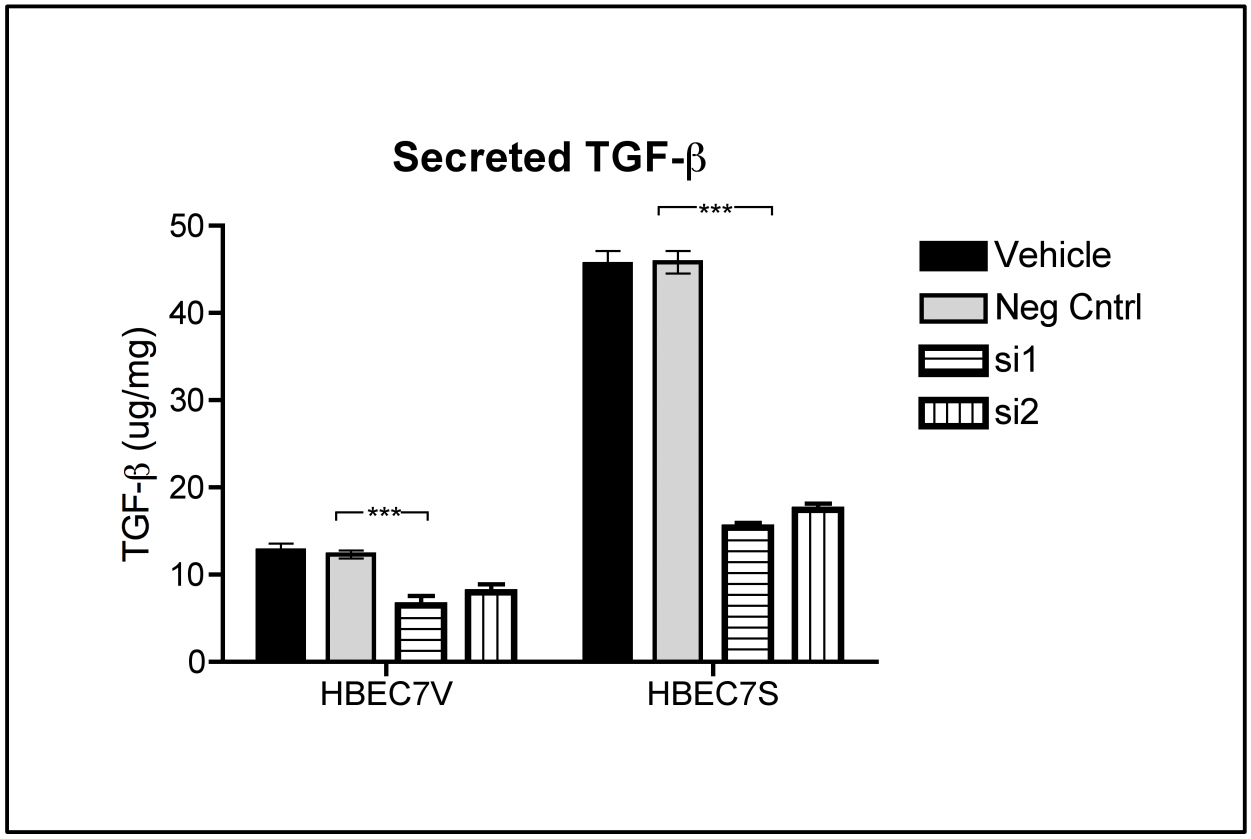

Figure 3.6. Transfection of TGF-β1 siRNA inhibits secretion of TGF-β1 by HBEC cell lines. Snail-overexpressing HBEC cell lines and vector controls were treated with single siRNA sequences targeting TGF-β1 (1,2,), a negative control siRNA (N), or untreated (C) for 24 hours in serum-free media. Lysates and supernatants were collected and the secreted protein levels of TGF-β1 were measured by ELISA and compared to appropriate vector controls. (*** = $p<0.0001$)

