1-1-2011

Hedgehog signaling: a potential therapeutic target for non-small cell lung cancer

Ma'in Yehya Maitah
Wayne State University,

HEDGEHOG SIGNALING: A POTENTIAL THERAPEUTIC TARGET FOR NON-SMALL CELL LUNG CANCER

by

MA'IN MAITAH

DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

2011

MAJOR: PATHOLOGY

Approved by:

_____

Advisor Date

_____

_____

_____

DEDICATION

I dedicate this research work to my beloved wife and my precious kids who share all the frustrations and happiness with me. I also dedicate this work to my parents, brothers and my sisters who have given me their endless love and encouragement.

ACKNOWLEDGMENTS

I am extremely grateful to my mentor and advisor, Dr. Fazlul H. Sarkar, who has guided me through this most challenging and fascinating research field. Without his guidance, support, and encouragement, this work would have never been possible. No words can completely express the appreciation and respect I have for him. He has taught me innumerable lessons and insights on academic research, and offered tremendous advice that helped towards the completion of this dissertation. I would like to express my sincere gratitude and appreciation to my committee members: Dr. Adhip P.N. Majumdar, Dr. Shijie Sheng and Dr. Shirish Gadgeel for their valuable advice, encouragement, and help. I sincerely thank Dr. Todd Leff, Graduate Officer of the Pathology Department and Dr. Wael A. Sakr, Chairman of the Pathology Department for their interest and support of the Pathology students. Furthermore, I would like to thank the former and present members of the Dr. Sarkar's laboratory, especially Dr. Yiwei Li, Dr. Sanjeev Banerjee, Dr. Wahidur Rahman, Dr. Ahmad Aamir, Mrs. Shadan Ali, Dr. Dejuan Kong, and Dr. Zhiwei Wang for sharing their knowledge and experience. Moreover, I would like to thank Dr. Ramzi M. Mohammad, Dr. Asfar Azmi, and Amro Aboukameel for their support and encouragement. Finally, I would like to thank my wife and kids, for their understanding and love. I also want to thank my parents, brothers, and my sisters, for their love, support, and encouragement.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Lung cancer is the leading cancer site in males, comprising 17% of total new cancer cases, and is the most common cause of cancer related deaths at 23%. In the United States, The American Cancer Society estimated that 222,520 Americans were diagnosed with lung cancer and 157,300 died of lung cancer in 2010 (Jemal et al. 2009, 225-249;Jemal et al. 2011, 69-90). Dreadfully, in United States death rates from lung cancer in women are almost twice that of breast cancer, and in men, the rate is three times greater compared to prostate cancer (Jemal et al. 2008, 71-96;Jemal et al. 2009, 225-249;Jemal et al. 2011, 69-90).

Increasing incidence of lung cancer is associated with several risk factors including: lifestyle choices, environmental factors, and genetic make-up, all of which contribute to the risk of developing lung cancer. Among many risk factors, tobacco smoke, Radon, asbestos, and arsenic exposure have been linked with increasing rates of lung cancer.

Lung cancers are divided into two major categories as follows (Table 1): non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC is the most common type, accounting for (85%) of all lung cancer cases. There are three histological subtypes of NSCLC:

a. Squamous cell carcinoma, usually found among patients with a history of smoking; it forms in the central lung near the large airways (bronchi).

b. Adenocarcinoma occurs in smokers and non-smokers; it forms in the outer region of the lung.
c. Large-cell undifferentiated carcinoma; it is a very aggressive cancer with poor prognosis and forms in any region of the lung.

SCLCs make up only about 10 to 15 percent of all lung cancer cases but tend to be highly aggressive. Lung cancer is difficult to treat effectively with cancer type, size, and location determining the choice of treatment. Currently, there are many strategies available for the treatment of lung cancer, some of which are discussed below (Table 2) but the outcome is still disappointing.

Treatment Options for Lung Cancer

Surgery is the first line of treatment for localized non-small cell lung cancer (NSCLC) and lung carcinoid cancers. For cancers that have grown more extensively in the lungs, more aggressive surgery is required to remove the cancer. If SCLC is caught before it spreads surgery is the best option. However, surgical procedures will not excise all cancer cells from the lung, and thus residual cancer cells must be treated with radiation therapy, chemotherapy or a combination of the two, known as adjuvant therapy. Current chemotherapy drugs are listed in Table 2. Additional drugs called targeted therapy have been added recently to be specially used in combination with traditional chemotherapy. Targeted-drug-therapy is an approach that blocks the ability of cancer cells to grow by targeting a mutated protein or other driver oncogenes within heterogeneous populations of cancer cells in the tumor mass. For example,

Erlotinib is a tyrosine kinase inhibitor that blocks the activity of the epidermal growth factor receptor (EGFR). Angiogenesis inhibitory drugs prevent cancers from acquiring new blood vessels; examples that have been used include Bevacizumab, which is known to block vascular endothelial growth factors (VEGF) that activate endothelial cells to develop new blood vessels. However, these targeted therapies only work effectively in a small subset of cancer patients suggesting that newer therapies must be developed (Herbst and Sandler 2008, 1166-1176;Ramalingam and Belani 2008, 5-13).

Despite all recent advances in lung cancer therapy the 5-year survival rate across all stages of lung cancer is approximately 15%. This is due to lack of reliable and cost-effective screening techniques for early detection of lung cancer, and thus many patients are diagnosed at advanced stages of the disease. This results in about 90% of cancer patient's deaths occuring from metastasis of their primary cancer. Furthermore, resistance to anti-cancer therapies (chemotherapy, radiation, and targeted therapies) both at the onset of therapy and following initial therapy is a major obstacle in cancer therapies. In other words, patients treated with conventional anti-cancer drugs may respond initially (e.g. shrinkage in tumor mass), but over the course of the treatment tumor mass re-grows with the acquisition of a drug-resistant phenotype. It is believed that both *de novo* and acquired drug resistance is the cause of treatment failure, which leads to tumor recur rence and subsequent metastasis that kills lung cancer patients. The combination of this lack of cost-effective early

diagnosis, and the intrinsic (*de novo*) and extrinsic (acquired) therapeutic resistance to conventional therapy that exist in some cancer patients contribute to dismal the outcome in the survival of patients diagnosed with lung cancer.

Therefore, to increase survival rate among lung cancer patients we need to target metastasic processes and also develop novel therapies targeting the surviving cancer cells after conventional existing therapy or it is necessary to develop and employ targeted drugs that must be found or discovered based on molecular knowledge. Hence, a deeper understanding of the molecular mechanisms involved in drug resistance, tumor progression, and metastasis of lung cancer cells will be the key to newer and more effective strategies for the treatment of lung cancer with better treatment outcomes (http://www.aacr.org/home/public--media/patients--family/fact-heets/organ-site-fact-sheets/lung-cancer.aspx).

Emerging evidence suggests that the phenomenon called Epithelial to Mesenchymal Transition (EMT), which shares some molecular characteristics with cancer stem-like cells, contributes to treatment failure. It is important to note that the EMT process has been implicated in the two important biological processes that are accountable for cancer-related deaths: the progressive growth of cancer cells to a distant organ and the acquisition of resistance to conventional cancer therapeutics. Therefore, further mechanistic understanding of the role of EMT in lung cancer would be very important, and such knowledge will help us in developing novel strategies that can would interfere with EMT induced drug

resistance and tumor metastasis. Moreover, such knowledge of which oncogenic targets is important would be invaluable and contribute to finding drugs that could destroy those targets. This would vastly improve the overall survival outcomes of lung cancer patients, in particular NSCLC, which accounts for the majority of lung cancer cases.

Epithelial to Mesenchymal Transition Phenomenon and its role in cancer:

Epithelial to Mesenchymal Transition (EMT) and Mesenchymal to Epithelial Transition (MET) are changes in cellular phenotype where cells transition between epithelial and mesenchymal states. Early on EMT was recognized to be important in embryonic development, including parietal endoderm formation and gastrulation (Thiery 2003, 740-746). EMT was also recognized as an important process in the progression of cancer, and has been been implicated in other human diseases.

EMT refers to a complex cellular and molecular program where epithelial cells lose their differentiated characteristics including cell–cell adhesion, planar and apical–basal polarity, as well as their lack of motile function. They then acquire mesenchymal features including increased motility, invasiveness, and resistance to apoptosis (Fig-1) (Turley et al. 2008, 280-290).

The process of EMT was first described as a cell culture phenomenon, but its role in physiological processes was not clear (Baum, Settleman, and Quinlan 2008, 294-308;Hugo et al. 2007, 374-383;Mani et al. 2008, 704-

715;Thiery and Sleeman 2006, 131-142). However, the increasing awareness of its roles in human cancers and the wealth of data that has been generated using animal models has contributed convincing evidence in support of the existence of EMT and MET, suggesting that the processes of EMT-MET are physiologically relevant in both normal embryogenesis and carcinogenesis (Morel et al. 2008, e2888;Sabbah et al. 2008, 123-151). In cancer, the processes of EMT were found to play key roles in invasion, metastatic dissemination and the acquisition of therapeutic resistance. In addition, EMT phenotype in cancers has been associated with poor clinical outcomes in multiple cancer types (Polyak and Weinberg 2009, 265-273). Moreover, EMT has been implicated in the alteration of early-stage cancers the led to their becoming invasive malignancies (Iwano et al. 2002, 341-350;Kang and Massague 2004, 277-279;Larue and Bellacosa 2005, 7443-7454;Yilmaz, Christofori, and Lehembre 2007, 535-541;Yu et al. 2007, 1109-1123). In addition, MET (reversal of the EMT process) is anticipated to play a role following the tumor cells dissemination and, consequently, the formation of distant metastases prior to rapid cancer cell growth at the metastatic sites. Recently, MET has been recognized for the extra cellular matrix protein versican *in vitro*, whereas the exogenous expression of the versican isoform V1 induced a N-cadherin to E-cadherin switch, resulting in epithelial-specific adhesion junctions in fibroblasts, while silencing of endogenous versican prevented MET (Sheng et al. 2006, 2009-2020). Interestingly, a different model for the role of EMT in cancer metastasis has been proposed in which EMT and

non-EMT cells cooperate to complete the entire process of spontaneous metastasis (Tsuji, Ibaragi, and Hu 2009, 7135-7139).

In general, the processes of EMT in cancer are regulated by the genomic instability of cancer cells, including genetic and epigenetic factors, and the effect of stromal cells signaling within the cancer microenvironment, which leads to cancer heterogeneity within the tumor mass (Fig-2) (Sabbah et al. 2008, 123-151).

The EMT-phenotypic cells have been shown to share characteristics with both stem cells and cancer stem-like cells. These findings has been supported by several studies, showing that EMT phenotype induced by TGF-β or expression of TWIST or SNAIL transcription factors shares stem-like cell features as demonstrated by cell surface antigenic profiles, gene expression patterns, ability of EMT cells to form mammospheres in culture and ductal outgrowth in xenotransplant assays (Morel et al. 2008, e2888;Sabbah et al. 2008, 123-151). This data was found to be correlated with the data showing that high levels of genes associated with invasion, metastasis, and angiogenesis were indeed found in CD44+CD24– breast cancer cells (Sheridan et al. 2006, R59;Shipitsin et al. 2007, 259-273) further enabling these cells to invade and metastasize (Sheridan et al. 2006, R59). Furthermore, breast cancer cells disseminated in circulation and by the bone marrow have been found to be CD44+CD24– antigen phenotype (Riethdorf and Pantel 2008, 140-148;Riethdorf, Wikman, and Pantel 2008, 1991-2006;Slade et al. 2009, 160-166). Moreover, Immunohistochemistry

analysis of 479 invasive breast carcinomas showed a high expression of EMT-induced markers such as vimentin, α-smooth muscle actin, neural cadherin (n-cadherin), cadherin 11, SpArC, laminin, and fascin, in addition to low levels of E-cadherin, all of which were found to be associated with basal-type breast cancers (associated with poor prognosis and increased capability to metastatisize) compared with other subtypes of breast cancer (Perou et al. 2000, 747-752;Sorlie et al. 2001, 10869-10874). Furthermore, the acquisition of EMT (gain of cancer stem-like cell characteristics) is also known to be associated with insensitivity to apoptosis (Iwano et al. 2002, 341-350;Robson et al. 2006, 254-264), suggesting that the reversal of these phenotypes would be important to make cancer cells sensitive to apoptotic stimuli. For example, EpH-4 and nMuMG murine mammary epithelial cell lines acquired resistance to ultraviolet-induced apoptosis after EMT conversion by treatment with TGF-β (Robson et al. 2006, 254-264). Similar findings were shown by down-regulation in the expression of the let-7 microRNA (miRNA) in breast cancer cell lines that led to increased metastatic capability and therapeutic resistance, associated with the acquisition of stem cell features consistent with EMT specific gene expression profiles.

Several mechanisms for EMT stimulation have been identified, and consequently numerous molecules and signaling pathways shown to be involved in the EMT process. Examples of positive regulators of EMT include matrix metalloproteinase 3 (MMP-3, also known as stromelysin 1) (Radisky et al. 2005,

123-127), hypoxia (Gort et al. 2008, 60-67), and 5-aza-cytidine (a DNA methyltransferase inhibitor), which increased MCF-7 breast cancer cells invasiveness and metastasis by up-regulation of pro-invasive EMT-associated genes (Ateeq et al. 2008, 266-278;Guo et al. 2002, 41571-41579). More examples include chronic exposure to EGFR-TKI (Rho et al. 2009, 219-226), HGF(Montesano et al. 1991, 901-908), and aromatic hydrocarbon (e.g. Benzo[a]payere) (Yoshino et al. 2007, 369-374), which lead to tumor aggressiveness and drug resistance.

Likewise, Wnt signaling can also lead to EMT by the inhibition of glycogen synthase kinase-3β (GSK3β)-mediated phosphorylation and the degradation of β-catenin in the cytoplasm, leading to increased β-catenin levels and its translocation to the nucleus, where it acts as a transcription factor inducing the expression of genes, resulting in the acquisition of EMT phenotype (Vincan and Barker 2008, 657-663). In addition, Notch signaling has been implicated in playing important roles in the regulation of EMT which occurs both during embryogenesis and carcinogenesis (Bailey, Singh, and Hollingsworth 2007, 829-839). The Notch signaling signally pathway, once activated, is known to cross-talk with other important signaling pathways such as Nuclear Factor-κB (NF-κB) pathway (Wang et al. 2006, 2778-2784) and TGF-β signaling pathways during the acquisition of EMT. In addition, emerging evidence suggests that certain miRNAs are important regulators of many genes that are involved in the acquisition of EMT. For example, miR-200 family (miR-200a, miR-200b, miR-

200c), miR-141, miR-429 and miR-205 are among many miRNAs that appear to play an important role in the acquisition of EMT (Gregory et al. 2008, 3112-3118;Gregory et al. 2008, 593-601;Park et al. 2008, 894-907).

Nevertheless, the Transforming Growth Factor-β (TGFβ) family of cytokines are the major and best characterized inducers of EMT, and are associated with many biological processes such as the development of embryos, wound healing, fibrotic diseases, and cancer progression (Ateeq et al. 2008, 266-278;Massague 2008, 215-230;Yang and Weinberg 2008, 818-829). Recent studies have shown involvement of TGF-β in regulating breast cancer stem cell phenotypes (Mani et al. 2008, 704-715;Morel et al. 2008, e2888).

It is equally important to note that Hedgehog (HH) signaling has been implicated in EMT, cancer metastasis, and regulates and maintains stem cell characteristics in niche-stem cell interaction. The niche–stem cell interaction is known to play an important role in triggering the EMT process (Bailey, Singh, and Hollingsworth 2007, 829-839). Therefore Hedgehog (HH) signaling pathway is becoming an important target in cancer therapy.

Epithelial to Mesenchymal Transition and Lung Cancer:

It is important to understand that invasion and metastatic processes vary depending on the biological and anatomic characteristics of the primary organ. For example, in non-small lung cancer (NSCLC) invasion is a complex process due to the anatomy of the lung which lacks the anatomic support for invasion.

Therefore, invasion in NSCLC is usually understood as cancer cells infiltrating into their own newly formed desmoplastic stroma.

Desmoplastic stroma is a scar-like, highly fibrotic tissue that comprises more than 50% of the tumor mass, and it differentiates to myofibroblast cells, called Tumor Associated Fibroblast (TAF) (Bissell and Radisky 2001, 46-54;Liotta 1986, 1-7). NSCLC progression from the adjacent desmoplastic stroma is facilitated by the acquisition of the EMT phenotype by NSCLC cells. As indicated earlier, the processes of EMT involves numerous changes in the levels, distribution, and/or function of proteins including E-cadherins, vimentin, fibronectin, matrix metalloproteinases and ZEB1/2 (Larue and Bellacosa 2005, 7443-7454). Importantly, in NSCLCs expression alterations of EMT-MET proteins have been reported in both desmoplastic stroma and carcinoma cells (Kodama et al. 2007, 269-274;Sasaki et al. 2001, 843-848).

Recent studies have employed gene expression, xenograft assays and proteomic profiling techniques in order to find biomarkers associated with sensitivity to erlotinib in panels of sensitive and insensitive NSCLC cell lines (Thomson et al. 2005, 9455-9462;Witta et al. 2006, 944-950;Yauch et al. 2005, 8686-8698). Authors have found that sensitive cell lines express the well-established epithelial markers E-cadherin and catenin as well as exhibit the typical cobblestone epithelial morphology and tight cell–cell junctions of epithelial cells. On the other hand, insensitive cell lines did not have epithelial markers, but they express protein characteristics of mesenchymal cells including vimentin,

fibronectin and Zeb-1 consistent with more fibroblastic scattered morphology; this trans-differentiation was found to be similar to cells undergoing the processes of EMT. These observations were also extended to other cancer types including pancreatic, colorectal (Buck et al. 2007, 532-541), head and neck (Frederick et al. 2007, 1683-1691), bladder (Shrader et al. 2007, 277-285) and breast cancers (Buck et al. 2007, 532-541) using EGFR antagonists.

Collectively, these results suggest that the processes of EMT may serve as an indicator of sensitivity to EGFR inhibitors. Moreover, a study by Prudkin *et al* has shown a clear association with these EMT markers in the sequential pathogenesis of squamous cell carcinoma (Prudkin et al. 2009, 668-678), suggesting that the combination of EGFR-TKI with the inhibitor of EMT-inducing-molecules could become a novel approach toward the treatment of lung cancer, especially NSCLC, for which better innovative treatment is urgently needed.

It is well known that the expression levels of EGFR and/or mutated EGFR are a predictor of the response to EGFR antagonists both in the clinic and in cultured cell lines (Ono et al. 2004, 465-472;Thomson et al. 2005, 9455-9462). In TRIBUTE, a NSCLC phase III randomized trial that compared a combination of erlotinib and chemotherapy to chemotherapy alone, they showed provocative findings in a retrospective analysis of E-cadherin expression by immunohistochemistry in a subset of tumor specimens from patients. The authors found that tumors with strong E-cadherin staining had a drastically longer time to progression (hazard ratio 0.37), without a significant increase in overall

survival when treated with the combination of erlotinib and chemotherapy as compared to chemotherapy alone, and these results were consistent with *in vitro* findings (Gillan et al. 2002, 5358-5364;Yauch et al. 2005, 8686-8698). These *in vitro* and clinical data indicate that the expression of E-cadherin or fibronectin, and the quantification of EMT-phenotypic cells could be an excellent biomarker in predicting the therapeutic response to EGFR inhibitors in NSCLC patients. These results also suggest that the reversal of EMT to MET by novel approaches and/or the sensitization of EMT-type cells with novel agents prior to treatment of NSCLC with conventional chemotherapeutic agents or targeted agents would be beneficial for the treatment of lung cancer. Hence, better understanding of the full extent of the mechanisms of EMT as a crucial biological phenomenon in NSCLC therapy is exetremely important.