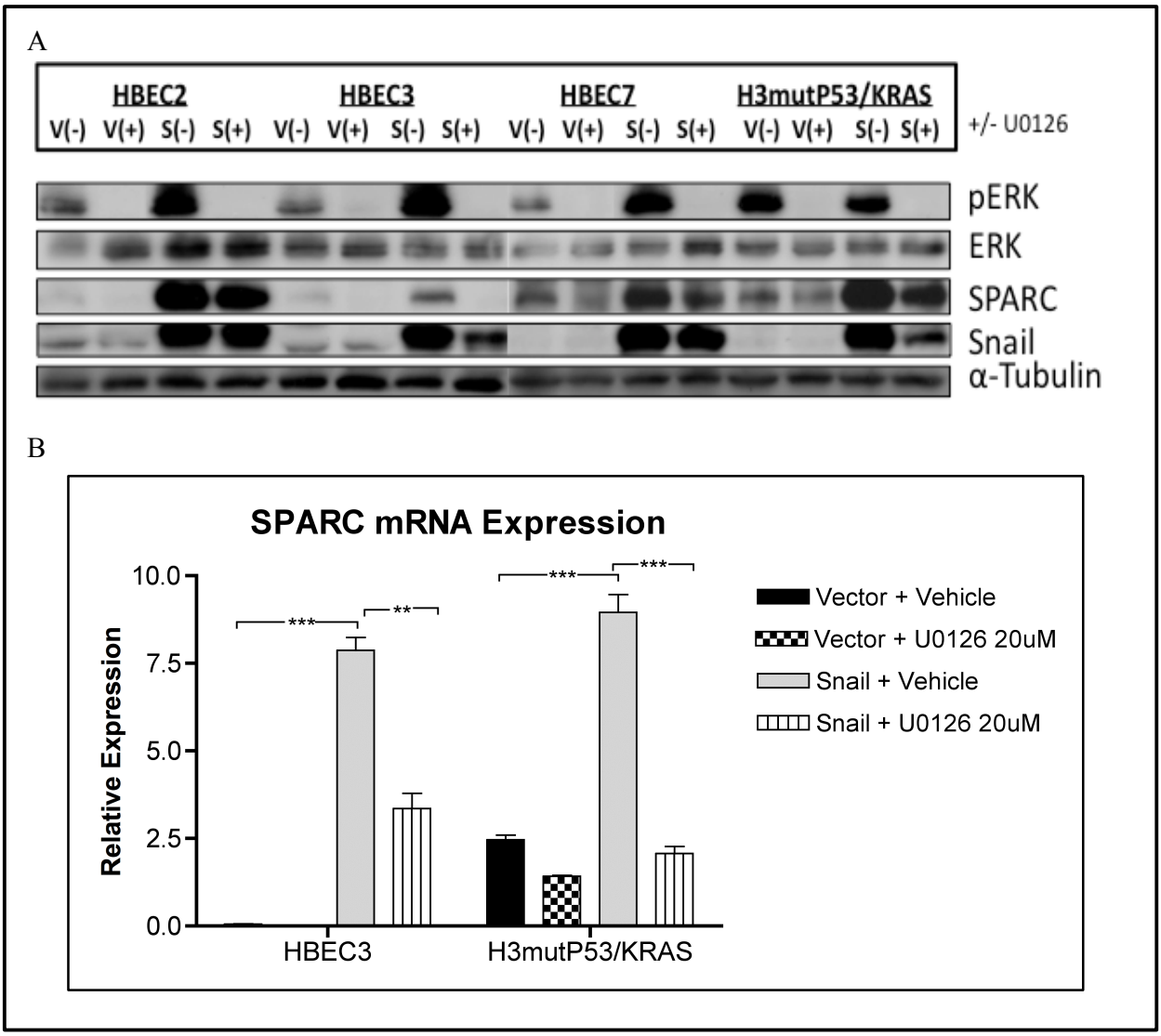

Figure 3.7. Inhibition of MEK/ERK signaling downregulated SPARC expression. (A) The cell lines HBEC2, HBEC3, HBEC7, and H3mutP53/KRAS with and without Snail overexpression (-V/-S) were treated with the MEK1/2 phosphorylation inhibitor U0126 and evaluated for SPARC protein expression. Membranes were incubated with antibodies against Snail, phosphorylated ERK1/2 (pERK), total ERK1/2, SPARC and α-tubulin. Protein levels were normalized against α-tubulin. **(B)** SPARC mRNA expression was evaluated following U0126 treatment as in (A). mRNA expression was normalized against GUSB. (** = $p<0.01$, *** = $p<0.0001$)

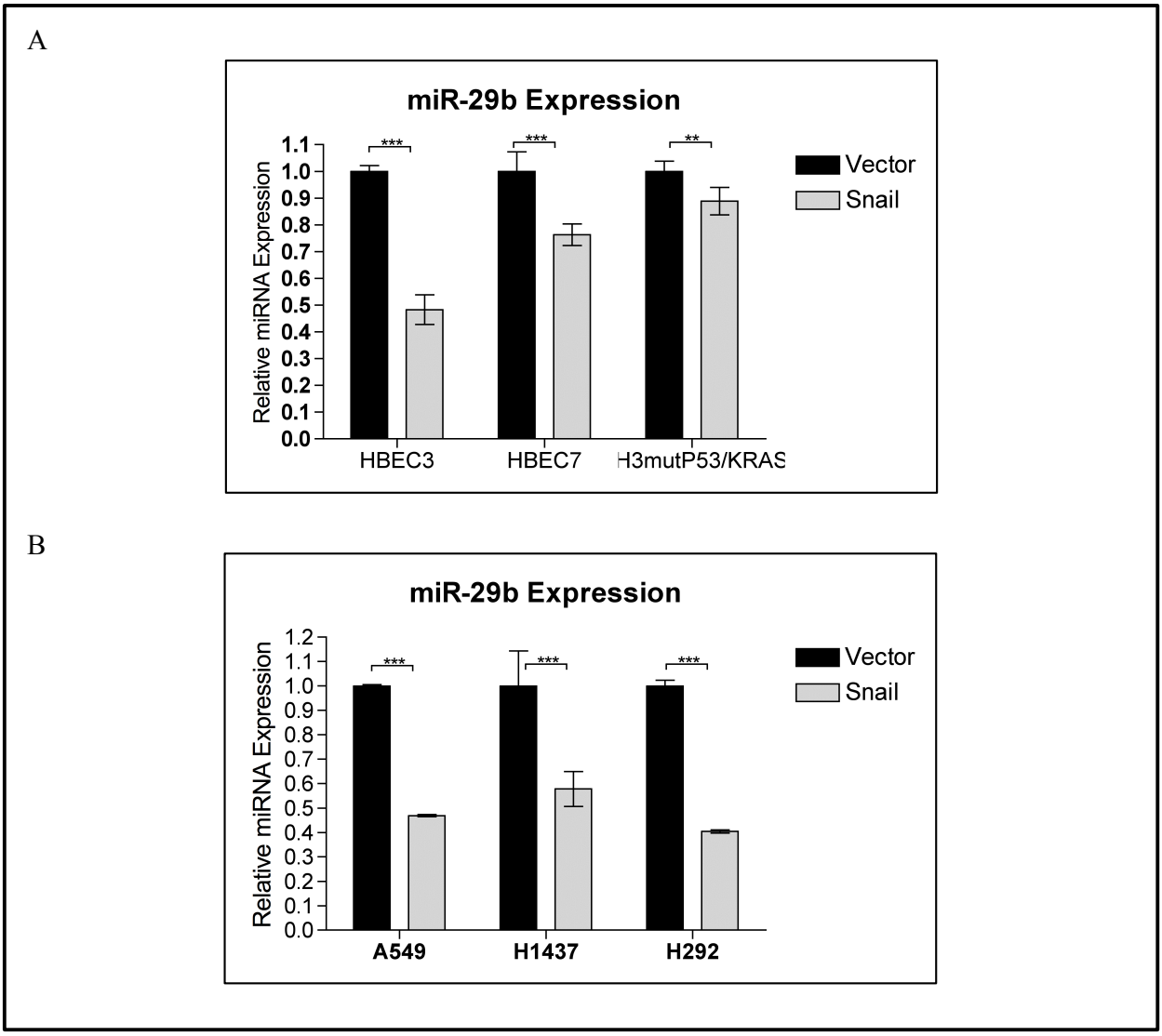

Figure 3.8. miR-29b is downregulated in cell lines over-expressing Snail. (A) Total RNA was isolated from HBEC3-V/S, HBEC4-V/S, and H3mutP53/KRAS-V/S cell lines. Expression levels of miR-29b were evaluated by qRT-PCR using TaqMan primers. miRNA levels were normalized to RNU6b. **(B)** Total RNA was isolated from A549V/S, H1437V/S, and H292V/S cell lines. Expression levels of miR-29b were evaluated by qRT-PCR using TaqMan primers. miRNA levels were normalized to RNU6b.

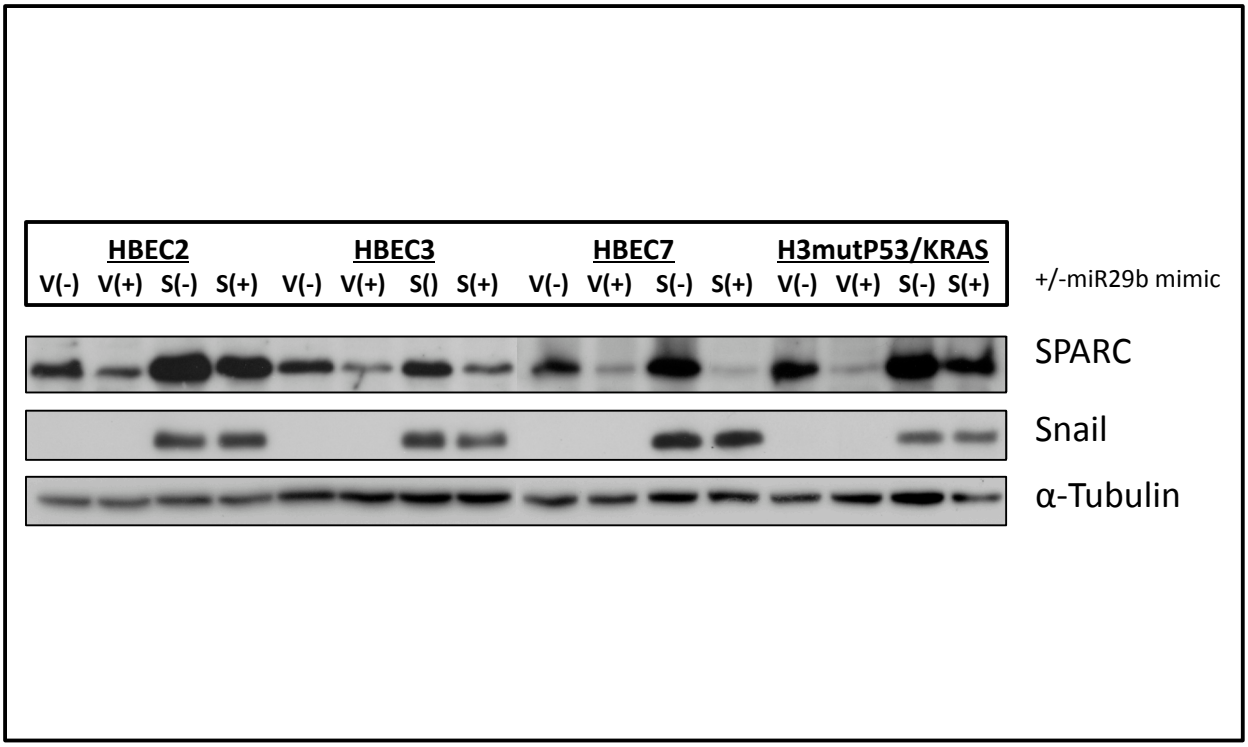

Figure 3.9. Overexpression of miR-29b downregulates SPARC expression. The HBEC cell lines HBEC2, HBEC3, HBEC7, and HBEC3mutP53/KRAS with and without stable Snail overexpression (-V/-S) were transiently transfected with a miR-29b precursor sequence. Lysates were collected 24 hours after transfection and evaluated for expression of miR-29b and SPARC by western blot. Protein levels were normalized to α-Tubulin.