Hedgehog (Hh) signaling pathway in Lung Cancer and Epithelial to Mesenchymal Transition

The Hh signaling pathway is required for normal lung embryogenesis, organogenesis, and is known to play important roles in stem cell maintenance. In mammals, Hh ligands consist of three members: Sonic Hh (SHH), Indian Hh (IHH), and Desert Hh (DHH). The hedgehog-ligands family consists of an N-terminal signal peptide, a Hedgehog core domain, and the C-terminal processing domain. Hedgehog precursors are autocatalyzed to cleave the C-terminal processing domain for cholesteroylation and are then further processed by

Hedgehog acyltransferase (HHAT) to cut off the N-terminal signal peptide for palmitoylation (Fig. 3). Mature Hedgehog proteins with lipid modifications are then transported to the cell surface for packaging into lipoprotein particles depending on Dispatched 1 (DISPI), or for multimerization via lipophilic tails. Mature Hedgehog proteins secreted from producing cells induce concentration dependent effects on target cells expressing Hedgehog receptor. PTCH1 and PTCH2, Patched family members, are Hedgehog receptors distantly related to Dispatch family members with multi-trans-membrane domains and a sterol-sensing domain with PTCH1 acting as the predominant Hh receptor. Smoothened is a seven trans-membrane type receptor which transduces Hh-signaling. CDON and BOC are trans-membrane proteins with extracellular immunoglobulin-like (Ig-like), and fibronectin type III (FNIII) domains, which enhance Hedgehog signaling activity as co-receptors. GASI is a GPI-anchored cell surface protein binding to Hedgehog ligands for the potentiation of Hedgehog signaling. On the other hand, HHIP is a negative regulator of Hedgehog signaling, it compete with Patched receptors for Hedgehog-ligand binding (Fig. 4). *GLI1/2* is a transcription factors functioning as a Hedgehog signaling effectors. *GLI1* gene initially cloned as an oncogene amplified in malignant glioma, and then characterized as a transcription factor of Hh signaling pathway. GLI1, GLI2, and GLI3 are human homologs of *Drosophila* Cubitus interruptus.

In the absence of Hh ligands, Patched family members inhibit Smoothened function, GLI1 is transcriptionally repressed, GLI2 is phosphorylated

by GSK3 and CKI of the FBXWll (BTRCP2)-mediated degradation, and GLI3 is processed to a cleaved repressor. Upon Hedgehog-ligand binding to PTCH1/2 is rapidly internalized and consequently Smoothened is released from Patched-dependent suppression. Then Smoothened induces MAP3KlO (MST) activation and SUFU inactivation for the stabilization and nuclear accumulation of GLI family members, respectively. GLI1 functions as transcriptional activator of Hedgehog target genes, while GLI2 and GLI3 function as transcriptional activator or repressor in a context-dependent manner. Hedgehog signaling activation leads to transcriptional activation of target genes through GLI-binding to the GACCACCCA motif. GLI1, PTCH1/2 and HHIP1 are up-regulated by Hedgehog signaling, while CDON, BOC and GASl are down-regulated. Hedgehog-dependent GLI1 up-regulation constitutes a positive feedback loop, while Hedgehog dependent regulation of PTCH1, HHIP1, CDON, BOC, and GASl constitutes a negative feedback network. Hedgehog signals induce transient up-regulation of target genes through the combination of positive and negative feedback mechanisms. Hedgehog signals up-regulate CCNDl and CCND2 for cell cycle acceleration, and similarly up-regulation of FOXA2, FOXC2, FOXE1, FOXF1, FOXL1, FOXP3, POU3F1, RUNX2, SOX13, and TBX2 are known to be important for cell fate determination. Hedgehog signals also up-regulate JAG2 to regulate Notch, and lNHBC/I/E to regulate Activin signaling cascades. In addition, Hedgehog signals up-regulate SFRPl at least in mesenchymal cells without its promoter CpG hypermethylation to inhibit canonical WNT signaling cascade in

epithelial cells (Fig.4) (Table 3.) (Krishnan et al. 1997, 1947-1950;Teh et al. 2002, 4773-4780;van Den Brink, de Santa, and Roberts 2001, 2115-2116).

Recently, the hedgehog (Hh) signaling pathway was reported to be active in many cancers including NSCLC (Katoh and Katoh 2008, 271-275;Thayer et al. 2003, 851-856;Watkins et al. 2003, 313-317;Yuan et al. 2007, 1046-1055). In addition, the Hh signaling blockade inhibits the growth, invasion and metastasis of cancer cells having activated Hh signaling, which was associated with the down-regulation of Snail and up-regulation of E-cadherin. Furthermore, over-expression of GLI-1 in epithelial cancer cells led to the aggressive phenotype with down-regulation of E-cadherin (Feldmann et al. 2007, 2187-2196;Fukaya et al. 2006, 14-29), suggesting that Hh signaling is an important regulator of EMT and important target for therapy. Moreover, it has been reported that an EMT regulator SIP1 is a target gene of the Hh signal in gastric pit cells and in diffuse-type gastric cancer, and interestingly SIP1 regulates mesenchymal-related genes (WNT5A, CDH2, PDGFRB, EDNRA, ROBO1, ROR2, and MEF2C) (Ohta et al. 2009, 389-398). In addition, it was found that Hh-signaling cascades cross-talk with WNT, EGFR, FGF, and TGF-β/Activin/NodallBMP signaling cascades, all of which have been implicated in EMT through the repression of E-cadherin expression. Moreover, RTK signaling potentiates GLI activity through PI3K/AKT-mTOR/P70S6K2-mediated GSK3β inactivation or RAS-STIL1-mediated SUFU inactivation. Further evidence in support of the role of Hh signaling in EMT, comes from recent studies showing that up-regulation of SNAI1 (Snail-1) and

PTCH1 mRNAs was induced after GLI1 expression (Katoh and Katoh 2008, 271-275) and that Hh signaling was found to be essential for the maintenance of cancer stem cells (Zhao et al. 2009, 776-779). Collectively, all of these studies clearly suggest that Hh signaling is a legitimate and novel target for the development of innovative therapeutic strategies for the treatment of invasive and metastatic cancer phenotypes with EMT or cancer stem-like cell characteristics in NSCLC. In the list below (table 4.) different types of Hedgehog pathway inhibitors, targeting different molecules in the Hh pathway are presented. It is important to understand which model of Hh signaling applies to each drug type because this has several implications for drug development and therapy, for example, if cancer does not express Hh ligands, it will not respond to Hh ligand blockers. Therefore, identification of Hh pathway elements and the pathways for its cross-talks in patient's tumor will provide a good predictive marker of response.

Interaction and cross-talk between Nuclear Factor-Kappa B (NF-κB), Transforming Growth Factor-beta (TGF-β), and Sonic hedgehog (Shh)

Hedgehog has been reported to cross-talk with NF-κB, a major cell growth and apoptotic regulatory pathway (Cui et al. 2010, 927-933;Kasperczyk et al. 2009, 21-33;Nakashima et al. 2006, 7041-7049;Umeda et al. 2010, 692-698;Yamasaki et al. 2010, 675-686). NF-κB plays important roles in the control of cell growth, differentiation, apoptosis, and inflammation (Baud and Karin 2009,

33-40;Campbell and Perkins 2006, 165-180;Grilli, Chiu, and Lenardo 1993, 1-62;Pikarsky and Ben-Neriah 2006, 779-784;Sethi, Sung, and Aggarwal 2008, 21-31). NF-κB is now known to induce and control a broad spectrum of genes including inflammatory cytokines, chemokines, cell adhesion molecules, growth factors, interferons, and viruses (Chen and Greene 2004, 392-401). NF-κB belongs to a family of proteins including members Rel A, c-Rel, Rel-B, Bcl-3, p100 and p105. Under non-stimulated conditions, NF-κB consists primarily of the Rel A (p65) and p50 heterodimer associated with cytosolic IκB inhibitory protein (Vallabhapurapu and Karin 2009, 693-733). Following stimulation with an inducer such as tumor necrosis factor α (TNF−α), Lipopolysaccharide (LPS) or H2O2, NF-κB is activated. The activation of NF-κB involves the phosphorylation of IκB, an inhibitory binding partner of NF-κB complex, for ubiquitination and degradation through the proteasome degradation pathway (Haefner 2005, 137-188;Karin and Greten 2005, 749-759;Nakanishi and Toi 2005, 297-309). The free NF-κB complex is then translocated into the nucleus, binds to the DNA consensus sequence and activates target genes (Chen et al. 2001, 1653-1657;Hacker and Karin 2006, re13). A key regulatory step in the NF-κB pathway is the activation of a high molecular weight IKK complex in which catalysis is thought to be done by kinases, including IKKα and IKKβ, which directly phosphorylate IκB proteins (Chen et al. 2011, 1172-1185;Miyamoto 2011, 116-130;Sun 2011, 71-85;Wajant and Scheurich 2011, 862-876). NF-κB mediates

survival signals that inhibit apoptosis and promote cancer cell growth (Chen et al. 2011, 1172-1185;Miyamoto 2011, 116-130;Sun 2011, 71-85;Sun 2011, 71-85;Wajant and Scheurich 2011, 862-876;Zhao et al. 2011, 367-381). NF-κB is constitutively activated in most human lung cancer tissues and cell lines but not in normal lung tissues and immortalized lung epithelial cells, suggesting that the activation of NF-κB is involved in the carcinogenesis of lung cancers (Chen et al. 2011, 1172-1185;Lin et al. 2010, 45-55;Saitoh et al. 2010, 263-270). It has been shown that NF-κB is activated in lung cancers through constitutive activation of IκB kinase (IKK) and degradation of IκBα. Inhibition of NF-κB by a super-inhibitor of NF-κB (delta-N-IκBα) results in impaired proliferation and induction of apoptosis, suggesting an important role of NF-κB in lung cancer tumorigenesis (Chen et al. 2011, 1172-1185;Lin et al. 2010, 45-55;Saitoh et al. 2010, 263-270). Moreover, evidence has shown that NF-κB participates in the process of lung cancer metastasis (Chen et al. 2011, 933-941;Gupta et al. 2010, 405-434). Also, number of K-Ras induced lung tumors are diminished, following the inactivation of the NF-kappaB subunit p65/RelA, in the presence and the absence of the tumor suppressor p53, suggesting that constitutive NF-κB activity plays a key role in lung cancer tumorigenesis and tumor progression (Basseres et al. 2010, 3537-3546;Meylan et al. 2009, 104-107). However, the precise molecular mechanism by which NF-κB is activated needs additional investigation.

Particularly, TGF-β is reported to induce the NF-κB translocation to the nuclei in activity-reporter assays (Arsura et al. 2003, 412-425;Gingery et al. 2008, 2725-2738;Yu et al. 2010, 869-878). Chow et al., found that TGF-β suppresses PTEN in pancreatic cancer cells through NF-κB activation, and enhances cell motility and invasiveness in a Smad4-independent manner that can be counteracted when TGF-β-Smad2/3 signaling restored. Their data suggests that the activation of NF-κB is by Smad2 and/or Smad3, and not Smad-4 (Chow et al. 2010, G275-G282). Furthermore, TGF-β ligand TGF-β1 activates NF-κB in human osteoclast, and promotes osteoclast survival. Furthermore, TGF-β1 transiently inhibits apoptosis in liver tumor through activation of NF-κB (Arsura et al. 2003, 412-425;Gingery et al. 2008, 2725-2738;Yu et al. 2010, 869-878), suggesting that TGF-β1 activates NF-κB in cancer cells.

Interestingly, monocytes stimulated by Inflammation produced Shh through activation of NF-κB signaling pathway (Yamasaki et al. 2010, 675-686). Also NF-κB found to activate the hedgehog signaling pathway via induction of Shh expression in pancreatic cancer specimens (Nakashima et al. 2006, 7041-7049). Also, it has been found that NF-κB binds to Shh promoter regions prior to the up-regulation of Shh expression. Authors have found that NF-κB activation results in increased Shh mRNA and protein expression *in vitro* and *in vivo* in an inducible NF-κB activity mouse model. Inhibition of NF-κB by super-repressor (inhibitory NF-κB alpha; IκBα) or by p65 knock-down inhibits induction of Shh

promoter activation and Shh expression by NF-κB indicating that Shh expression is regulated by NF-κB and that NF-κB-induced Shh involved in NF-κB-mediated proliferation and apoptosis resistance both *in vitro* and *in vivo* (Kasperczyk et al. 2009, 21-33).

TGF-β, NF-κB and Hh pathways are key regulators of numerous cellular events such as proliferation, differentiation and apoptosis (Chen et al. 2011, 1172-1185;Inman 2011, 93-99;Massague 2008, 215-230;Zhao et al. 2009, 776-779). TGF-β and NF-κB mediated cell growth and apoptotic resistance could in part be mediated via up-regulation of Shh. Therefore, agents that inhibit Shh signaling could be beneficial in the prevention or treatment of cancer.

Overall hypothesis

Lung is the leading cancer site in male, comprising 17% of the total new cancer cases, and the most common cause of cancer related deaths with 23% of the total cancer deaths. In the United States, The American Cancer Society estimated that 222,520 Americans were diagnosed with lung cancer and 157,300 died of lung cancer in 2010 (Jemal et al. 2009, 225-249;Jemal et al. 2011, 69-90).

Emerging evidence is accumulating suggesting that the phenomenon called Epithelial-to-Mesenchymal Transition (EMT), which shares similar molecular characteristics with cancer stem-like cells, contributes to lung cancer treatment failure. EMT process has been implicated in the two important

biological processes that are accountable for cancer-related deaths; the progression of cancer cells to a distant organ and the acquisition of resistance to conventional cancer therapeutics (Leng et al. 2011, 145-155;Shih and Yang 2011;Zhang et al. 2011). Therefore, further mechanistic understanding of the role of EMT in lung cancer aggressiveness and treatment failure is very important.

Recently, studies have suggested that Hh signaling is an important regulator of EMT and a crucial target for therapy. Among all major cancers studies have shown that Hh signaling blockades inhibits the growth, invasion and metastasis of cancer cells having activated Hh signaling which was associated with the down-regulation of Snail and up-regulation of E-cadherin. Furthermore, over-expression of GLI-1 in epithelial cancer cells leads to aggressive phenotype with down-regulation of E-cadherin (Feldmann et al. 2007, 2187-2196;Fukaya et al. 2006, 14-29),

Therefore, we hypothesize that the Hedgehog (Hh) signaling pathway functions as a positive regulator of epithelial-to-mesenchymal transition (EMT) in NSCLC; thus, it is an excellent target for lung cancer therapy. We also hypothesize that Hh inhibitors sensitizes NSCLC cells to cisplatin and/or EGFR-TKI by reversing the processes of EMT to MET.

We set out to test our hypotheses by accomplishing the following specific aims.

Specific Aims:

1. Examine the role of Hh signaling in the processes of EMT *in vitro* and *in vivo*, especially because EMT has been shown to be associated with tumor progression and cause resistance of non-small lung cancer (NSCLC) to conventional therapeutics.

2. Assess the molecular mechanism of Hh signaling in the processes of EMT and deregulation of miroRNAs (miRNAs).

3. Investigate whether down-regulation of Hh signaling could reverse EMT phenotype and lead to the sensitization of NSCLC cells to conventional therapeutics.

4. Investigate the expression of EMT markers (protein and mRNA) that are involved in drug resistance of NSCLC cell lines before and after treatment with Hh inhibitors.

Table 1		
Cancer Type	Subtype	percent of all lung cancer cases
NSCLC	• Squamous cell carcinoma • Adenocarcinoma • Large-cell undifferentiated carcinoma	85%
SCLC		10-15%

Table 1: Lung cancers major categories

Table 2.	
1	*Surgery*
2	*Radiation therapy*
3	*Chemotherapy:* Cisplatin, Carboplatin, Paclitaxel, Docetaxel, Gemcitabine, Vinorelbine, Irinotecan, Etoposide, and Vinblastine
4	*Targeted-drug-therapy:* Gefatinib, Erlotinib, Bevacizumab

Table 2: Treatment Options for Lung Cancer

Table 3.	
Function of target genes	**gene**
HH Signaling cascade	GLI1 PATCH1,2
Cell cycle regulation	CCND1 CCND2 N-Myc FOXM1
Cell-Fate Determination	FOXA2 FOXC2 FOXE1 FOXF1 FOXL1 FOXP3 SOX13 TBX2 RUNX2 POU3F1 SNAI1,2 ZEB1,2
Stem cell signaling network	JAG2 INHBC INHBE SFRP1 WNT2B WNT5A BMP4

Table 3. Downstream target genes of Hedgehog Signaling Pathway and their functions

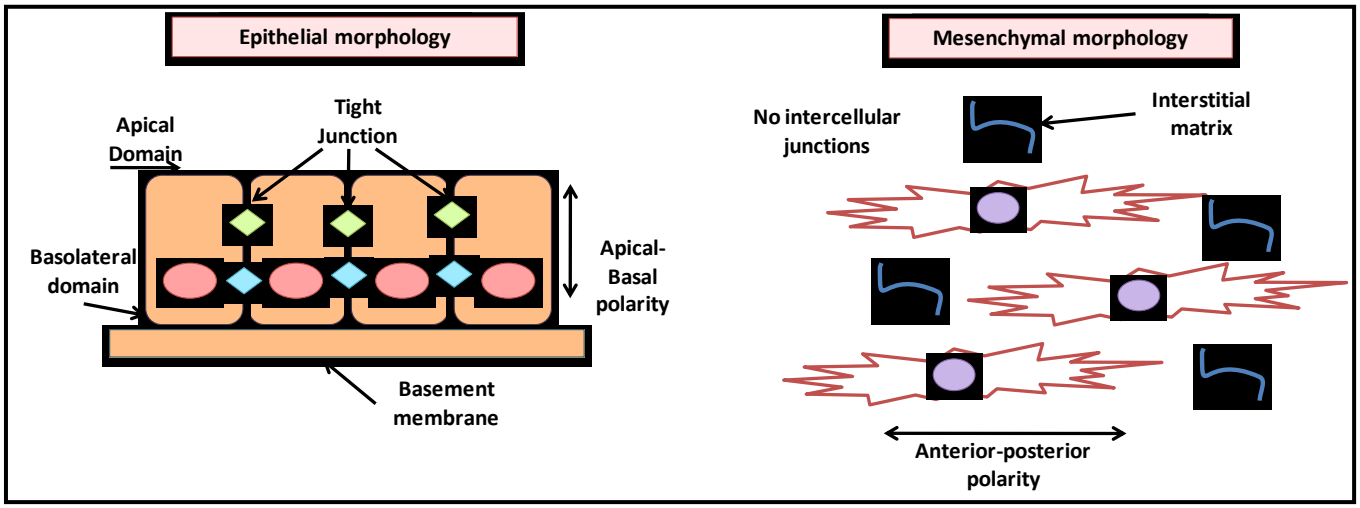

Figure 1.

Morphologic hallmarks of epithelial and mesenchymal cells. Epithelial morphology presented by an apical–basal polarity, joined with a basal basement membrane and formation of extensive cell–cell contacts, including tight junctions. While mesenchymal morphology featured by loss of anterior–posterior polarity, if any, cell–cell junctions within a more unstructured interstitial matrix.

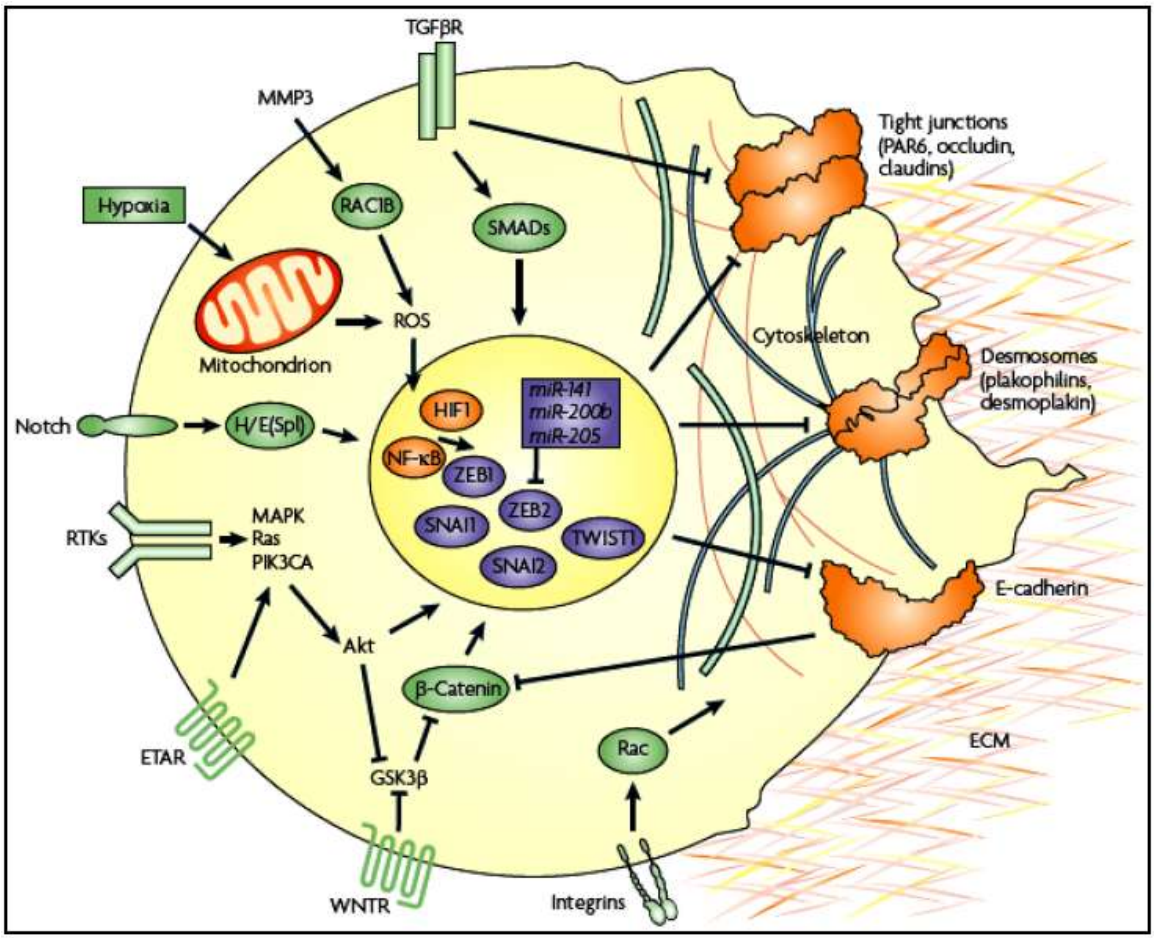

Figure 2.
A simplified sketch of epithelial-to-mesenchymal transition regulating signaling networks. Receptor tyrosine kinases (RTKs), transforming growth factor-β (TGFβ), Notch, endothelin A receptor (ETAR), integrins, Wnt, hypoxia and matrix etalloproteinases (MMPs) can induce EMTs through multiple different signaling pathways, and the relative importance of each of these may depend on the particular cellular context.

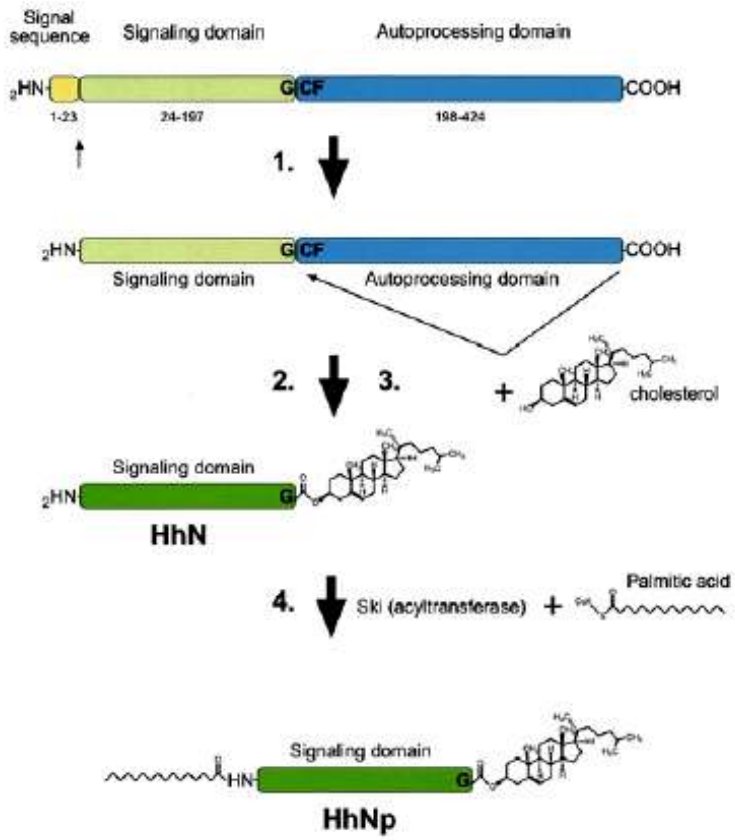

Figure 3.
Hedgehog ligands family consist of N-terminal signal peptide, Hedgehog core domain, and C-terminal processing domain. Hedgehog precursors are Auto-catalyzed to cleavage the C-terminal processing domain for cholesteroylation, and then further processed by Hedgehog acyltransferase (HHAT) to cut off the N-terminal signal peptide for palmitoylation.

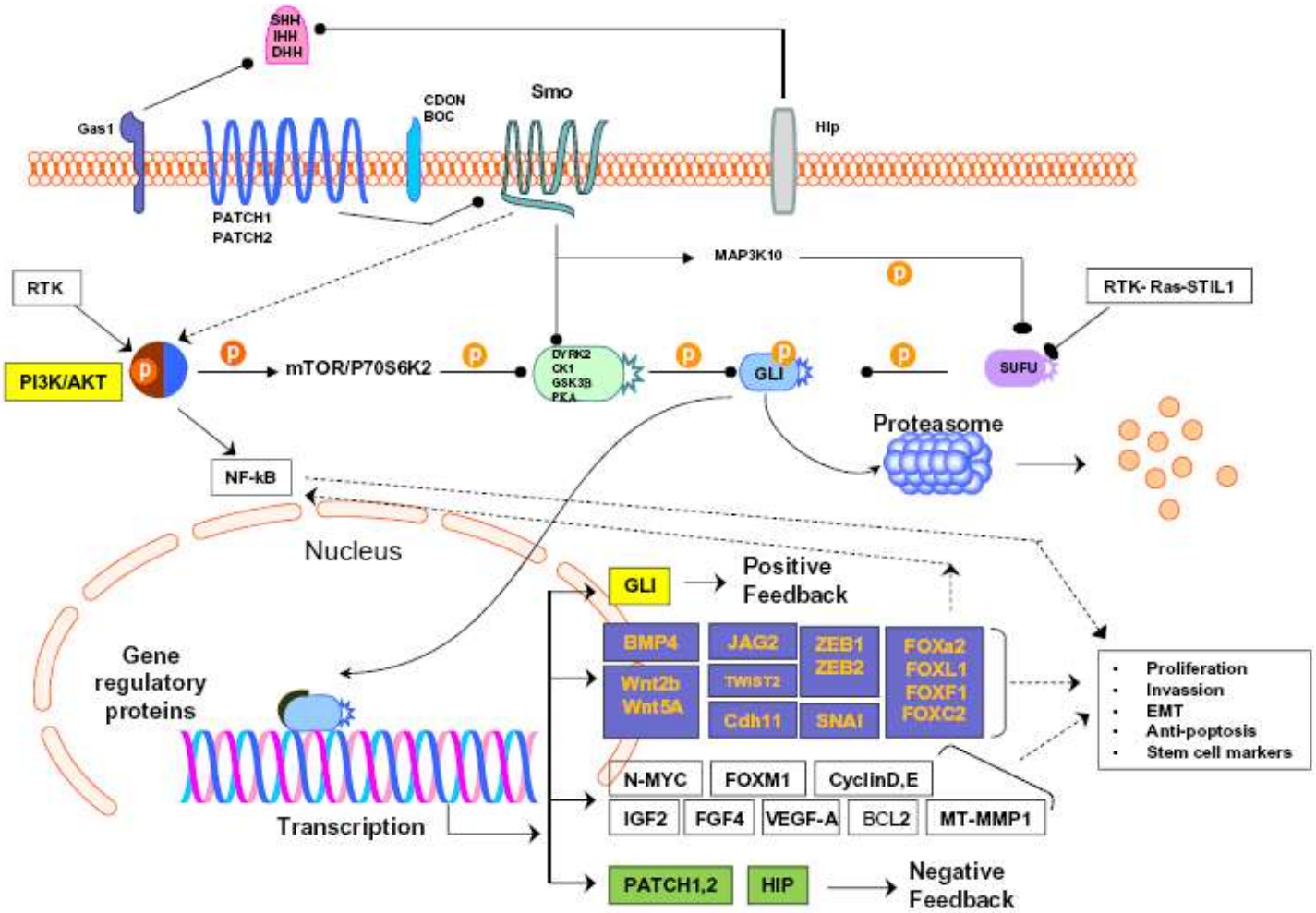

Figure 4.
Illustration of Hedgehog Signaling Pathway in Cancer; Canonical and noncanonical activation of Hedgehog pathway and it's cross-talk among key important signaling molecules.

CHAPTER 2

MATERIALS AND METHODS

Cell culture and experimental reagents

The human lung adenocarcinoma cell lines, A549, H2030, H1299, H1650, and mouse fibroblast NIH-3T3 cells were purchased from the American Type Culture Collection (Manassas, VA) and maintained according to the American Type Culture Collection's instructions. The normal lung epithelial cell line (NHBE cells) was purchased from Lonza. NHBE cells where maintained and cultured according to Lonza's instructions. All the cell lines have been tested and authenticated using the Karmanos Cancer Center, Wayne State University core facility (Applied Genomics Technology Center at Wayne State University) on March 13, 2009, and these authenticated cells were frozen for subsequent use. The method used for testing was short tandem repeat profiling using the PowerPlex 16 System from Promega. A549 cells were treated with TGF-β1 (5 ng/ml) for 21 days before experiments were conducted. Cells were treated with GDC-0449 (20 nM) or Cyclopamine (2 mM) for 72 hours, before conducting assays.

Reagents and antibodies

Anti-Shh N-terminal peptide antibody and recombinant human TGF-β1 protein was purchased from R&D Systems (Minneapolis, MN). Cyclopamine was

purchased from Sigma (San Louis, MO) and diluted in dimethyl sulfoxide as a control vehicle. GDC-0449 (20 nM) was obtained from Genentech. Rabbit anti-GLI1 was purchased from Abcam. Rabbit anti-fibronectin was obtained from Santa Cruz biotechnology (CA, USA). Antibodies to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Affinity BioReagents (Golden, CO.). Mouse anti-bactin was obtained from Sigma (St. Louis, MO). β-tubulin rabbit mAb was obtained from cell signaling (Danvers, MA).

Plasmids, cloning and transfections

The Shh promoter plasmid encoding the Shh promoter region fused to RenSP (optimized luciferase gene) reporter was purchased from switchgeargenomics. Mutant shh-promoter-luciferase was generated by Site-Directed Mutagenesis kit obtained from Oragene. The following primers were used: sence 5'- GGT GGG GAG CGG TCG AGA GTC CGC CGC AGC CGC GGC -3', antisence 5'- GCC GCG GCT GCG GCG GAC TCT CGA CCG CTC CCC ACC -3'. Afterward, Cells were transfected with wild type Shh-promotor or mutant Shh-promoter, respectively, using Fugene 6 transfection reagent (Roche). All plasmids were controlled by sequencing.

Luciferase assays

Renilla luciferase activities determined using The Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Using

Fugene 6 Transfection Reagent (Roche), cells in 6-well plates were transfected with *Renilla* luciferase vector under control of the ubiquitin promoter per well. After 24 hrs, cells were stimulated as by TGF-β1 for 24 hrs. Control cells were not treated with TGF-β1. Then cells were lysed with Passive Lysis Buffer (Promega). The luminance of each well was measured in Ultra 26 Multifunctional Microplate Reader (Tecan, Durham, NC). Results were plotted as means ± SD of three separate experiments having three determinations per experiment for each experimental condition.

Cell growth inhibition studies by MTT assay

Cells were seeded at (5×10^3) cells per 100 μl of culture medium per well in 96-well plates. The number of viable cells was assessed in six wells using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma) according to the manufacturer's instructions, and then with isopropanol at room temperature for 1 h. Cells were treated with Hh inhibitor (GDC-0449 or Cycopamine) for 72 hour treatments, or knock-down with siRNA specific for Shh (si-Shh) for 48 hours. Next, cells were treated for 72 hrs with either Cisplatin or Erlotinib, at the concentration specified in the text. Control cells received 0.1% DMSO or 0.5 mM Na2CO3 in culture medium. After treatment, the cells were incubated with MTT reagent (0.5mg/ml; Sigma) at 37°C for 2 h and then with isopropanol at room temperature for 1 h. Spectrophotometric absorbance of the samples was determined by an Ultra-26 Multifunctional Microplate Reader

(Tecan, Durham, NC). Results were plotted as means ± SD of three separate experiments having six determinations per experiment for each experimental condition.

Western blot analysis

Cells were lysed in lysis buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 0.5% Triton X-100, 2.5 mM sodium orthovanadate, 10 µl/ml protease inhibitor cocktail and 1 mM PMSF) by incubating for 20 min at 4°C. The protein concentration was determined using the Bio-Rad assay system (BioRad, Hercules, Calif). Total proteins were fractionated using SDS-PAGE and transferred onto nitrocellulose membrane. The membranes were blocked with 5% nonfat dried milk or BSA in 1 x TBS buffer containing 0.1% Tween 20 and then incubated with appropriate primary antibodies. Horseradish peroxidase conjugated anti-rabbit or anti-mouse IgG was used as the secondary antibody and the protein bands were detected using the enhanced chemiluminesence detection system (Amersham Pharmacia Biotech, Piscataway, NJ). Quantification of Western blots was performed using ImageJ software and the results are presented as the mean of three independent experiments with error bars representing standard errors. For reprobing, membranes were incubated for 30 min at 50°C in buffer containing 2% SDS, 62.5 mM Tris (pH 6.7), and 100 mM 2-mercaptoethanol, washed and incubated with desired primary antibody.

Real-time reverse transcription-PCR analysis for gene expression studies

Quantitative real-time RT-PCR analysis was conducted; 1 μg of total RNA from each sample was subjected to reverse transcription using the High-Capacity RNA-to-cDNA Kit (Applied Biosystems) according to the manufacturer's protocol. Real-time PCR reactions were then carried out in a total volume of 25 μl reaction mixture (2 μl cDNA, 12.5 μl of 2 x SYBR Green PCR Master Mix from Applied Biosystems, 1.5 μl of each 5 μmol/L forward and reverse primers, and 7.5 μl distilled H_2O) using a SmartCycler II (Cepheid). The PCR program was started by 10 min at 95^0C before 40 thermal cycles, each at 15 s at 95^0C and 1 min at 60^0C. Data were analyzed according to the comparative Ct method. Data was normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression in each sample. GLI1 primers have been previously described [25]. Shh primers (Shh-forward: GTGGCCGAGAAGACCCTA, Shh-reverse: CAAAGCGTTCAACTTGTCCTTA. GAPDH, ZEB1, and E-cadherin primers were previously described (Kong et al. 2009, 1712-1721). Experiment was repeated at least, three times independently. The primers were checked by running a virtual PCR, and primer concentration was optimized to avoid primer dimer formation. Also, dissociation curves were checked to avoid nonspecific amplification Data reported here is one representative experiment

Nuclear Extract Preparation

In brief, cells were washed with cold phosphate-buffered saline and collected in conical centrifuge tubes. After a 5-min centrifugation, the pellet was resuspended in a lysis buffer (100 mM HEPES, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 5 mM PMSF, 0.02 mg/ml leupeptin, 0.02 mg/ml aprotinin, and 5 mg/ml benzamidine) and incubated on ice for 15 min. 12.5 µl of 10% NP-40 for every 400 µl of cell suspension was added and the cells were lysed. The disrupted cells were then centrifuged for 3 min at 20,000 x *g* at 4 °C, and the supernatant was saved as a cytosolic extract. The pellet was resuspended in extraction buffer (22.5 mM HEPES, 452 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1 mM DTT, 0.1 mM PMSF, 0.2 µg/ml leupeptin, 0.2 µg/ml aprotinin, 0.05 mg/ml benzamidine). The suspension was incubated on ice for 30 min with gentle shaking and centrifuged at 20,000 x *g* for 5 min. The supernatant was stored as a nuclear extract at –80°C. Nuclear protein (10 µg) was subjected to EMSA.

Electrophoretic mobility shift assay (EMSA) for measuring NF-κB activity

A non-radioisotopic EMSA was used for measuring NF-κB activity. NF-κB standard consensus double-stranded oligonucleotide and the Shh-promoter NF-κB binding region of wild-type and mutants double-stranded oligonucleotide was 5' end-labeled with IRDye (IDT Integrated DNA Technologies), and used as a probe, and the assays were performed in a final volume of 20 µl containing 20 mM HEPES, pH 7.9, 0.4 mM EDTA, pH 8, 0.4 mM DTT, 5% glycerol, 1% NP-40,

60 mM NaCl, 2 µg poly (dI-dC), 10 µg nuclear extract, 2 pmol NF-κB oligonucleotide. The samples were incubated for 30 min at 37°C and were then electrophoresed through 8% polyacrylamide gel, followed by scanning with The Odyssey® Imaging System (LI-COR, Lincoln, NE). EMSA experiments were done by additional 30 minutes incubations with polyclonal supershift antibodies against p65 before the addition of labeled probe. Supershift assay using NF-κB p65 antibody was conducted to confirm the specificity of NF-κB DNA-binding activity. For loading control, 10 µg of nuclear proteins from each sample were subjected to Western blot analysis for retinoblastoma protein, which showed no difference in loading between samples. Images were scanned using laser densitometry.

***In vivo* experiment**

Four-week-old female ICR-SCID mice were obtained from Taconic Laboratory (Germantown, NY). The mice were adapted to animal housing. Each mouse received 1.5 x 10^6 A549 cells (control cells) and A549-M cells (in serum-free F-12) via tail-vein (Intravenous, IV) route. Mice were checked three times per week for any sign of distress. Mice in the control and treated group were followed for changes in body weight. Two Animals were sacrificed at four weeks, five weeks, and finally three animals were sacrificed at six week following tumor cells injection. Tumor tissues cut into two-halves, one-half was fixed in bouin's solution, and other half was formalin-fixed for histological staining with

Hematoxylin and Eosin. Tumors foci were counted and pictures of tumor cells taken under microscope at 4x magnification. Tumor foci count was plotted for three animals, sacrificed at six weeks of each group. All studies involving mice were done under Animal Investigation Committee–approved protocols.