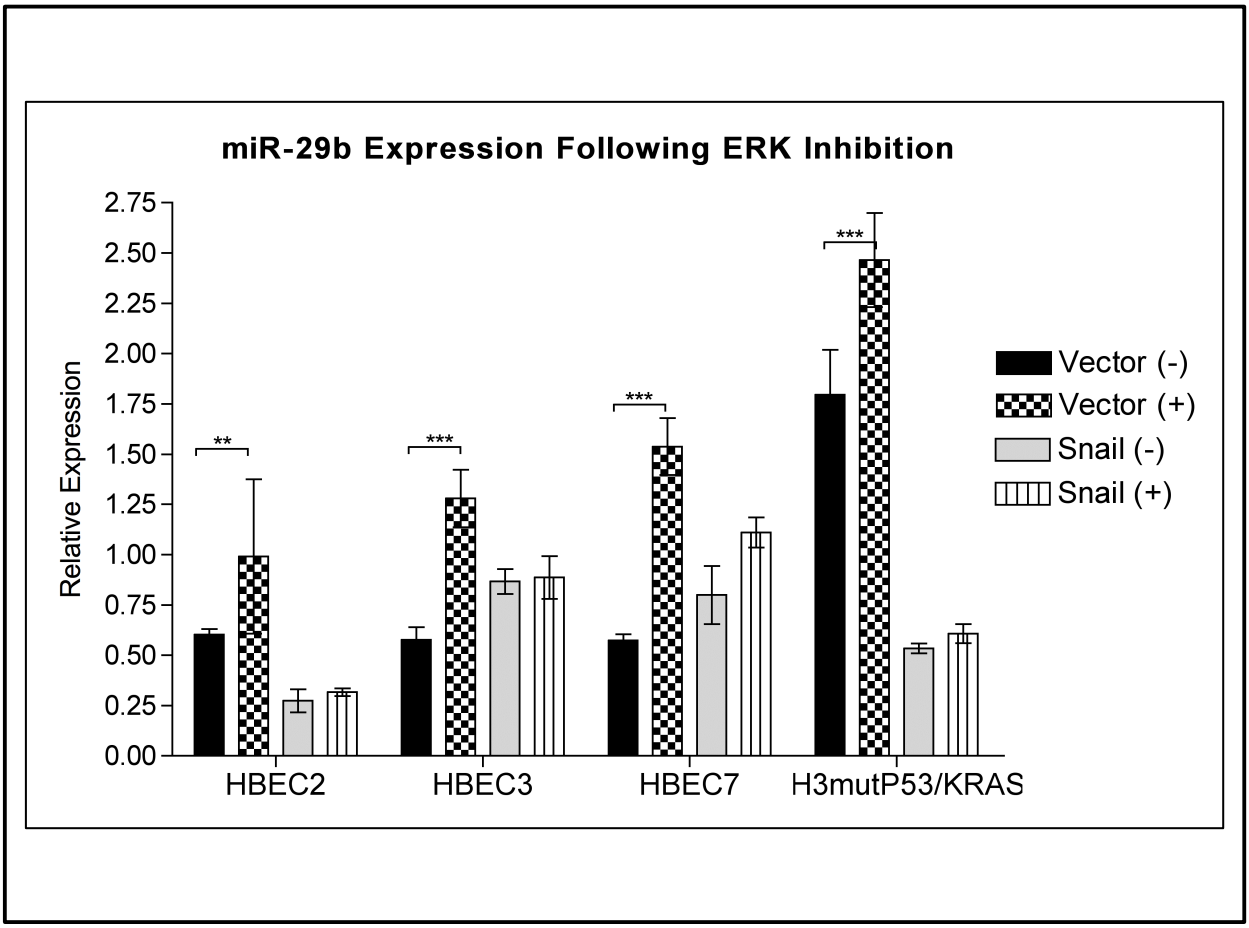

Figure 3.10. Inhibition of MEK/ERK signaling increases miR-29b expression. The HBEC cell lines HBEC2, HBEC3, HBEC7, and HBEC3mutP53/KRAS with and without stable Snail overexpression (-V/-S) were treated with the MEK1/2 phosphorylation inhibitor U0126 for 24 hours. Expression of miR-29b was evaluated following U0126 treatment. miRNA expression was normalized against RNU6b. (** = $p<0.01$, *** = $p<0.0001$)

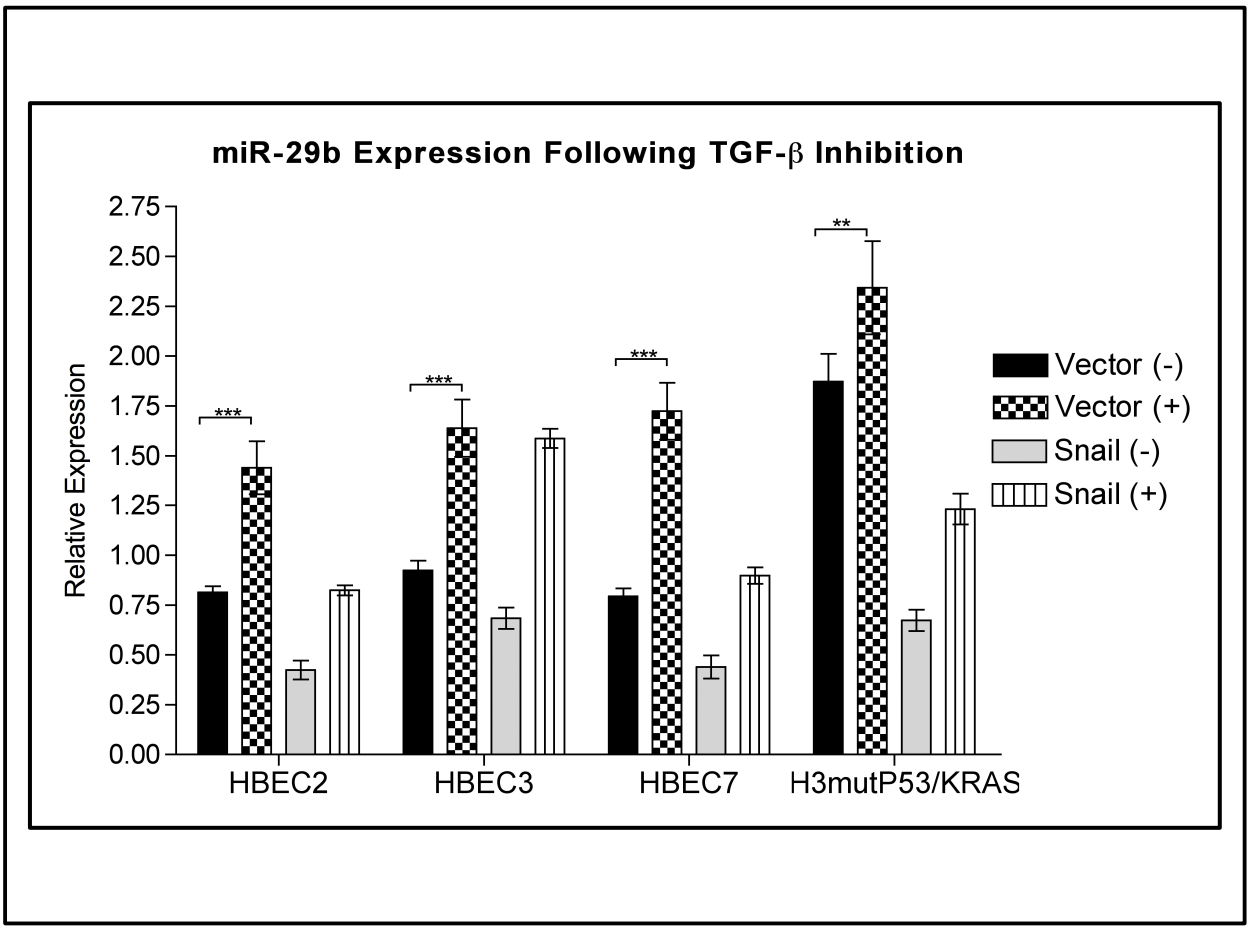

Figure 3.11. Inhibition of TGF-β signaling increases miR-29b expression. The HBEC cell lines HBEC2, HBEC3, HBEC7, and HBEC3mutP53/KRAS with and without stable Snail overexpression (-V/-S) were transiently transfected with TGF-β1 siRNA (+) or a negative control (-) for 24 hours. Expression of miR-29b was evaluated following TGF-β inhibition. miRNA expression was normalized against RNU6b. (** = $p<0.01$, *** = $p<0.0001$)

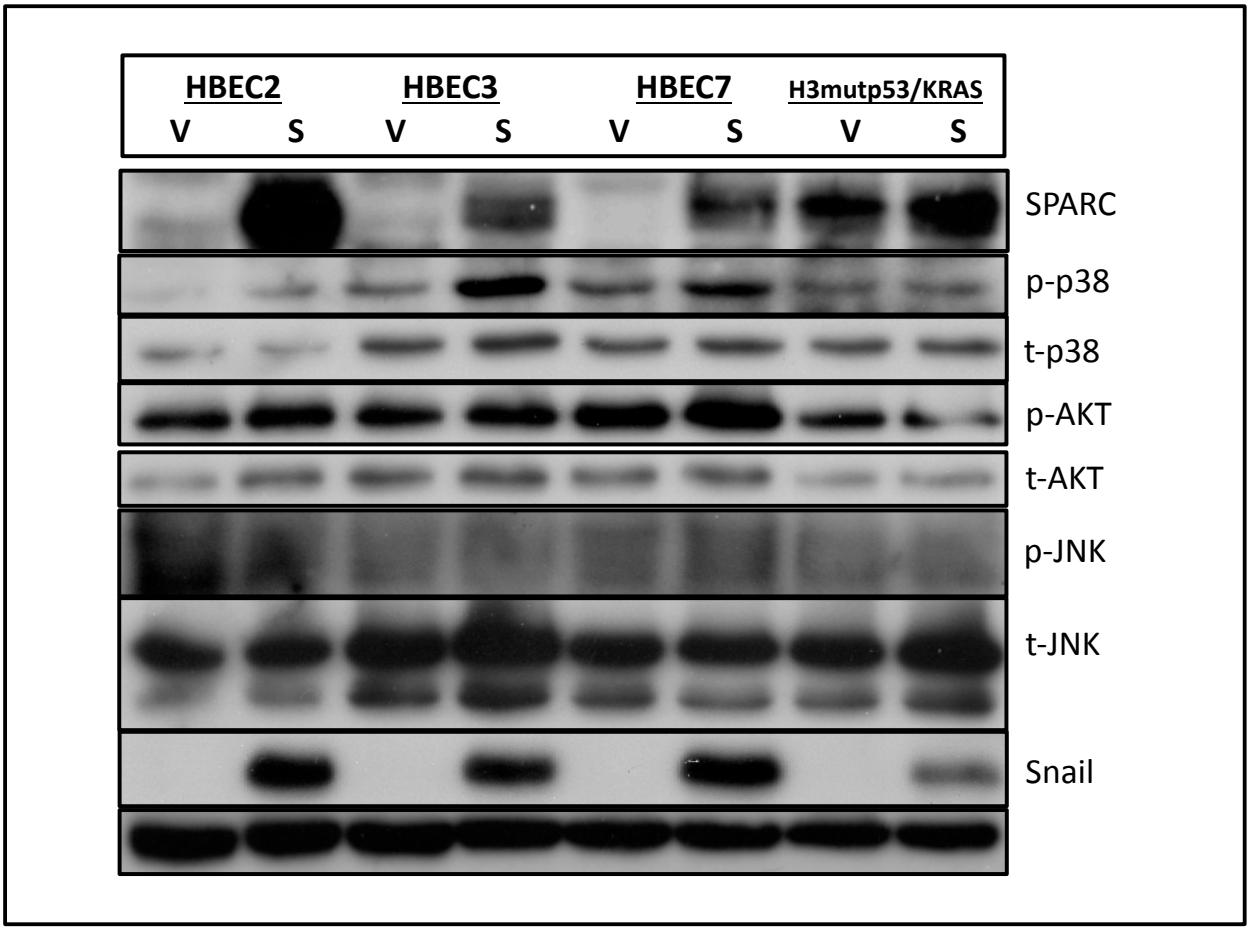

Figure 3.12. TGF-β signals through p38 but not AKT or JNK downstream of Snail. Levels of Snail, SPARC, phospho-p38, -AKT, -JNK, and total-p38, AKT, and JNK protein were evaluated by Western blotting in HBEC2-V/S, HBEC3-V/S, HBEC7-V/S, and H3mutP53/KRAS-V/S cell lines. Protein levels were normalized by α-Tubulin.