Chromatin immunoprecipitation (ChIP) and Real-time polymerase chain reaction (PCR) analysis

The ChIP assay kit (Upstate, Lake Placid, NY, USA) was used and the assay was performed according to the manufacturer's instructions, using rabbit anti-p65 polyclonal antibody or IgG isotype as negative control antibody (Santa Cruz Biotechnology) or mouse Anti-RNA polymerase II was used as positive control for immunoprecipitation. Next, real-time PCR reactions were then carried out in a total volume of 25 μl reaction mixture (2 μl DNA, 12.5 μl of 2 x SYBR Green PCR Master Mix from Applied Biosystems, 1.5 μl of each 5 μmol/L forward and reverse primers, and 7.5 μl distilled H_2O) using a SmartCycler II (Cepheid). the following primers: 5'-GAGCTCCACAAGCTCTCCAGGCTTGC-3' and antisense 5'-CTCGAGTCCTCGCTCCGGCTCGCCCGC-3', previously Described (Kasperczyk et al. 2009, 21-33). GAPDH primer was used as internal control and the samples input were used as reference control.

Wound healing assay

Cells were treated with TGF-β1 for 21 days, Hh inhibitor for three-72 hour treatments, or knock-down with siRNA specific for Shh (si-Shh) for 48 hours. Prior to treatment, cells were seeded at $1x10^6$ cells per well in a 6-well plates. Upon 90% confluence, cells were scraped across the cell monolayer using a plastic 200 μl tip. Photomicrographs were taken with Phase contrast objective microscope 4 x magnifications, at zero time point and after 24 hours. The measured ratio of the remaining wound area relative to the initial wound area was Quantified and reported. Quantification of the wound area using the NIH Image-J program was performed, and the results are expressed as the percentage of wound area change. Experiment was repeated at least three times, independently. Data reported here is representative of an experiment.

Matrigel invasion assay

Cells were treated with TGF-β1 for 21 days, Hh inhibitor for three-72 hour treatments, or knock-down with siRNA specific for Shh (si-Shh) for 48 hours. Following seeding cells at $5x10^4$ cells/well, invading cells at the bottom of the membrane and media in the lower chamber were detected by pre-labeled with DiIC12 (3) Fluorescent Dye or by post-staining using immune-staining Diff-QuickTM staining kit after removal of noninvasive cells. Cells were seeded in the upper chamber of a 24-insert with serum-free medium. Upper chambers coated with Matrigel (fluoro-block insert and MATRIGELTM Invasion Chamber; BD Biosciences, USA). Lower chamber contained 10% FBS plus regular media.

After 24 h of incubation, invading cells were examined by using a fluorescence microscope and photographed. The transfection efficiency was photographed at 10X, whereas invading cells was photographed at 4X magnification. TECAN Ultra imaging system was used to measure the fluorescence of invading cells. Immunestained cells were also counted under phase contrast objective microscope (10 X magnifications). The experiment was repeated at least three times independently. Data reported here is one representative experiment.

Small interfering RNA (siRNA) transfection

Small interfering RNA (siRNA) specific for Shh (SHH Stealth RNAiTM siRNA) was purchased from Invitrogen. As a nonspecific control siRNA, scrambled siRNA duplex was used which was also purchased from Invitrogen. Transfection was done using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) following the manufacturer's instruction. Shh was silenced by siRNA in A549-M, H1650 and H1299 for 48 hrs prior to assay or treatment. Experiment was repeated at least, three times independently. Data reported here is representative of an experiment.

Clonogenic assay

Cells were treated with TGF-β1 for 21 days, Hh inhibitor for three-72 hour treatments, or knock-down with siRNA specific for Shh (si-Shh) for 48 hours. Prior to treatment, cells were plated at a density of $1x10^3$ cells in 100-mm Petri

dishes. Then the cells were incubated for 10–14 days at 37^0C in a 5% CO_2/5% O_2/90% N_2 incubator. Next, colonies were stained with 2% crystal violet and quantified using NIH Image-J software. The experiment was repeated at least three times independently. Data reported here is representative of one experiment.

microRNA (miRNA) Expression Profiling Microarray

Small RNA was isolated with the mirVana-miRNA Isolation Kit. Expression levels of pooled miRNAs were measured by LC Sciences (Houston, TX, USA) using miRHuman_10.0_070802 miRNA array chip, based on Sanger miRBase Release 10.0 compared the miRNA expression of A549 samples to miRNA expression data for A549-M. Preliminary statistical analysis was performed by LC sciences on raw data normalized by Locally-weighted Regression (LOWESS) method on the background-subtracted data. Then, Student's t-test was performed to identify the different miRNA expression. MiRNAs with $P < 0.01$ was considered as having significant difference between.

Densitometric and statistical analysis

The optical densites of Smad and Psmad and GAPDH proteins on the films were quantified and analyzed with ImageJ software (NIH).

The ratios of Smad and PSmad against GAPDH were calculated. The cell growth inhibition by transfection or by GDC-09440 or Cyclopamine was statistically

evaluated using ImageJ software (NIH). Comparisons were made between control and transfection or treatment. The two-tailed v2 test was performed to determine the significance of the difference among the covariates. $P<0.05$ was used to indicate statistical significance.

CHAPTER 3

Up-regulation of Sonic Hedgehog contributes to Epithelial to Mesenchymal transition, and tumor progression of non-small lung cancer (NSCLC)

The work described in this chapter has been published

in the **PIoS ONE** (2011), 6(1): e16068.

Lung cancer as the leading cancer site in male covers 17% of total new cancer cases, and is the most common cause of cancer related deaths with 23% of the total cancer deaths. As indicated earlier, The American Cancer Society estimated that 222,520 Americans were diagnosed with lung cancer and 157,300 died of lung cancer in 2010 (Jemal et al. 2009, 225-249;Jemal et al. 2011, 69-90). Epithelial-to-mesenchymal transition phenomenon plays critical role in tumor invasion, metastatic dissemination and the acquisition of resistance to conventional therapies and this phenotype in cancers has been associated with poor clinical outcome in multiple cancer types including NSCLC (Baum, Settleman, and Quinlan 2008, 294-308;Hugo et al. 2007, 374-383;Mani et al. 2008, 704-715;Morel et al. 2008, e2888;Sabbah et al. 2008, 123-151;Thiery and Sleeman 2006, 131-142;Turley et al. 2008, 280-290). Interestingly, Hedgehog (Hh) signaling pathway is an important mediator of carcinogenesis and cancer metastases (Bailey, Singh, and Hollingsworth 2007, 829-839;Yoo et al. 2008, 480-490) and recent studies have shown that the acquisition of EMT phenotype

is consistent with up-regulation of marker SNAI1 (Snail-1) and PTCH1 mRNAs, and was induced after the induction of GLI1 expression (Katoh and Katoh 2008, 271-275). Moreover, Hh signaling was found to be essential for the maintenance of cancer stem cells (Zhao et al. 2009, 776-779). This evidence supports the role of Hh signaling in EMT and cancer metastasis. Inhibitors of the Hedgehog signaling pathways have shown some early promise in preclinical and clinical settings based on results showing that the inhibition of Hh signaling could inhibit cell growth, invasion, and metastasis of cancer cells (Dolgin 2011, 523;Feldmann et al. 2007, 2187-2196;Fukaya et al. 2006, 14-29;Ohta et al. 2009, 389-398).

Although epithelial-to-mesenchymal transition (EMT) phenomenon and hedgehog signaling pathway have been demonstrated to result in tumor metastases and invasiveness the exact role of Hh in EMT regulation in NSCLC still undefined. To test my hypothesis whether Hh is a positive regulator of EMT in NSCLC and whether Hh plays a role in EMT-induced tumor metastasis A549 and H2030 cells where exposted to TGF-β1 cytokine, especially because TGF-β1 is the most well known inducer of EMT, for both two weeks and three weeks. The chronic exposure was intended to recapitulate and mirror the *in vivo* circumstance where tumor cells are exposed chronically to cytokines in patients. Afterward, Hh signaling components expression were measured at mRNA and protein levels in A549, H2030, H1650, and H1299 cell lines. Furthermore, EMT markers were investigated in those cells, to confirm their EMT acquisition. In addition, inhibition of Shh signaling was inhibited concomitant with TGF-β

treatment, to investigate whether Shh is required for the EMT induction. Next, I investigated cellular assays such as invasion, migration, and tumorigenic ability of these cells before and after Hh inhibition. Tumor invasiveness was also measured *in vivo*.

Data showed for the first time that chronic exposure of A549 cells (NSCLC cells) to TGF-β1 lead to the acquisition of EMT phenotype concomitant with up-regulation of sonic hedgehog (Shh) both at the mRNA and at the protein levels, which was consistent with findings in another NSCLC cell line (H2030). The up-regulation of sonic hedgehog was consistent with increased cell motility, invasion, and tumor cell aggressiveness. Additionally, I found that this process was attenuated by Shh siRNA as well as by Hh signaling inhibitors such as cyclopamine and GDC-0449. Moreover, I found that the inhibition of Hh signaling by pharmacological inhibitors led to the reversal of EMT phenotype, as confirmed by the reduction of mesenchymal markers such as ZEB1 and Fibronectin, and induction of epithelial marker E-cadherin. This data clearly suggested that the acquisition of EMT phenotype by chronic exposue of NSCLC cells to TGF-β1 is mechanistically mediated by the activation of Shh signaling, especially because the knockdown of Shh by Shh specific siRNA attenuated TGF-β1-induced EMT phenotype which establishs a mechanistic role of Shh with EMT.

Induction of epithelial-to-mesenchymal transition (EMT) in A549 NSCLC cells by chronic exposure to TGF-β1:

It has been reported that A549 cells undergo EMT phenotypic changes upon exposure to TGF-β1 for 48-72 hrs (Kim et al. 2007, 898-904;Polyak and Weinberg 2009, 265-273). In an attempt to recapitulate the *in vivo* situation where cells are chronically exposed to TGF-β 1 in the tumor microenvironment, we exposed A549 cells to TGF-β 1 for up to three weeks. After 21 days of exposure to TGF-β 1, A549 cell's morphology was found to be completely changed to a mesenchymal phenotype (we named these cells as A549-M cells), with an elongated and disseminated appearance (Fig. 5A). I confirmed the acquisition of mesenchymal phenotype by assessing EMT molecular markers and ZEB1 mRNA, which was previously reported as a molecular marker of EMT (Sanchez-Tillo et al. 2010, 3490-3500;Singh and Settleman 2010, 4741-4751), and I found that ZEB1 was up-regulated while the expression of E-cadherin mRNA, an epithelial marker, was down-regulated (Fig. 5B). Fibronectin protein, a mesenchymal marker (Thomson et al. 2005, 9455-9462) was also found to be highly up-regulated in A549-M cells (Fig. 5C) - all of which is consistent with the acquisition of EMT phenotype.

A549-M cells showed significant increase in cell migration and invasive characteristics compared to the A549 parental cells *in vitro* and *in vivo:*

Previous studies have shown that tumor cells with EMT phenotype aquire invasiveness (Kim et al. 2007, 898-904;Singh and Settleman 2010, 4741-4751;Thomson et al. 2005, 9455-9462;Tsuji, Ibaragi, and Hu 2009, 7135-7139).

To characterize A549-M cells a wound healing assay was performed. This showed an increase in cell migration of A549-M cells compared to parental cells (Fig. 6A). In addition, matrigel-coated chamber assay and clonogenic growth assay showed that A549-M cells acquired more invasive and tumorigenic characteristics compared to parental A549 cells (Fig. 6B, 6C). Furthermore, both cell lines were injected through the tail vein of NOD/SCID mice where A549-M cells showed enormous increase in the development of tumor foci compared to A549 cells (Fig. 7) suggesting that EMT phenotypic cells acquired a mesenchymal phenotype with increased cell motility and invasiveness both *in vitro* and *in vivo*.

A549-M cells showed up-regulation of sonic hedgehog mRNA, and protein expression:

Since Hh signaling has been implicated in EMT induction, metastasis and invasion (Bailey, Singh, and Hollingsworth 2007, 829-839;Katoh and Katoh 2008, 271-275;Syn et al. 2009, 1478-1488;Watkins et al. 2003, 313-317;Yoo et al. 2008, 480-490;Yuan et al. 2007, 1046-1055) I sought to evaluate role of Hh signaling pathway in TGF-β1-induced EMT and characterize tumor cell aggressiveness. Interestingly, there was a dramatic increase in the expression of Hh pathway ligand Shh both at the mRNA and protein levels in A549-M cells compared to parental A549 cells. Parental A549 cells showed undetectable levels of Shh mRNA (Fig. 8A and Fig. 8B), consistent with previously published

data (Sato, Leopold, and Crystal 1999, 855-864). Furthermore, I confirmed the up-regulation of Shh by TGF-β 1 treatment and the induction of EMT in another NSCLC cell lines, H2030. H2030 cell line was treated with TGF-β 1 for two weeks, and I found a significant increase in the expression of Shh mRNA, which was consistent with the induction of EMT marker ZEB1 and down-regulation of epithelial marker E-cadherin (Fig. 9A). These results suggest that TGF-β 1-induced EMT is associated with transcriptional up-regulation of Shh. This novel finding is the first such report in the literature. Surprisingly, GLI1 levels in both A549-M and parental cells were higher compared to normal human bronchial epithelial cells (NHBE cells) although the expression of GLI1 was much more increased in A549-M cells (Fig. 8C and Fig. 8D). The high levels of Hh signaling target gene GLI1 in both A549-M and A549 cells despite the undetectable levels of Shh in A549 parental cells, implies that the expression of GLI1 could be ligand independent in the parental A549 cells. Moreover, to investigate the possiblitiy of GLI1 expression via autocrine or juxtacrine mechanisms, I cultured NIH-3T3 cells, mouse fibroblast cells, with conditioned medium collected from A549-M cells or A549 parental cells. Data showed increased Hh signaling, which was consistent with increased expression of GLI1 (Fig. 8E) in NIH-3T3 cells cultured in the presence of conditioned medium collected from A549-M but not from the parental A549 cells. These results suggest that A549-M cells secrete active Shh, which is responsible for the activation of Hh signaling (activation of GLI1) in NIH 3T3 cells, resulting in the activation of GLI1.

Shh up-regulation is required in EMT induction by TGF-β1:

I further investigated the on the mechanism in which Shh up-regulation mediates EMT induction after TGF-β1 treatment. Knock-down of Shh by siRNA significantly attenuated TGF-β1 induced EMT as confirmed morphologically and molecularly. The data is as follows: A549 cells transfected with Shh siRNA (A549-siShh) 24 hrs prior to treatment with TGF-β1 for 48 hours maintained epithelial morphology, while scrambled siRNA (A549-si-ve) showed transformation to mesenchymal morphology (Fig. 9B left panel) as expected. Likewise, scrambled siRNA (A549-si-ve) showed more EMT induction following re-transfection with Shh siRNA and treatment with TGF-β1 compared to Shh siRNA (A549-siShh) [Total six days of Shh siRNA transfection and five days of TGF-β1 treatment; please follow details under figure legend (Fig. 9B right panel]. The Shh siRNA trasfection resulted in a significant knock-down of Shh expression as shown by qRT-PCR (Fig. 4C). These results are consistent with significant attenuation in the induction of EMT markers subsequent to TGF-β1 treatment. A549 cells with scrambled siRNA transfection showed down-regulation of epithelial marker, E-cadherin and significant induction of ZEB1 expression, as expected (Fig. 9D, 48 hrs TGF-β1), whereas TGF-β1 failed to show any effect on these markers in A549 with Shh knock-down (A549-siShh), suggesting the mechanistic role of Shh in the induction of EMT induced by TGF-

β1. Moreover, further attenuation in the induction of EMT following second round of Shh siRNA transfection and TGF-β1 treatment was observed. A549-si-ve cells showed a significant increase in ZEB1 expression consistent with significant down-regulation of E-cadherin (Fig. 4D, 5 days TGF-β1) whereas TGF-β1 failed to show any effect on these markers in scrambled A549-si Shh cells. These results demonstrated for the first time that Shh up-regulation by TGF-β1 is required for TGF-β1 induced EMT in NSCLC cells.

Up-regulation of Shh in A549-M cells contributes to EMT-induced tumor cell migration and metastatic characteristics:

Next, I investigated the role of increased expression of Shh in the aggressive behavior of tumor cells such as cell migration and metastatic potential in A549-M cells. After treating A549-M cells with Smoothened inhibitors such as cyclopamine or GDC-0449, A549-M cells showed significant reduction in cell migration and invasion capacity (Fig. 10A–C and Fig. 14). To further confirm the role of up-regulated Shh in A549-M cells after TGF-β1 treatment, I assessed the cells apptidtude for cell migration, invasion and tumorigenesis. We initially assessed the transfection efficiency of cells after transfection with Shh-specific siRNA, and we found high transfection efficiency (Fig. 11A) and were associated with knock-down of Shh protein in these cells. The knock-down of Shh protein in A549-M cells also demonstrated significant reduction in cell migration, invasion,

and tumorigenic characteristics (Fig. 11B–D). These data further confirmed that the inhibition in cell migration, invasion, and tumorigenic potential of A549-M cells is mechanistically mediated through the inhibition of Shh autocrine signaling.

Down-regulation of Shh autocrine signaling in other NSCLC cell lines lead to the reduction in tumor cell migration, invasion, and tumorigenic characteristics:

I also further investigated the role of Shh autocrine signaling inhibition in the reduction in cell migration, invasion, and tumorigenic potential in other NSCLC cell lines that endogenously express Shh. For this purpose, I chose H1299 and H1650 cell lines, both of which were derived from lung metastasis of NSCLC patients. Both H1299 and H1650 cell lines showed resistance to chemotherapy and targeted therapy, e.g. Erlotinib (Johansson et al. 2010, 383-391;Teraishi et al. 2005, 6681-6687;Thomson et al. 2005, 9455-9462). Results confirmed that both the cell lines expressed Shh as documented by qRT-PCR and Western blot analysis (Fig. 12A). Treatment of both cell lines with Shh inhibitors GDC-0449 or knock-down of Shh showed decrease in cell migration, invasion and tumorigenic characteristics (Fig. 12B–C and Fig. 14, Fig. 13B-C and Fig 15A-B). These results clearly provide strong experimental evidence in support of the key-role of Shh in the induction of EMT phenotype associated with tumor cell aggressiveness.

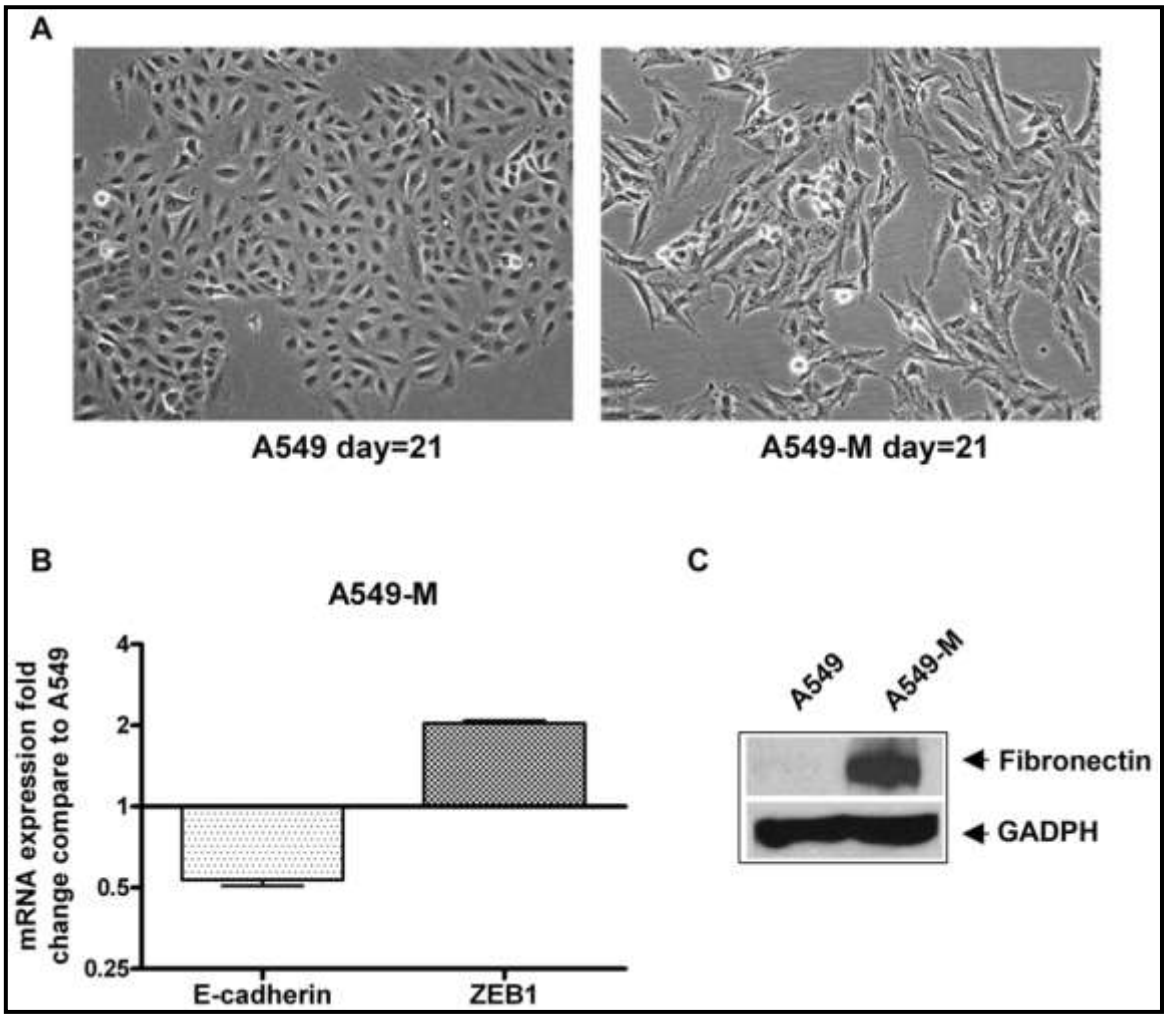

Figure 5. Induction of epithelial to mesenchymal transition (EMT) in A549 cells by chronic exposure to TGF-β1: TGF-β1 was added to A549 cells in culture media and maintained for 21 days with changing medium every third day with freshly added TGF-β1. A) Phase contrast microscopic pictures at 10X magnifications. A549 cells morphology changed to mesenchymal phenotype (A549-M cells). Cell shape appears elongated and non-polarized. B) qRT-PCR of A549 and A549-M cells. A549-M cells showed lower expression of E-cadherin ‘‘epithelial marker,’’ and a higher expression of ZEB1 ‘‘EMT marker’’, at the mRNA levels. Delta-delta-CT was calculated, considering GAPDH as internal control and A549 parental as reference control. C) Western blot analysis where A549-M cells showed up-regulation of fibronectin which is a ‘‘mesenchymal’’ marker compared to A549 parental cells.

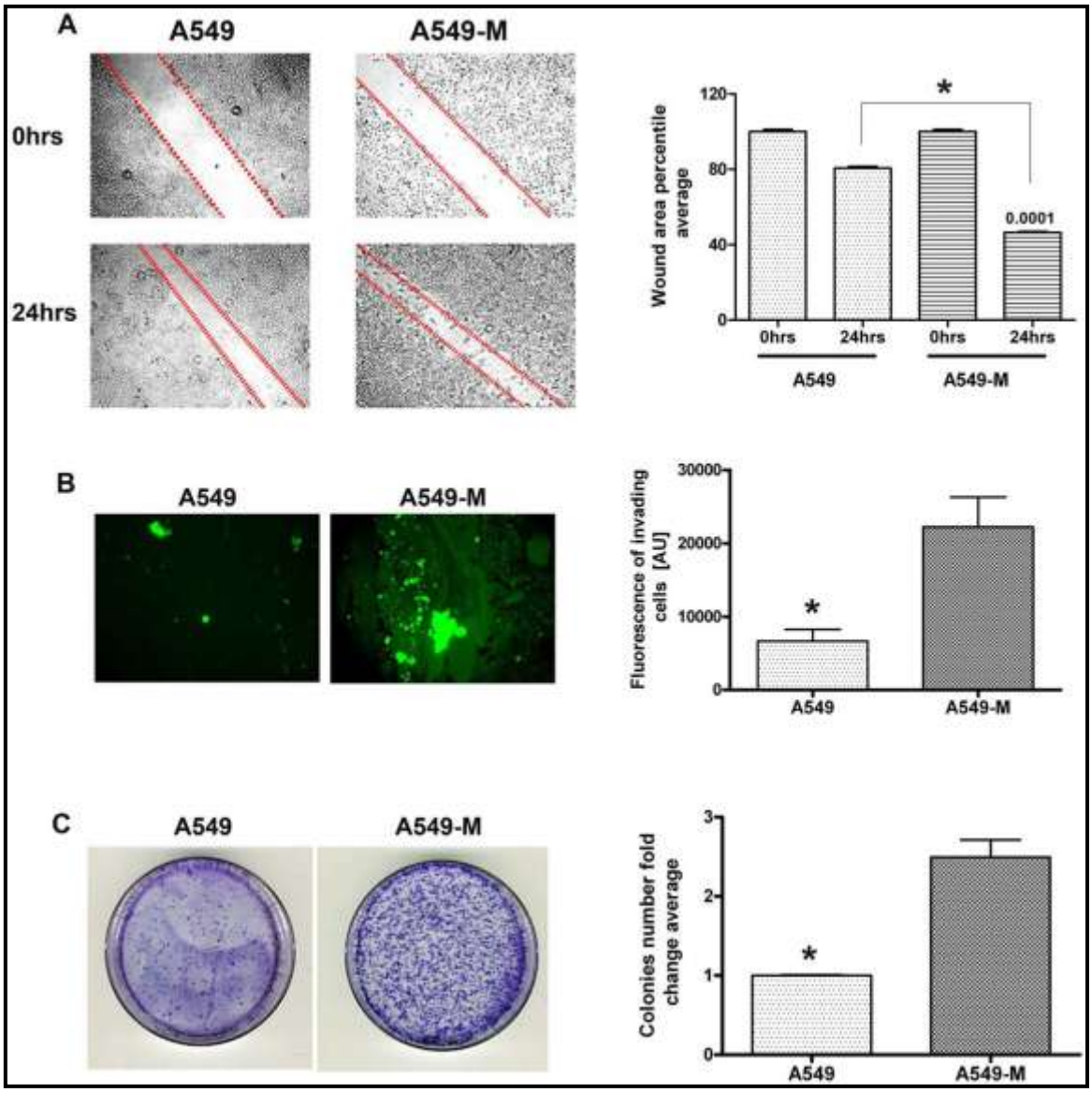

Figure 6. A549-M cells showed significant increase in cell migration, invasive, and tumorigenic characteristics compared to A549 parental cells: TGF-β1-induced EMT phenotypic cells (A549-M cells) were generated as discussed under ‘‘Materials and Methods’’ section. A: showed wound healing assay results with its quantitative analysis. A549-M cells showed much higher motility compared to A549 parental cells. B and C are showing the results of matrigel-coated membrane and colony formation assays, respectively with its quantitative analysis. Significant increase was observed in the invasion and clonogenicity of A549-M cells compared to parental A549 cells. (* = $p < 0.05$).

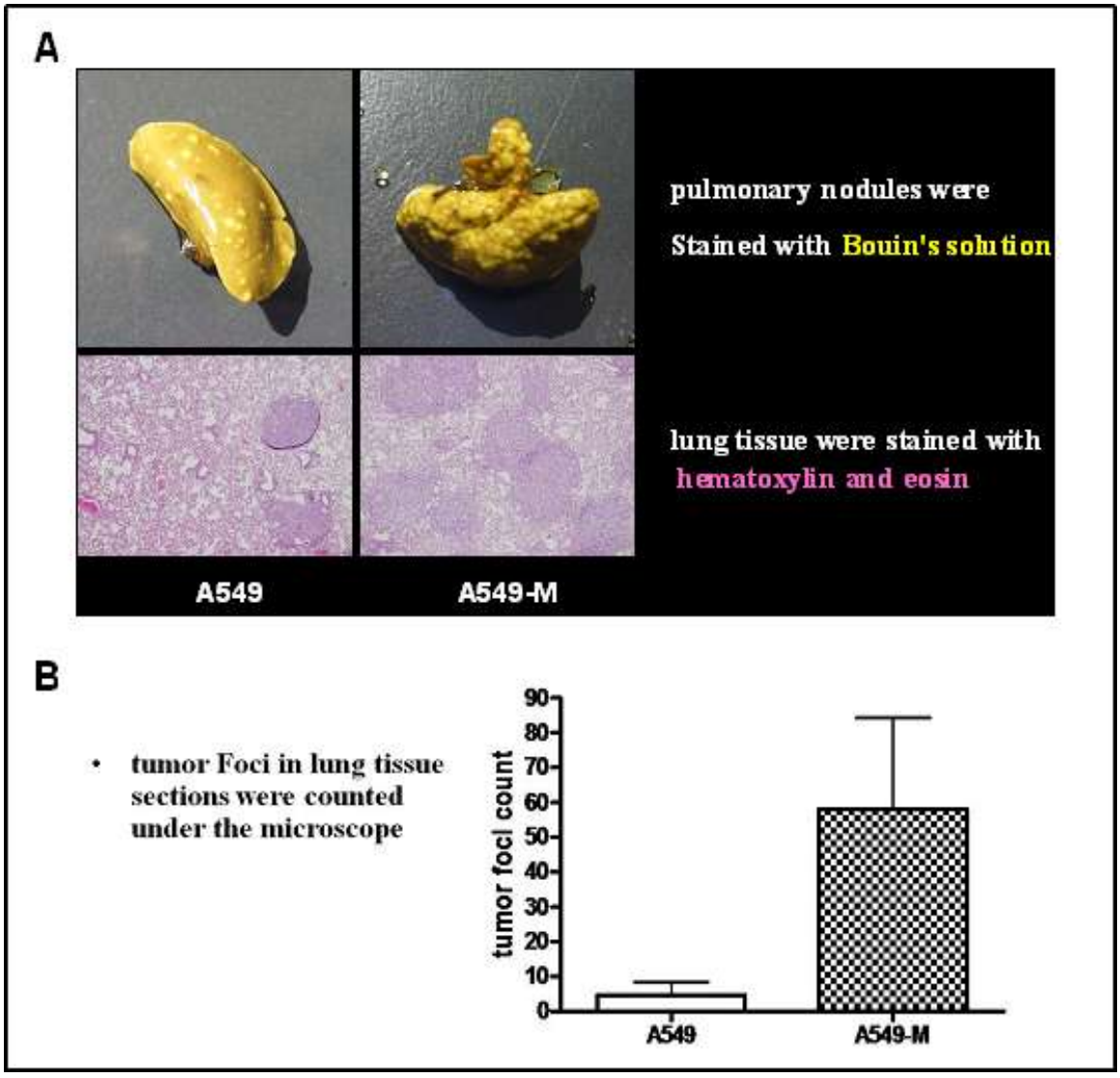

Figure 7. A549-M aquired remarkably increased invassivness *in vivo*: A549 and A549-M cell lines were injected through the tail vein of NOD/SCID mice, tumors were harvested from the lung tissue. A549-M cells showed drastic increase in the development of tumor foci compared to A549 cells. A, lung tissue photographs after 6 weeks of tumor cell injection, top panel, tissue fixed with bouin's solution, bottom panel tissue crosssection stained with H&E. B, plot of tumor foci count of H&E stained slides under microscope-4X, tumor foci count in slides of three animals of each group after six weeks, and +/-SD represent the error bars.

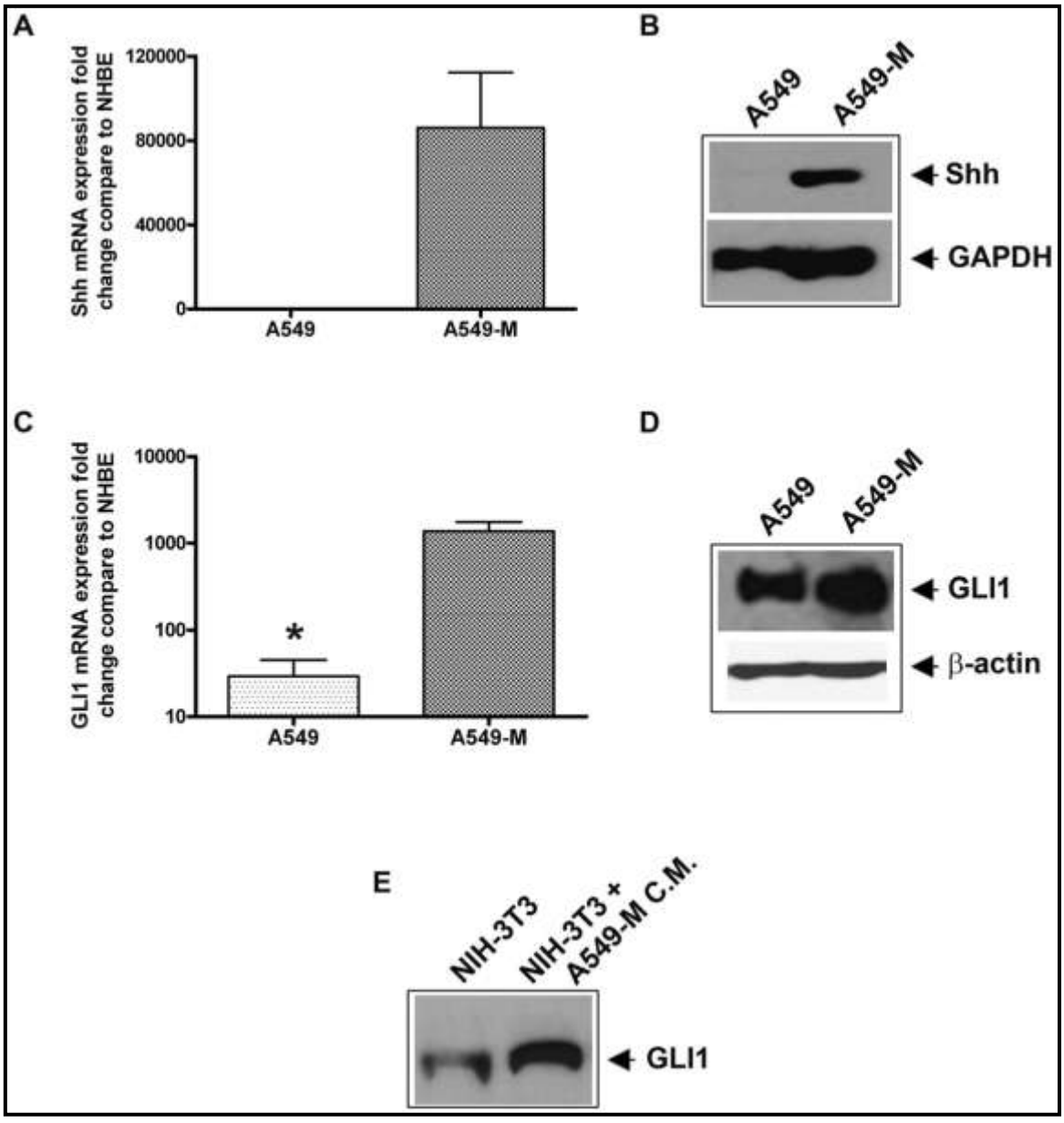

Figure 8. A549-M cells showed up-regulation in the expression of sonic hedgehog (Shh) and GLI expression both at the mRNA and protein levels: A and B showing qRT-PCR and Western blot results, respectively for the expression of Shh whereas C and D represent the expression status of GLI at the mRNA and protein levels, respectively in A549-M cells compared to parental A549 cells. E represent Western blot data of GLI1 expression in NIH-3T3 cell after culturing them in the presence of conditioned medium collected from A549-M cells showing higher levels of GLI1 expression. (* = $p>0.05$).

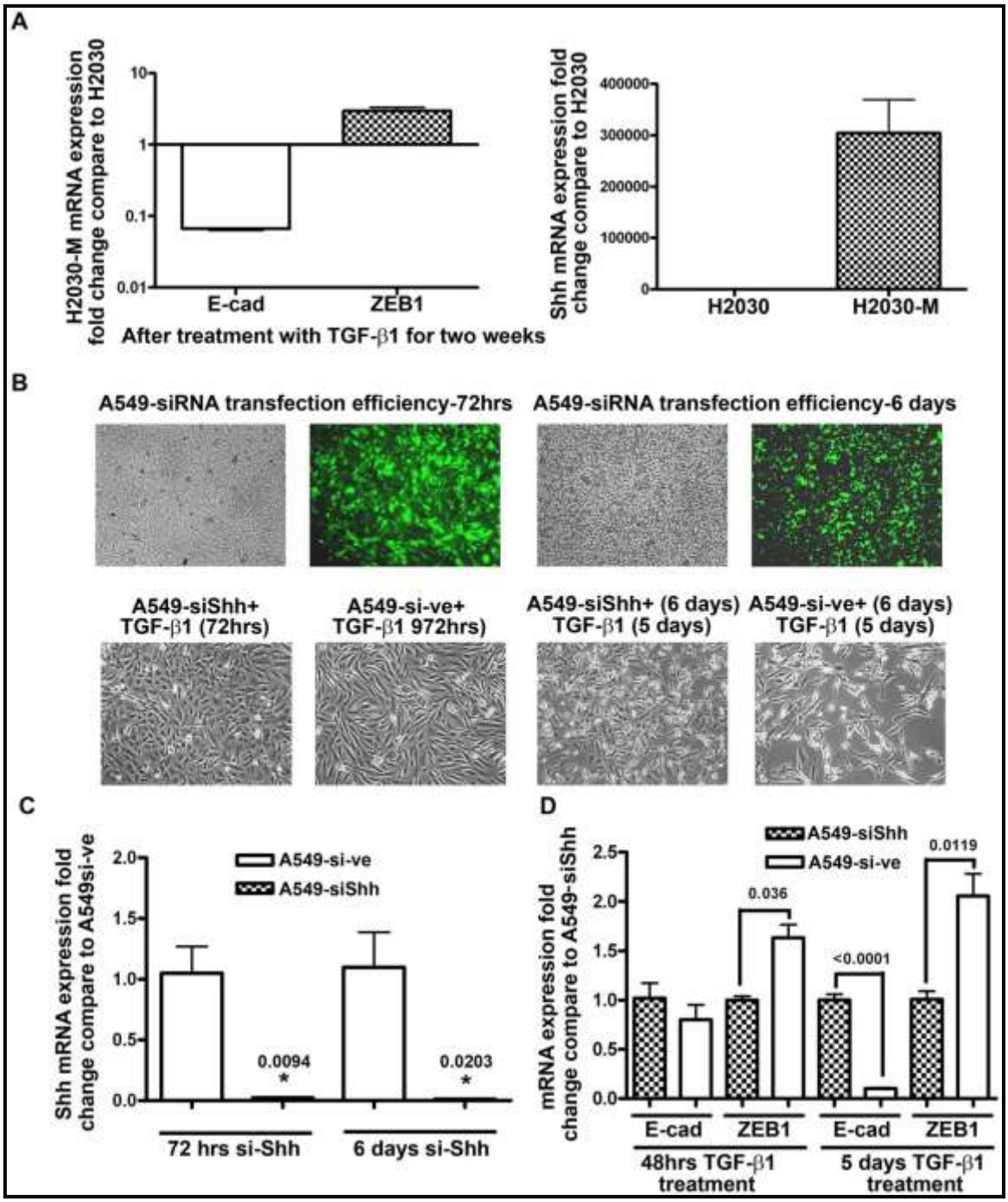

Figure 9. Shh up-regulation was concomitant with TGF-β1-induced EMT in NSCLC cell lines. The up-regulation of Shh contributes to the EMT induction through TGF-β1. (A) H2030 cell line was treated with TGF- β1 (5 ng/ml) for two

weeks, and the media was changed every three days. The qRT-PCR data showed induction in the expression of EMT marker ZEB1 mRNA, and reduced expression of epithelial marker E-cadherin mRNA, which was consistent with up-regulation of Shh mRNA similar to those observed in A549 cells exposed to TGF-β1. (B, C and D) A549 cells was transfected with Shh siRNA (A549-siShh) or scrambled siRNA (A549-si-ve) for 24 hrs prior to treatment with TGF-β1 (5 ng/ml) for 48 hrs, then the cells where collected for assays or re-transfected for the second time with siRNA or scrambled siRNA for 24 hrs (total 6-days after siShh transfection) prior to the second time treatment with TGF-β1 (5 ng/ml) for another 48 hrs (total 5-days of TGF-β1 treatment). (B) Upper panel shows transfection efficiency, and lower panel shows cellular morphology following treatments. A549-siShh maintained epithelial morphology after treatment with TGF-β1 at both time points as shown in left and right panels, respectively. (C) qRT-PCR expression of Shh mRNA showing significant down-regulation following Shh siRNA transfection. (D) qRT-PCR expression of ZEB1 and E-cadherin mRNA. A549-si-ve cells showed down-regulation of epithelial marker, E-cadherin consistent with significant induction in the expression of ZEB1 as expected whereas TGF-β1 failed to show any effect on these markers in A549-siShh cells.