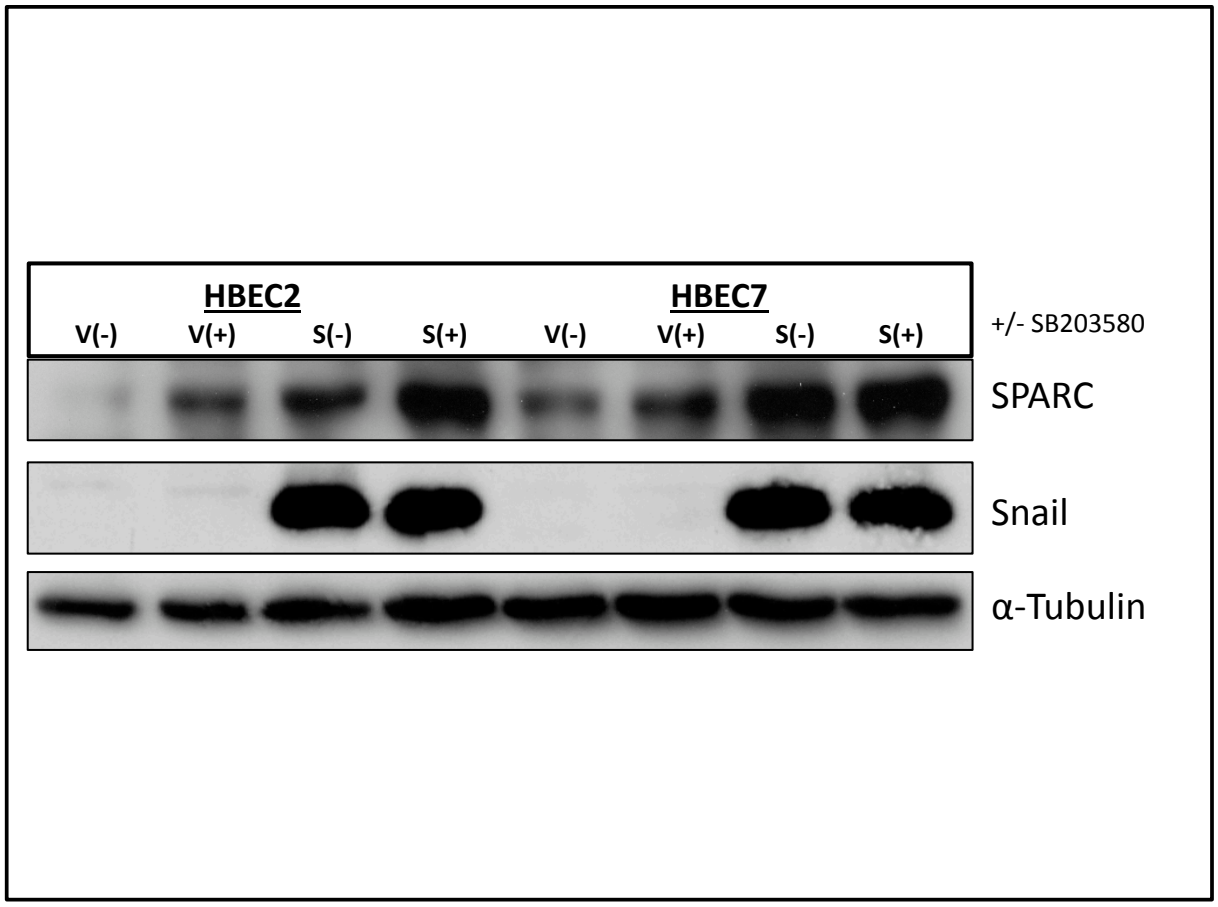

Figure 3.13. p38 signaling does not upregulate SPARC. The cell lines HBEC2 and HBEC7 with and without Snail overexpression (-V/-S) were treated with the p38 signaling inhibitor SB203580 for 24 hours and evaluated for SPARC protein expression. Membranes were incubated with antibodies against Snail, SPARC, and α-tubulin. Protein levels were normalized against α-tubulin.