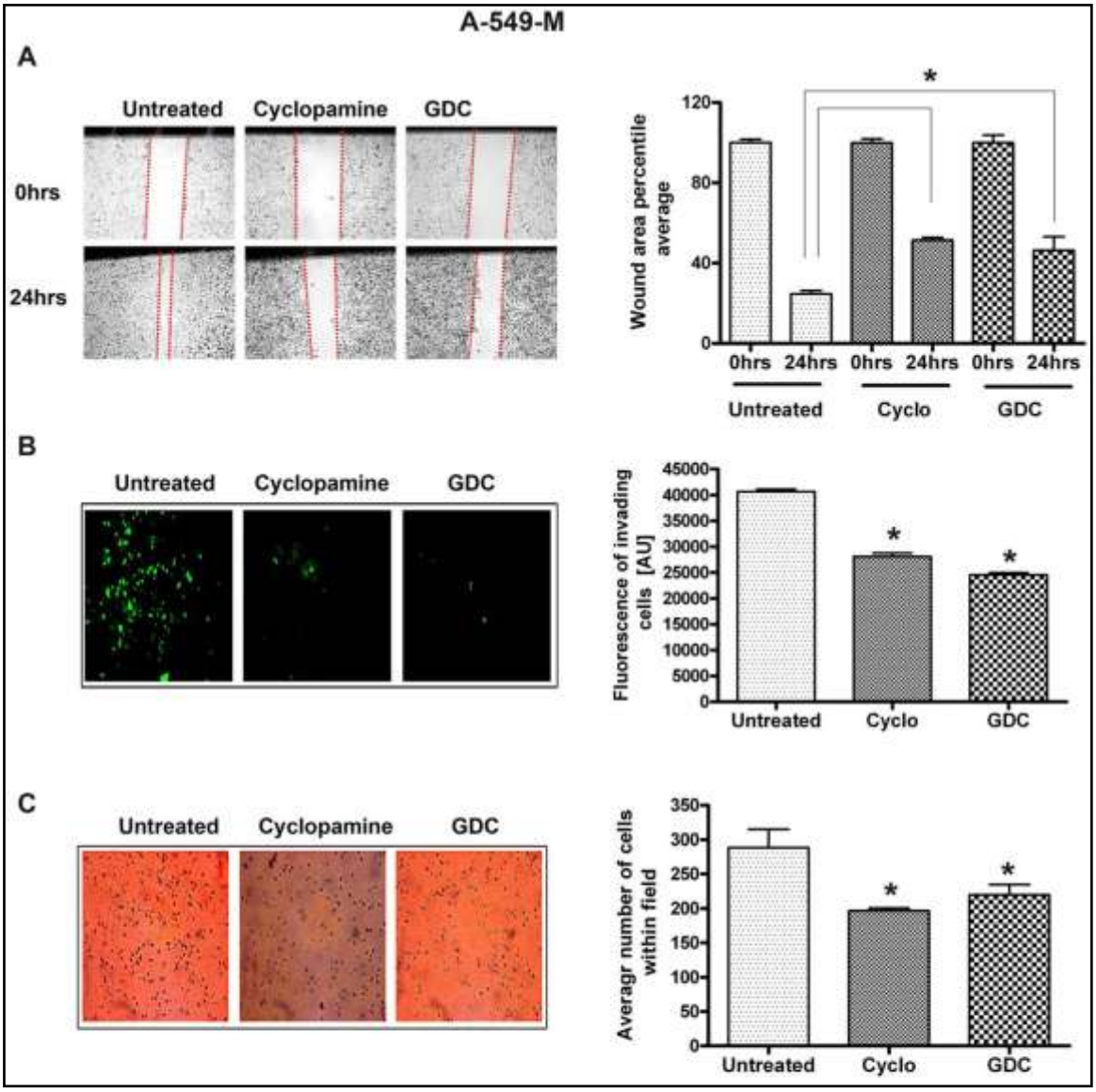

Figure 10. Up-regulation of Shh in A549-M cells contributes to increased tumor cells migration and metastatic characteristics: A549-M cells were treated with Shh inhibitors such as Cyclopamine (2 mM) and GDC-0449 (20 nM) and assayed for wound healing (A), invasion (B) and clonogenic growth (C), and performed quantitative analysis showing attenuation of invasion by the treatment of cells with Shh inhibitors.

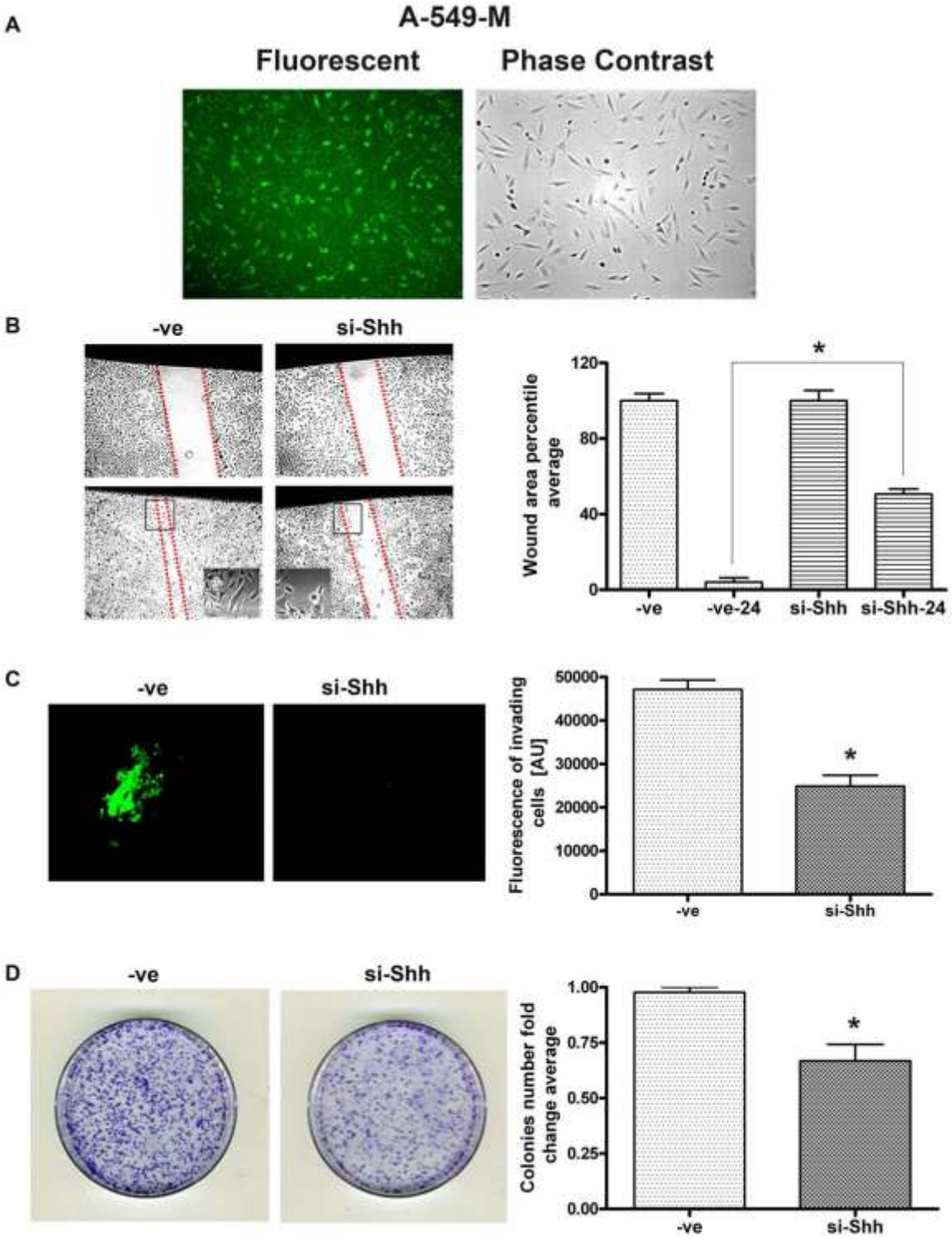

Figure 11. Reduction in A549-M cells motility, invasiveness, and tumorigenesis by specific knock-down of Shh using Shh-specific siRNA:

A549-M cells were transfected with Shh-specific siRNA (A): Transfection efficiency as assessed by GFP. The effect of knock-down of Shh was assessed by cell motility (wound healing) (B), invasion (C) and clonogenic growth (D) and further quantitated as detailed under ‘‘Materials and Methods’’ section. The results are showing a significant inhibition by Shh specific siRNA. (* = $p>0.05$).

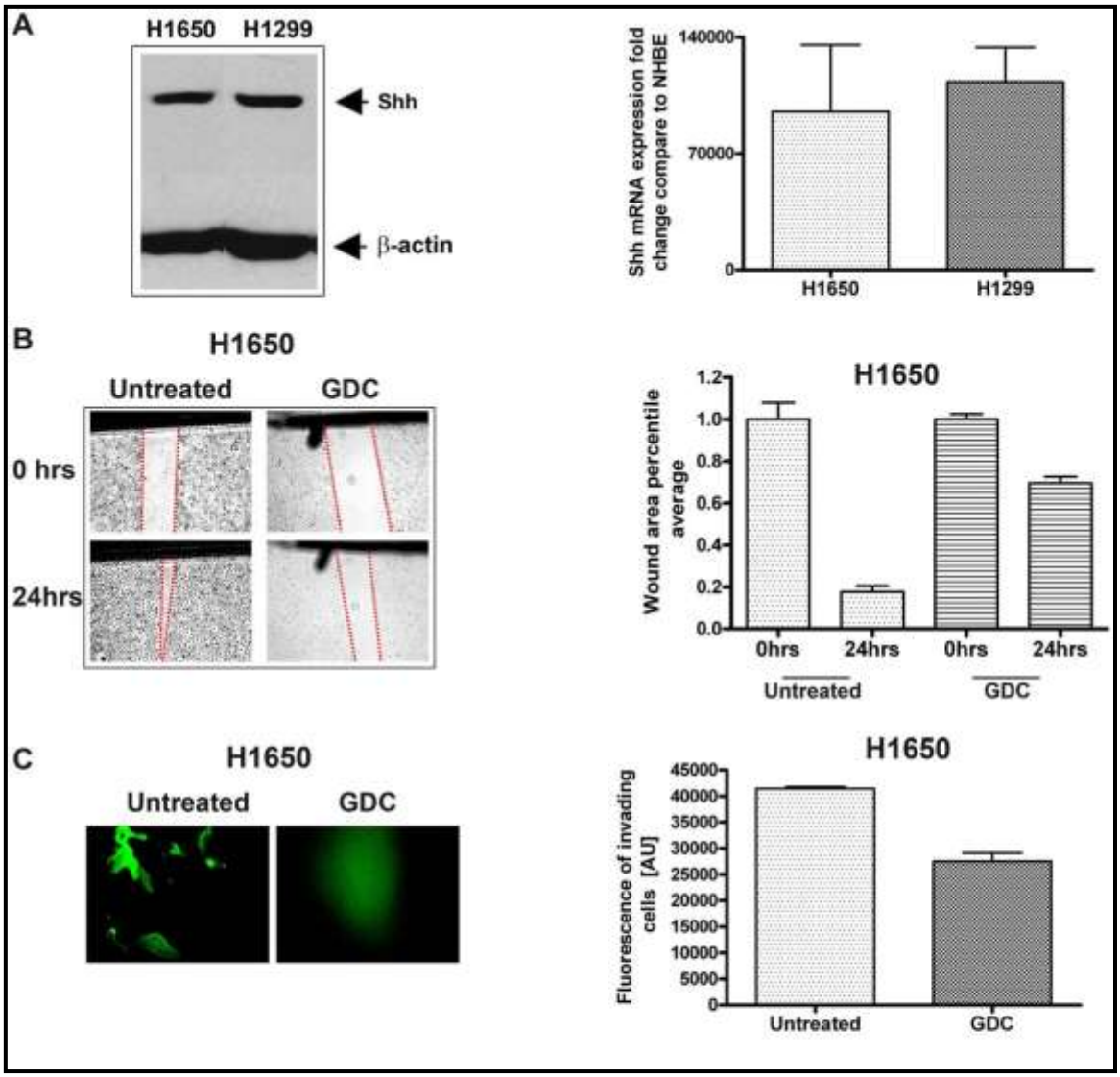

Figure 12. Down-regulation of Shh autocrine signaling in NSCLC cell lines led to the reduction in tumor cell migration, invasion, and tumorigenesis: A; both H1650 and H1299 cells expresses high levels of Shh mRNA compared to NHBE cells, and both cell lines have high Shh protein expression. B and C shows reduction in cell invasion and the colony-forming ability of H1650 cells following treatment with Shh inhibitors such as GDC-0449 (20 nM).

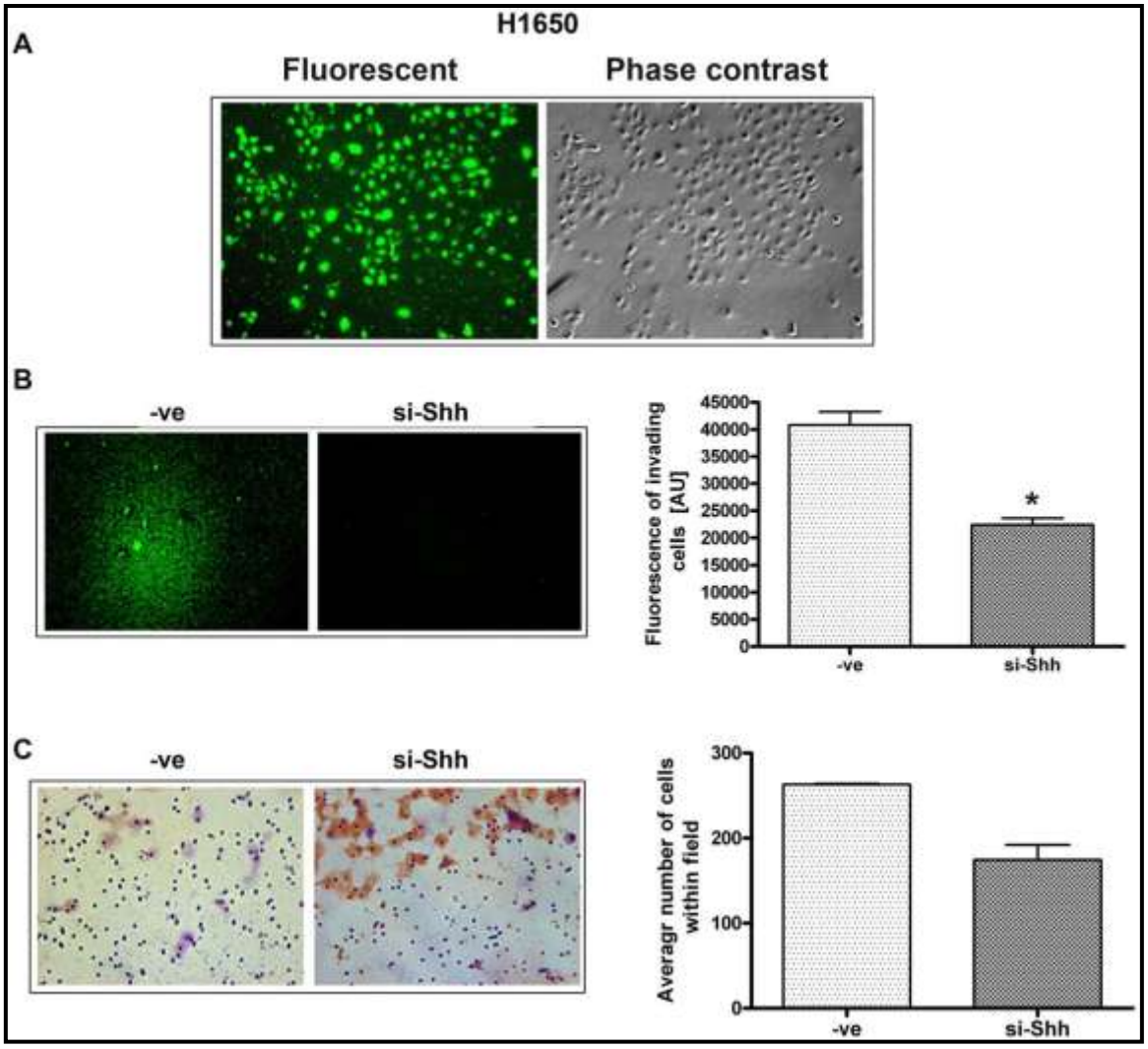

Figure 13. Down-regulation of Shh signaling in NSCLC cells lines (H1650 cells) led to reduced cell motility and invasion. (A): Transfection efficiency was assessed by GFP. (B) Matrigel-Coated membrane assay where cells were labeled with DiIC12 fluorescent dye. (C) Matrigel-Coated membrane assay where cells were labeled with immune-staining kit (Quik staining kit). (B and C right panel) also show quantitative data analysis. (*= p>0.05).