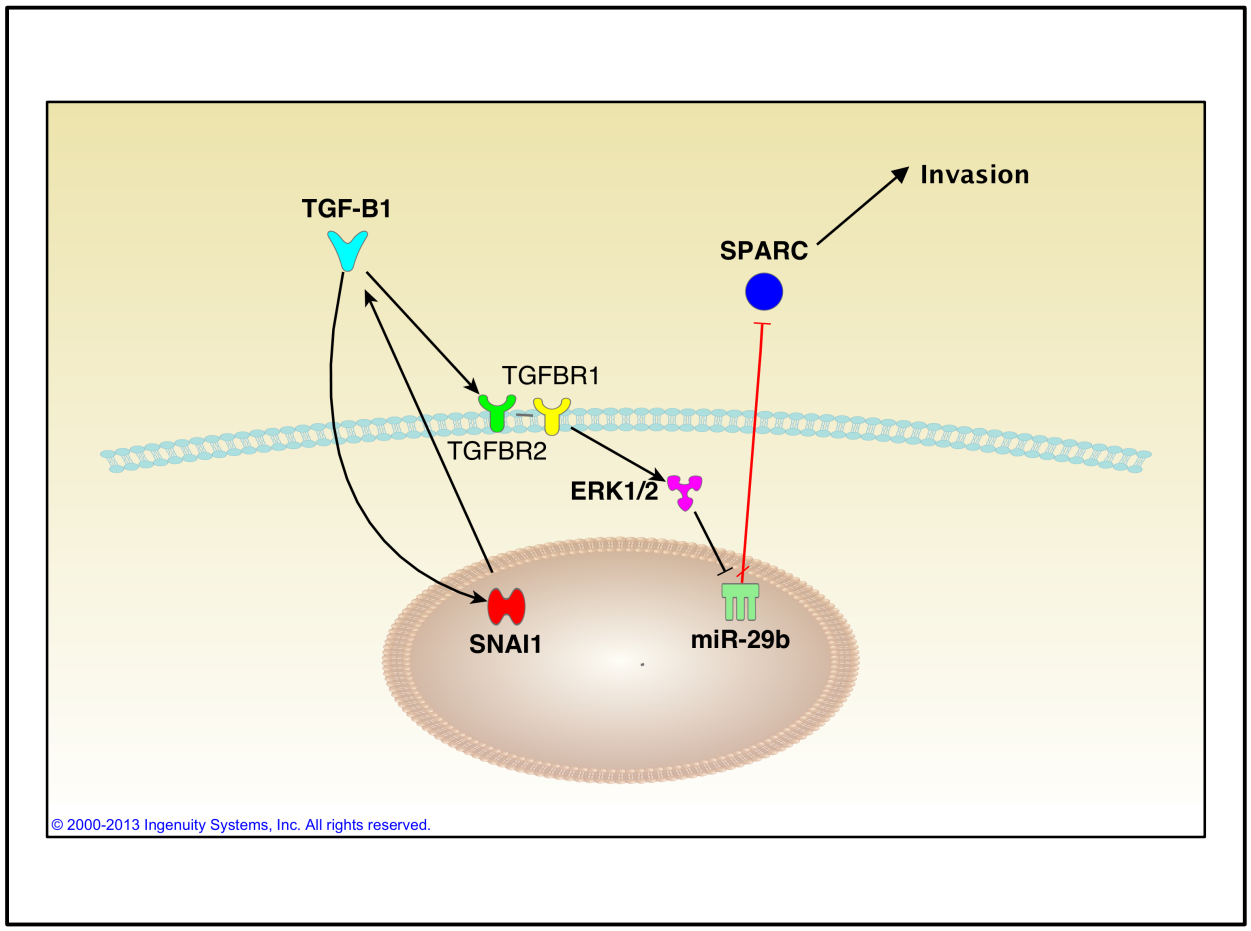

Figure 3.14. Proposed mechanism for Snail-mediated, SPARC-dependent invasion

Discussion

The preceding experiments identify a number of molecules not previously described as being intermediate in the Snail-to-SPARC pathway. Specifically, the mechanism for SPARC upregulation by Snail includes activation of the TGF-β and MEK/ERK signaling pathways and the downregulation of miR-29b. While Section II focused on the functional contribution of Snail-mediated upregulation of SPARC to NSCLC invasion, this section determines the partial molecular mechanism for this phenotype. As chronic inflammation is known to contribute to tumor initiation as well as EMT, identification of the molecular determinants in this pathway is crucial to understanding the pathogenesis of NSCLC. While the pathway described here is independent of mutation status, mutations in EGFR and KRAS would likely only compound the effects of Snail given the concurrence of signaling pathways. For example, EGF potentiates TGF-β1-mediated COX-2 induction in HBECs in an EGFR- and ERK-dependent manner [7], suggesting that constitutive EGF signaling would result in increased Snail and SPARC expression due to the relationship between TGF-β1, Snail, ERK, and SPARC described here. In addition, the induction of COX-2 would lead to further upregulation of Snail, as the COX-2 metabolite, PGE2 has been shown to induce Snail expression in NSCLC [44].

In addition, activating mutations in the KRAS gene lead to constitutive Ras signaling [8], leading to activation of the MEK/ERK signaling pathway, which, as we have shown here, leads to upregulation of SPARC through downregulation of miR-29b. Another transcriptional target of KRAS is the proinflammatory mediator IL-8, which is also upregulated by activating EGFR [8]. Secretion of IL-8 by the epithelial cells in the lungs can induce an innate immune response, contributing to further inflammation and ultimately upregulation of Snail, leading to tumorigenesis. As KRAS mutations are associated with poor prognosis and TKI resistance, it has

been identified as a likely therapeutic target. However, efforts to develop a direct KRAS inhibitor, including farnesyl transferase inhibitors, have been unsuccessful [8,10]. Identification of the downstream molecules involved in Ras-dependent pathogenesis, some of which are described here, could yield additional therapeutic targets.

One surprising observation was the differential activation of TGF-β signaling pathways, namely p38 and MEK/ERK over AKT and JNK. Due to the commonality of activators between the MAP kinases, the different MAPK groups are often coactivated. However, specific activation of a MAP kinase group (e.g. p38 over JNK) can be achieved by different MAP3Ks [92,93]. Likewise, selective deactivation of a MAP kinase group can be achieved by dephosphorylation by a MAP kinase phosphatase (MKP), of which several have been identified [94,95]. Further investigation into the mechanism of this selective activation is warranted. In addition, the contribution of p38 to NSCLC progression should be studied. Despite its activation downstream of Snail, p38 was determined to not contribute to SPARC upregulation. However, it may be contributing to progression, given its known roles in inflammation, cell death, growth, and differentiation [88,89].

The molecules and pathways described here are likely not the only intermediaries in the Snail-to-SPARC pathway and further studies could describe additional pathways as well as epigenetic changes involved. While we propose a linear pathway for Snail to SPARC expression here, it is likely that the interactions between all the involved molecules are more complex. For example, SPARC is known to increase expression of Snail in melanoma [154]. TGF-β and SPARC are known to cooperate in an autocrine-feedback loop [72]. As we demonstrated here, TGF-β1 and Snail also cooperate in an autocrine or paracrine feedback loop.