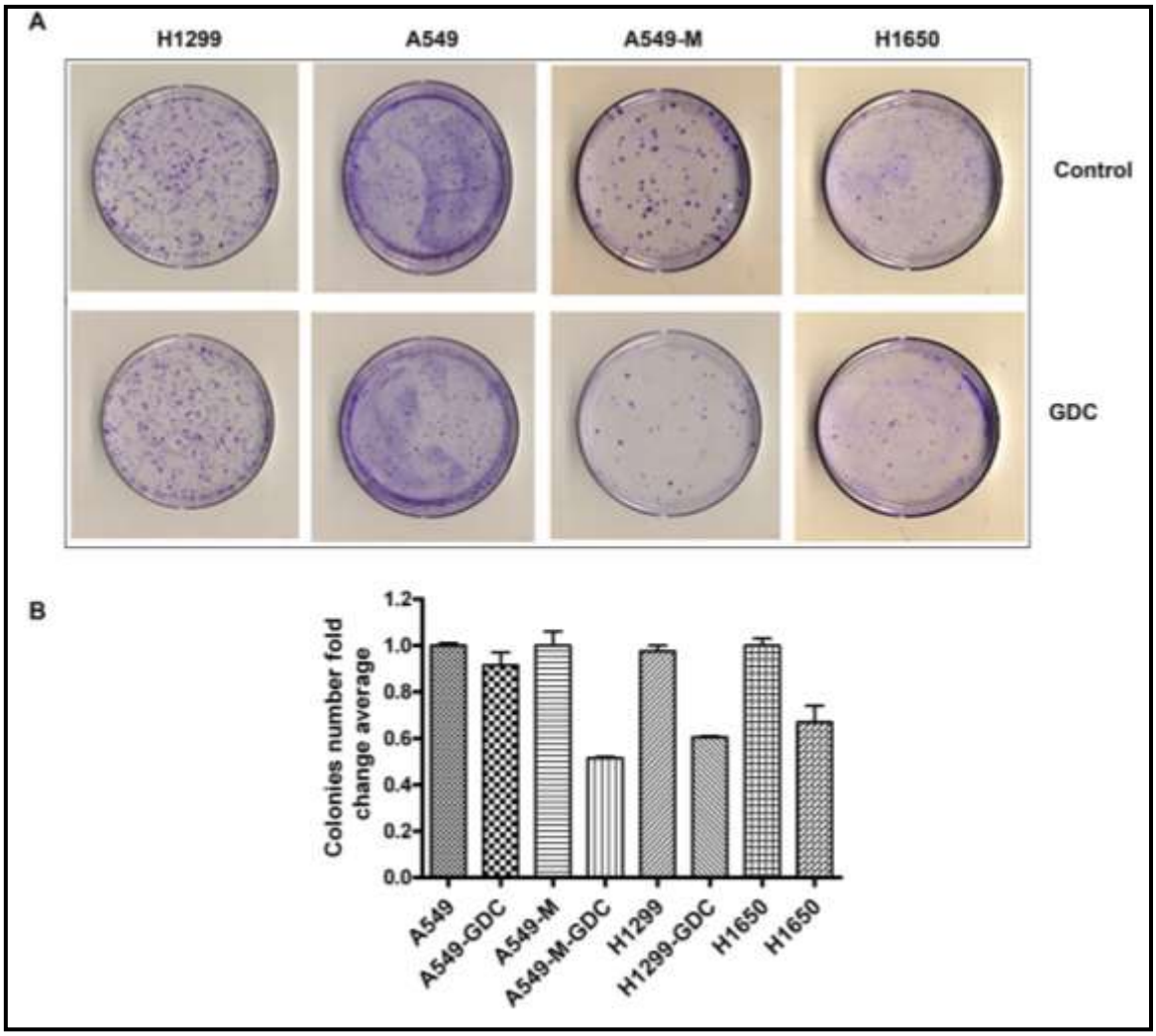

Figure 14. Shh signaling inhibition decreases tumorigenic potential of NSCLC cells: (A) clonogenic growth assay of three parental NSCLC cell lines was compared to A549-M cells before and after treatment with Hh inhibitor GDC-0449 (20 nM), and (B) represent quantitative data analysis of the data presented in panel-A (* = $p>0.05$).

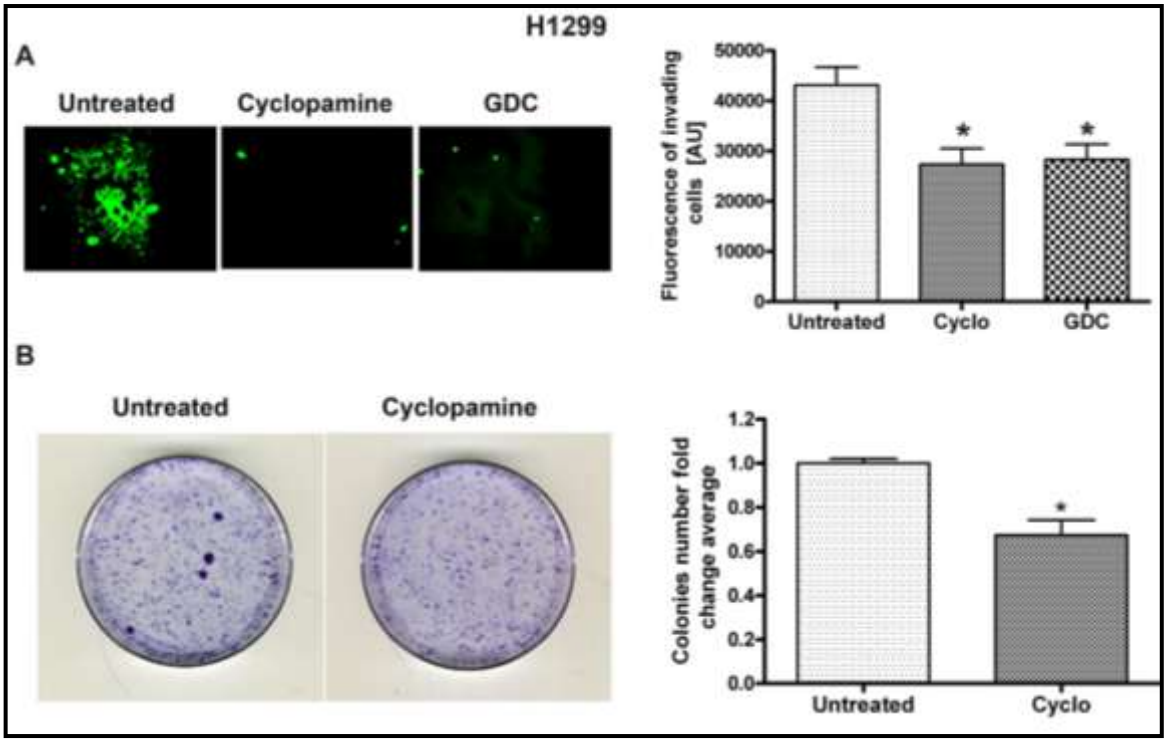

Figure 15. Inactivation of Shh signaling by cyclopamine and GDC-0449 (20 nM) led to the reduction in tumor cell invasion (A), and tumorigenic characteristics (B) of H1299 NSCLC cell line. Right panel shows quantitative analysis. (* = $p>0.05$).

CHAPTER 4

TGF-β1 up-regulated sonic hedgehog in EMT-undergoing cells though activation of NF-κB, and microRNA (miRNA) machinery in NSCLC

As indicated earlier that lung is the leading cancer site in male covers 17% of the total new cancer cases, and the most common cause of cancer related deaths with 23% of the total cancer deaths. According to The American Cancer Society estimates documenting that 222,520 Americans were diagnosed with lung cancer and 157,300 died of lung cancer in 2010 (Jemal et al. 2009, 225-249;Jemal et al. 2011, 69-90). The Epithelial-to- mesenchymal transition (EMT) phenomenon plays a critical role in tumor invasion, metastatic dissemination and the acquisition of resistance to conventional therapies, and the presence of EMT phenotype in cancers has been associated with poor clinical outcome in multiple cancer types including NSCLC (Baum, Settleman, and Quinlan 2008, 294-308;Hugo et al. 2007, 374-383;Mani et al. 2008, 704-715;Morel et al. 2008, e2888;Sabbah et al. 2008, 123-151;Thiery and Sleeman 2006, 131-142;Turley et al. 2008, 280-290). Interestingly, Hedgehog (Hh) signaling pathway is an important mediator of carcinogenesis and cancer metastases (Bailey, Singh, and Hollingsworth 2007, 829-839;Yoo et al. 2008, 480-490), and transforming growth factor-beta (TGF-β) is the most important inducer of EMT (Kalluri and Weinberg 2009, 1420-1428). We previously reported the up-regulation of sonic Hedgehog

(Shh) following treatment with TGF-β1 was a prerequisite for the induction of EMT. However, the extact mechanism of TGF-β-Shh up-regulation is not clear. Understanding of this process would therefore lead to specific therapeutic treatments in a subset of NSCLC patients. Thus, I focused my investigation on understanding the molecular mechanisms involving up-regulation of Shh gene expression in NSCLC following TGF-β1 treatment. I have characterized human Shh promoter and its activation. Using combination of electrophoretic mobility shift assay (EMSA), site-directed mutagenesis, luciferase reporter transient transfection, and chromatin immunoprecipitation assays, I found the the activation of NF-κB in A549-M cells (A549 cells following treatment with TGF-β1), which was consistent with increased expression of Shh. Furthermore, I investigated the posttranscriptional regulation of Shh by microRNAs (miRNA), well known negative regulators of gene expression at the posttranscriptional level, which are involved in tumorigenesis (Cho 2007, 60;Krutovskikh and Herceg 2010, 894-904;Mocellin, Pasquali, and Pilati 2009, 70-80). To address this question, I performed miRNA microarray using total RNA harvested from A549 and A549-M cells. Two miRNAs, miR-15a and miR-16, were found to be down-regulated in A549-M cells compared to A549 cells. Both, miR-15a and miR-16 are located at chromosome 13q14, and have been implicated in cell cycle control and apoptosis. Both are frequently deleted or down-regulated in squamous cell carcinomas and adenocarcinomas of the lung (Bandi et al. 2009, 5553-5559;Palamarchuk et al. 2010, 3916-3922). Interestingly, in computational

analysis of miRNA profiles and their targets, using UCSC, TargetScan and PITA to predict potential target genes of miR15a and miR-16, Shh **3'** untranslated regions (3' UTRs) was identified as a target gene. This combination of miRNA microarray and miRNA target gene data clearly show a consistent correlation with up-regulation in the expression of Shh gene and down-regulation of miR-15a and miR-16, suggesting that Shh up-regulation in A549-M is partly regulated by miR-15a and miR-16. These findings could help in the development of new therapeutic strategies that would allow re-expression of the lost miRNA such as miR-15a and miR-16, suggesting that further in-depth research is warranted.

Identification of TGF-β1 downstream regulatory molecule:

TGF-β has been reported to induce NF-κB translocation to the nuclei in activity-reporter assays (Arsura et al. 2003, 412-425;Gingery et al. 2008, 2725-2738;Yu et al. 2010, 869-878). Chow et al., found that TGF-β suppresses PTEN in pancreatic cancer cells through NF-κB activation and enhances cell motility and invasiveness in a Smad4-independent manner that can be counteracted when TGF-β-Smad2/3 signaling is restored. These results suggest that the activation of NF-κB by Smad2 and/or Smad3, and not Smad-4 (Chow et al. 2010, G275-G282). Furthermore, TGF-β ligand TGF-β1 activates NF-κB in human osteoclast, and promotes osteoclast survival. Furthermore, TGF-β1 transiently inhibits apoptosis in liver tumor through activation of NF-κB (Arsura et al. 2003,

412-425;Gingery et al. 2008, 2725-2738;Yu et al. 2010, 869-878), suggesting that TGF-β1 activates NF-κB in cancer cells.

To investigate whether TGF-β1 chronic treatment activates NF-κB in A549-M cells, DNA-binding activity using an authentic NF-κB–binding DNA was assessed (Gupta et al. 2005, 125-143;Libermann and Baltimore 1990, 2327-2334) as shown in figure 16A. A549-M cells showed greater activation in the DNA-binding activity of NF-κB compared to parental A549 cells, suggesting the induction of NF-κB activity following TGF-β1 treatment.

DNA-binding activity of NF-κB in the consensus sites in the 5' upstream sequences in the Shh promoter:

It has been reported earlier that monocytes stimulated by inflammation produce Shh through activation of NF-κB signaling pathway (Yamasaki et al. 2010, 675-686). Moreover, in pancreatic cancer specimens, NF-κB was found to activate the hedgehog signaling pathway via induction of sonic hedgehog expression (Nakashima et al. 2006, 7041-7049), and it was further shown that NF-κB binds to Shh promoter regions prior to the up-regulation of Shh expression. These authors have found that NF-κB activation results in increased Shh mRNA and protein expression *in vitro,* and *in vivo* in an inducible NF-κB activity mouse model. Inhibition of NF-κB by super-repressor (inhibitory NF-κBalpha; IκBα) or by p65 knock-down showed inhibition in the induction of Shh

promoter activation and Shh expression, suggesting that Shh expression is regulated by NF-κB, and that NF-κB-induced Shh is involved in the NF-κB-mediated cells proliferation and resistance to apoptosis, both *in vitro* and *in vivo*. In addition, the NF-κB binding site at position +139 is required for NF-κB-mediated up-regulation of Shh transcriptional activity (Fig. 16B) (Kasperczyk et al. 2009, 21-33). To investigate whether NF-κB binds to 5' upstream region in the Shh promoter, nuclear extracts obtained from A549 or A549-M cells were mixed with infrared-labeled oligonucleotides specific for the concensus NF-κB binding site (Fig. 16B), or the mutated oligonucleotide, and the resulting NF-κB binding complexes were resolved through EMSA. Interestingly, putative NF-κB binding sites within the human Shh promoter region formed NF-κB complexes, and further showed that NF-κB complexes have the lowest binding activity to mutant three (MUT-3) of Shh promoter region (Fig. 13A). To examine the specificity of NF-κB DNA binding complexes, I performed EMSA supershift experiments. Antibodies recognizing p65 subunits caused a significant supershift of NF-κB DNA binding complexes using authentic NF-κB oligonucleotide bound to the Shh promoter (Fig.17B).

Next, I performed ChIP experiments using nuclear extracts from A549 cells in order to investigate whether NF-κB binds to the Shh promoter *in vivo* following TGF-β1 treatment, using an antibody to p65 subunit of NF-κB. TGF-β1 stimulated NF-κB activation in A549 cells resulted in increased binding of p65 to

the Shh promoter region (Fig. 18). Together, this set of experimental data clearly suggests that NF-κB binds to the Shh promoter following treatment with TGF-β1 resulting in the induction of Shh expression. This is more direct evidence establishing the the role of NF-κB in the activation of Shh during TGF-β1-induced acquisition of EMT phenotype in NSCLC cells compared to the loss of miR-15a and miR-16 which also contributes to the induction of EMT.

Transcriptional activation of the Shh promoter by NF-κB:

Next, to address the question whether NF-κB transcriptionally activates the Shh promoter, a 5000-bp fragment of DNA that contains the human Shh promoter and 5' upstream region with the +139 putative NF-κB binding sites, was fused to upstream Renilla luciferase gene. This construct was obtained from Switchgear genomics. I employed site-directed mutagenesis to generate mutants in the putative NF-κB binding region, with the same mutation as mutant three of the oligonucleotide experiment as presented Fig. 17. Next, A549 cells were transfected with wild type Shh promoter-luciferase plasmid or the mutant Shh promoter-luciferase plasmid and subsequently treated with TGF-β1, while control cells were transfected with wild type Shh promoter-luciferase without TGF-β1 stimulation. Luciferase enzyme activity was measured; A549 cells treateted with TGF-β1 after they were transfected with wild type Shh promoter (Shh+TGF-β1) showed a four fold increase in luciferase activity compared to control, while A549

cells with mutant construct had an insignificant increase in luciferase activity (Fig. 19). This suggested that NF-κB transcriptionally activates Shh promoter following treatment with TGF-β1 in NSCLC.

Constitutive phosphorylation of Smad2 following chronic stimulation with TGF-β1:

Chow et al. group reported that in pancreatic cancer cells TGF-β1 activate NF-κB and enhances cell motility and invasiveness in a Smad4-independent manner which can be cancelled out when TGF-β-Smad2/3 signaling restored. Their data suggests that the activation of NF-κB is by Smad2 and/or Smad3, and not Smad-4 (Chow et al. 2010, G275-G282). Therefore, I investigated the involvement of NF-κB upstream molecule, Smad2, in TGF-β1 stimulation of NF-κB activity prior to Shh up-regulation. The Smad phosphorylation kinetics in TGF-β1-stimulated A549 cells shows highest levels of phospho-Smad (pSmad) expression after five hours of treatment, and after 24 hours pSmad dropped down to its basal levels (Fig. 20). Interestingly, A549-M cells had constitutive phosphorylation of Smad, even after TGF-β1 discontinuation. These results suggests that Smad, as downstream signaling molecule of TGF-β1, may be involved initially in the activation of NF-κB, consistant with previous reports (Chow et al. 2010, G275-G282) and subsequent induction of Shh in EMT phenotypic cells, and that there is a feedback mechanism that kept sustained

activation of Smad2 in A549-M cells. Interestingly, it has been reported that phosphorylation of Smad 3 was enhanced by treatment with N-Shh (Yoo et al. 2008, 480-490); however, further in-depth research is warranted.

Shh is a possible target gene of miRNA-15a and miRNA-16:

Further investigation was focused on miRNAs because the posttranscriptional regulation of Shh by miRNAs could play an important regulatory role. miRNAs are especially known to function as negative regulators of gene expression at the posttranscriptional level and are critically involved in tumorigenesis (Cho 2007, 60;Krutovskikh and Herceg 2010, 894-904;Mocellin, Pasquali, and Pilati 2009, 70-80). As previously indicated I performed miRNA microarray using total RNA extracted from A549 and A549-M cells. Two miRNAs, miR-15a and miR-16, were found to be down-regulated in A549-M cells compared to A549 cells. Both miR-15a and miR-16 are located at chromosome 13q14 and have been implicated in cell cycle control and apoptosis. Both miRNAs are frequently deleted or down-regulated in squamous cell carcinomas and adenocarcinomas of the lung (Bandi et al. 2009, 5553-5559;Palamarchuk et al. 2010, 3916-3922). Interestingly, computational analysis of miRNA profiles and their targets, using UCSC, TargetScan and PITA to predict potential target genes of miR15a and miR-16 what that the Shh 3' untranslated regions (3' UTRs) were their potential target (Fig. 21). A Combination of miRNA microarray data and miRNA target genes data correlates with Shh gene up-regulation, suggesting that

Shh up-regulation in A549-M can be partly due to the loss of miR-15a and miR-16 expression.

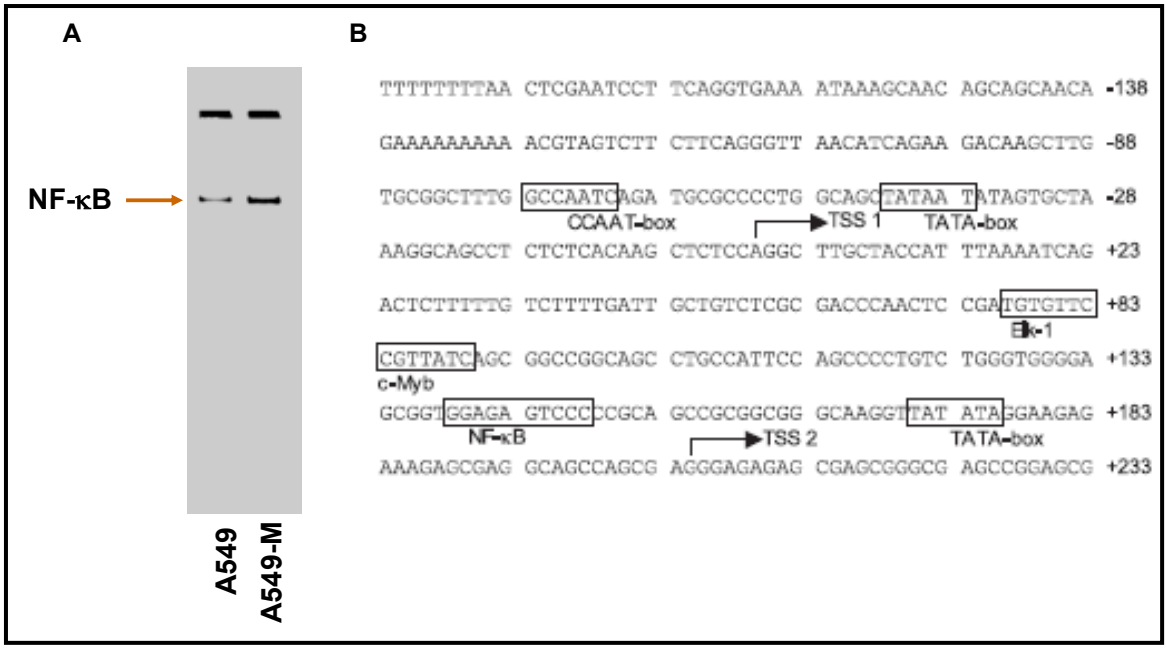

Figure 16. Increaced activation of NF-κB following chronic treatment with TGF-β1: (A), A549-M cells have greater activation in NF-κB compared to A549 cells as assessed by electrophoretic mobility shift assay (EMSA) (B) Promoter and 5' upstream region of the human Shh gene, Nucleotide sequence of the human Shh promoter. Arrows correspond to the two transcriptional start sites, TSS 1 and TSS 2. TATA and CCAAT boxes, and putative sites for transcription factors binding, are indicated by boxes. Positions were counted relative to the first TSS (Figure 12B. courtesy of Kasperczyk group).