Considering the role the tumor microenvironment is known to play in progression, further investigation in an *in vivo* model would give additional clues to the role of this pathway in parallel progression. For example, tumor-derived TGF-β transdifferentiates fibroblasts at the invasive front of tumors into myofibroblasts, which share characteristics of mesenchymal cells and smooth muscle cells [60]. The presence of myofibroblasts has been correlated with invasion and progression in breast, colon, and lung cancers [61]. In reaction to signals received from the tumor cells, the fibroblasts in the tumor stroma also secrete ECM remodeling factors to enhance degradation, including collagen type I & IV, MMPs, and SPARC [60-62]. Given the importance of SPARC in the inflammatory response to injury and collagen deposition, it is unsurprising that it has been found to play a role in tissue fibrosis. For example, SPARC has been found to be upregulated in fibrotic tissue in cirrhotic livers, specifically secreted by hepatic stellate cells (HSC) in these tissues. In an *in vivo* model of cirrhotic liver fibrosis, SPARC knockdown in HSCs decreased fibrosis through increased fibronectin adhesion and decrease chemokine-mediated migration [72].

CONCLUDING REMARKS

This is the first report demonstrating that Snail upregulates SPARC in models of both early and established NSCLC. The necessity of SPARC for Snail-mediated invasion in both models suggests a role for both Snail and SPARC in the pathogenesis of NSCLC. Furthermore, we have demonstrated a number of critical molecules intermediate in this pathway. The deregulation of both the TGF-β and MEK/ERK pathways in cancers are well known, though this is the first description of their deregulation in this context. By demonstrating deregulated expression of the protein SPARC, the downregulation of a critical microRNA, as well as aberrant activation of two highly influential signaling pathways, we have identified novel opportunities for clinical targeting of the most aggressive subset of malignant cells.

Understanding the molecular profile of the invasive phenotype may enhance the diagnostic and therapeutic processes. While no therapies targeting Snail signaling or Snail-expressing cells have yet been developed, therapies targeting the other molecules are in various stages of clinical development. Albumin-bonded paclitaxel (nab-paclitaxel), an injectable form of the mitotic inhibitor drug paclitaxel, is known to accumulate preferentially in tissues expressing SPARC, due to SPARC's albumin-binding properties [78]. In a retrospective study of monotherapy nab-paclitaxel in head and neck cancer patients, response to treatment correlated positively with SPARC expression due to the SPARC-albumin interaction [80]. Additional studies in pancreatic, adrenocortical, and advanced breast cancers confirm the enhanced efficacy of nab-paclitaxel therapy in SPARC-expressing tumors [81-83]. A number of MEK inhibitors have been developed for the clinic, including the MEK 1 and 2-specific small molecule selumetinib. Selumetinib has been shown to be efficacious in KRAS-mutant NSCLC, though our results suggest a MEK inhibitor may have broader application as the MEK/ERK pathway is

activated downstream of Snail, independent of KRAS mutation [107]. Clinical studies targeting various TGF-β pathway molecules are underway, including an autologous tumor cell vaccine carrying a TGF-β2 antisense transgene [115]. Restoration of tumor-suppressor miRNA expression in patients has been successfully evaluated in the clinic [126,137,138]. Intratumoral injection of miR-29 miRNA mimics in xenograft models of human liver cancer, rhabdomyosarcoma, and AML resulted in tumor regression, though this method has not been evaluated in human patients. Use of adenovirus associated vectors to efficiently transduce tumors intravenously has been proposed as an improved delivery mechanism that will allow miRNA mimic use in the clinic [139].

In addition, our results and the literature suggest that Snail and SPARC may play a role in the tumor initating cell (TIC) population. As potential drivers of both oncogenesis and metastatic progression, the TIC population is an obvious target for therapeutic intervention. A high-throughput screen of 16,000 small molecules identified salinomycin as a selective inhibitor of breast cancer stem cells [37]. The tumor cells remaining after salinomycin treatment had a distinctly differentiated and epithelial phenotype when compared to standard chemotherapy, suggesting preferential targeting of cells that had undergone, or were capable of undergoing, EMT. Importantly, salinomycin-treated cells no longer expressed stem cell markers and were unable to form tumorspheres. Salinomycin has subsequently been shown to be efficacious in cancer stem cell populations of many tumor types and is in early clinical evaluation [38-40].

Identifying the molecular determinants of tumor initiation and progression is an important step in identifying novel targets for not only clinical intervention of established disease, but also prevention and early detection of lung cancer. The clinical response to surgery suggests that parallel metastases are a frequent and major clinical problem: as many as 40% of

patients will have recurrence of lung cancer at metastatic sites following lung cancer resection [1]. This is generally agreed to be due to micrometastatic disease that is below the level of detection by imaging studies. We have here identified a number of molecules and signaling pathways that have altered genetic expression or activation in a model of premalignant disease. These molecules could be used as biomarkers for detection of premalignant disease and also for therapeutic prevention of progression to malignancy and metastatic disease.

Finally, while we propose a linear pathway for Snail to SPARC expression here, it is likely that the interactions between all the involved molecules are more complex. For example, SPARC is known to increase expression of Snail and therefore repress E-cadherin in melanoma progression [154]. TGF-β and SPARC are known to cooperate in an autocrine-feedback loop, with SPARC knockdown reducing TGF-β1 gene expression and secretion and TGF-β1 treatment increasing SPARC gene expression [72]. As we demonstrated here, TGF-β1 and Snail also cooperate in an autocrine or paracrine feedback loop **(Figures 3.2-3.4)**. These signaling pathways are known to have numerous effects on processes independent of invasion and metastatic progression, including cell cycle regulation, proliferation, and survival. Additionally, the microRNA described here, miR-29b likely has many targets in addition to SPARC, with as many as 7,000 predicted by computational algorithms. Understanding the contribution of the pathway described here to these phenotypes would improve development of the therapies described above as well as detection techniques.

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