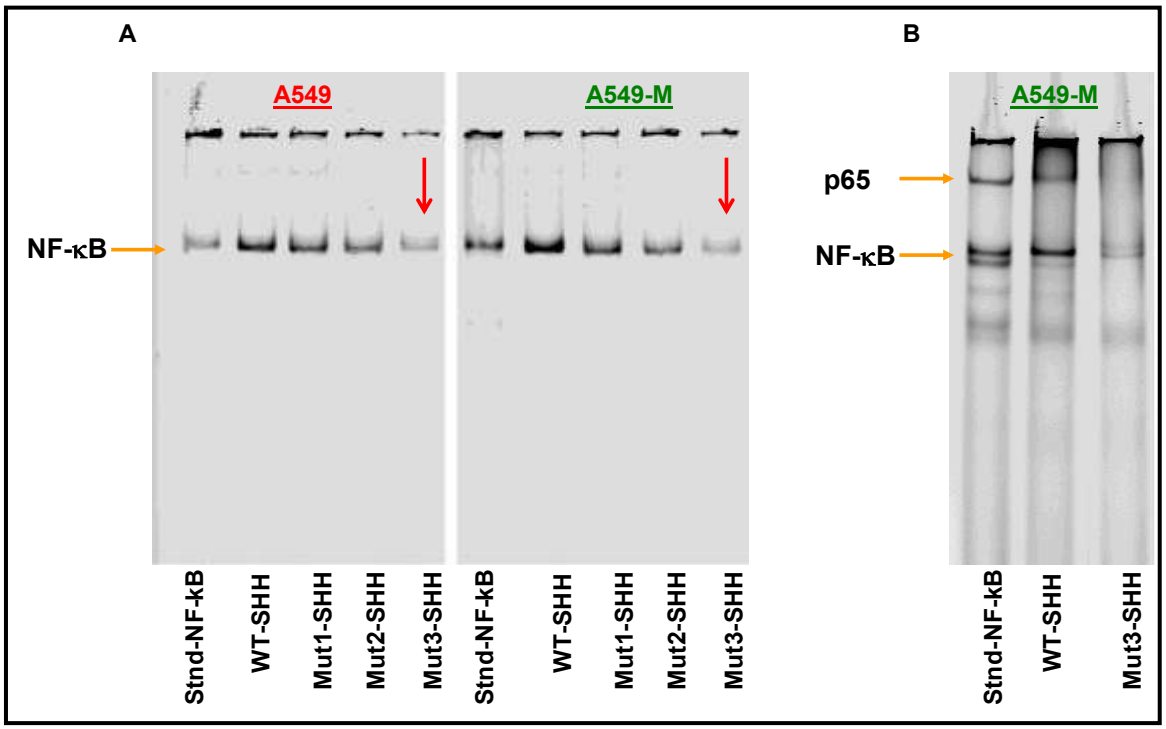

Figure 17. NF-κB complexes bound to NF-κB binding site within the human Shh promoter region. (A), putative NF-κB binding site within the human Shh promoter region bound NF-κB complexes, and NF-κB complexes showed the lowest binding activity to mutant three of Shh promoter region, in A549 and A549-M cells (B), Antibodies recognizing p65 subunits caused a significant supershift of NF-κB DNA binding complexes documenting the specificity of the complex.

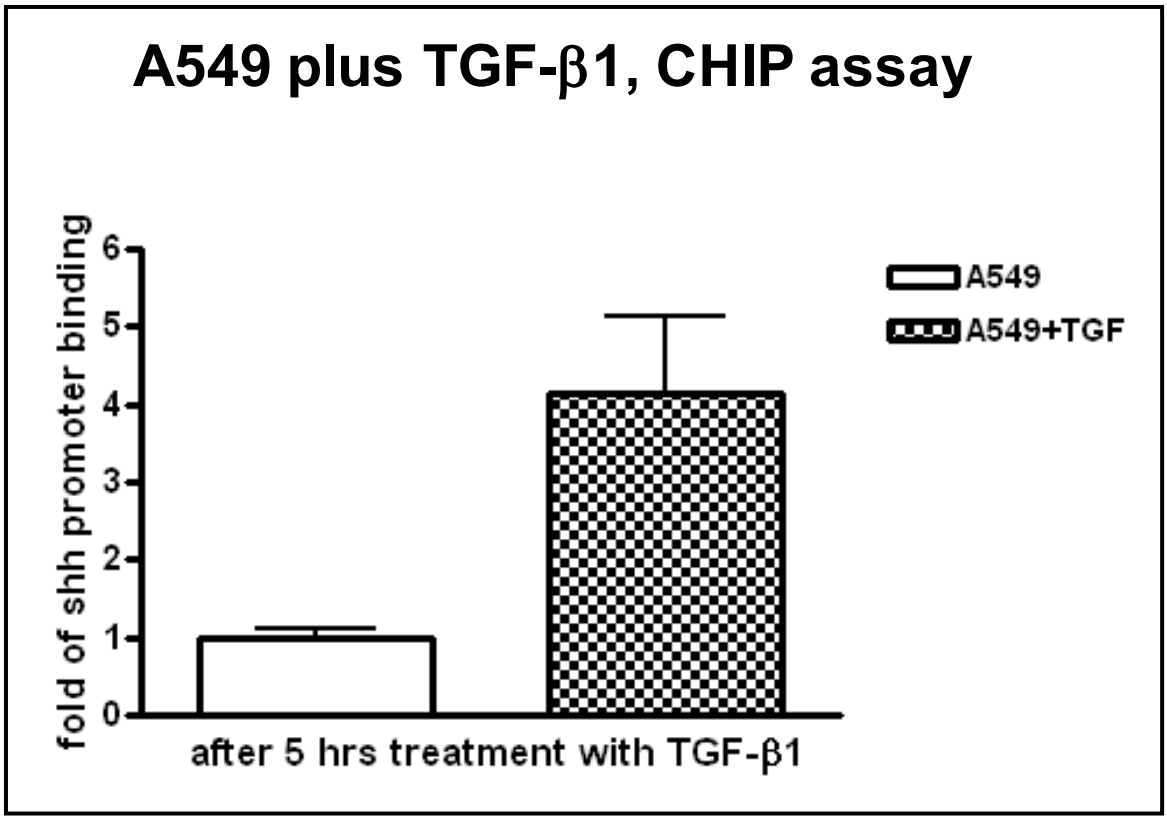

Figure 18. NF-κB binds to the Shh promoter following treatment with TGF-β1. Using an antibody to p65 subunit of NF-κB, TGF-β1 treatment showed stimulation in the NF-κB activation in A549 cells, resulting in increased binding of p65 to the Shh promoter region.

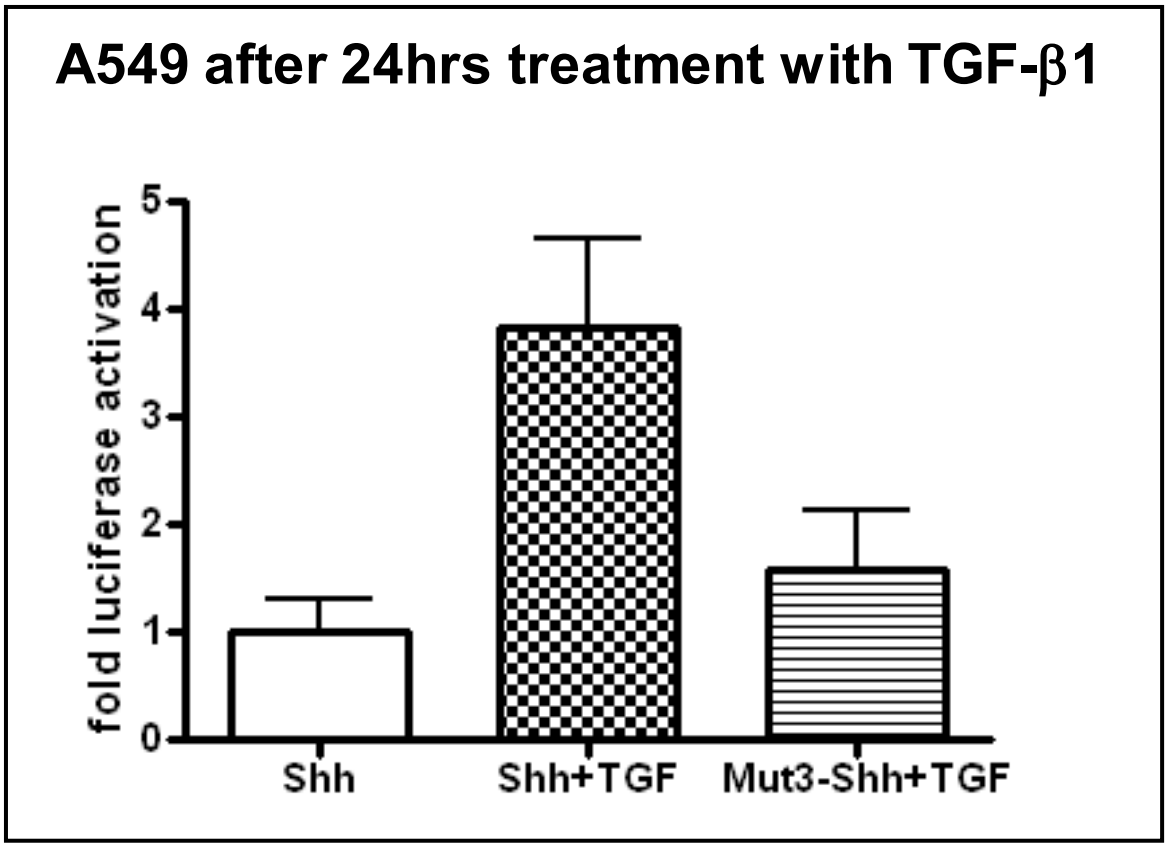

Figure 19. NF-κB transcriptionally activates Shh promoter following treatment with TGF-β1 in NSCLC cells. A549 cell were transfected with wild type Shh promoter-luciferase plasmid or the mutant Shh promoter-luciferase plasmid and subsequently treated with TGF-β1, while control cells was transfected with wild type Shh promoter-luciferase, without TGF-β1 treatment.

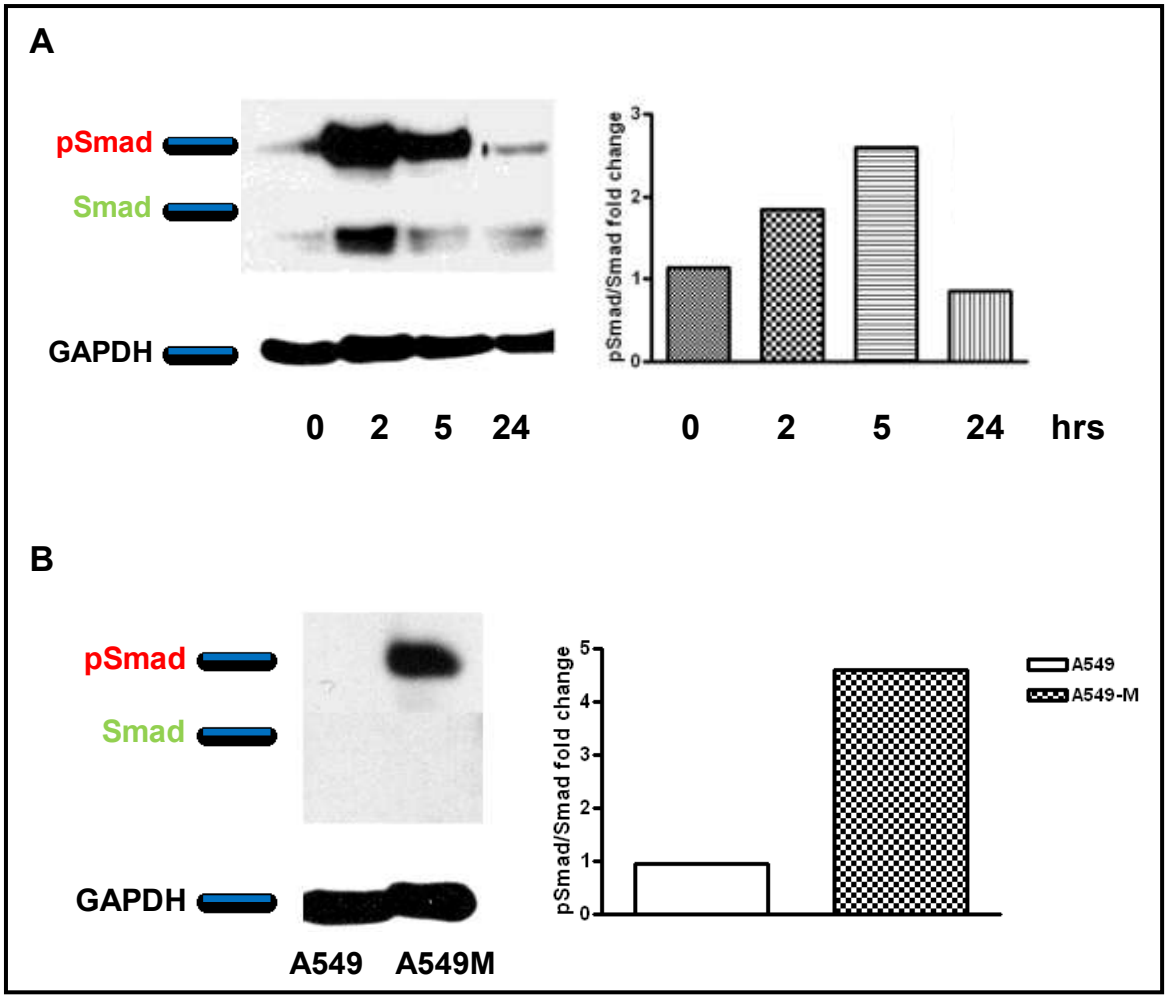

Figure 20. Constitutive phosphorylation of Smad2 following chronic treatment with TGF-β1. (A) Smad phosphorylation Kinetics in TGF-β1-treated A549 cells showing highest levels of phospho-Smad (pSmad) expression after five hrs of treatment; however, after 24 hrs pSmad levels drop-down to basal levels. (B), A549-M cell had constitutive phosphorylation of Smad, even after TGF-β1 treatment discontinuation.

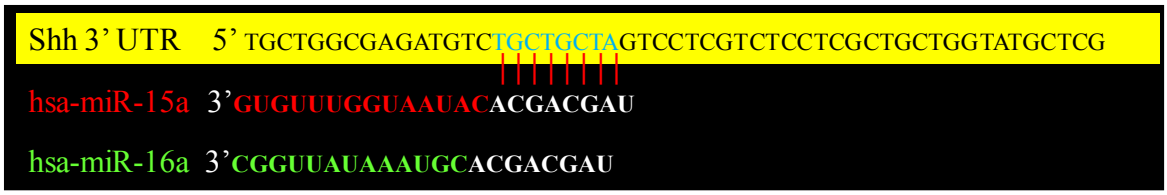

Figure 21. Shh is a possible target gene of miRNA-15a and miRNA-16. using UCSC, TargetScan and PITA to predict potential target genes of miR15a and miR-16, Shh **3'** untranslated regions (3' UTRs) was identified as a potential target, suggesting that Shh gene is regulated through miR-15a and miR-16.

CHAPTER 5

Hedgehog inhibitors sensitizes EMT-phynotypic cells to conventional therapeutics in NSCLC cells

Lung cancer causes the highest mortality rate among cancers and also results in short survival rate because most of the patients are in later stages and have a high incidence of metastatic disease at the time of diagnosis which lacks effective therapy. The standard therapeutic approaches of advanced disease have failed miserably in this case (Herbst et al. 2011, 1846-1854;Jemal et al. 2008, 71-96;Jemal et al. 2009, 225-249;Jemal et al. 2011, 69-90). Recent studies employed gene expression, xenograft assays and proteomic profiling techniques to find biomarkers associated with sensitivity to erlotinib in panels of sensitive and insensitive NSCLC cell lines (Thomson et al. 2005, 9455-9462;Witta et al. 2006, 944-950;Yauch et al. 2005, 8686-8698). These authors have found that sensitive cell lines express the well-established epithelial markers E-cadherin and catenin as well as exhibit the typical cobblestone epithelial morphology and tight cell–cell junctions of epithelial cells. On the other hand, insensitive cell lines did not have epithelial markers and they express protein characteristics of mesenchymal cells, including vimentin, fibronectin, and Zeb-1 consistent with more fibroblastic scattered morphology; this trans-differentiation is similar to cells undergoing the processes of EMT. Collectively, these results suggest that the processes of EMT may serve as an indicator of sensitivity to EGFR inhibitors.

Moreover, a study by Prudkin *et al.* has shown a clear association with these EMT markers in the sequential pathogenesis of squamous cell carcinoma (Prudkin et al. 2009, 668-678), suggesting that the combination of EGFR-TKI with the inhibitor of EMT-inducing-molecules could become a novel approach toward the treatment of lung cancer, especially NSCLC.

Recently, the hedgehog (Hh) signaling pathway was reported to be active in many cancers including NSCLC (Katoh and Katoh 2008, 271-275;Thayer et al. 2003, 851-856;Watkins et al. 2003, 313-317;Yuan et al. 2007, 1046-1055). In addition, the Hh signaling blockade inhibits the growth, invasion and metastasis of cancer cells having activated Hh signaling, which was associated with the down-regulation of Snail and up-regulation of E-cadherin. Furthermore, over-expression of GLI1 in epithelial cancer cells led to aggressive phenotype with down-regulation of E-cadherin (Feldmann et al. 2007, 2187-2196;Fukaya et al. 2006, 14-29) suggesting that Hh signaling is an important regulator of EMT and important target for therapy. Moreover, Hh signaling was found to be essential for the maintenance of cancer stem cells (Zhao et al. 2009, 776-779). Collectively, all of these studies clearly suggest that Hh signaling is a legitimate and novel target for the development of innovative therapeutic strategies for sensitization of invasive and metastatic cancer with EMT or cancer stem-like cell characteristics to standard therapies in NSCLC.

To address this question, A549-M and H1299, mesenchymal phenotypic cell lines, were treated by Hh inhibitor GDC-0449 (table. 4) for three days. Next,

cells were treateted with cisplatin (Cis) or Erlotinib (Erlo), the epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI), and then cell viability was assessed. Furthermore, Shh was knocked-down by siRNA in A549-M or H1299 cells and then treated with Cis or Erlo, and cell viability was determined with the change in the half-maximal inhibitory concentration (IC_{50}). The data showed a significant decrease in IC_{50} for both Cis and Erlo, suggesting that Hh inhibitor can sensitize resistant NSCLC cell lines with mesenchymal phenotype to standard therapies.

A549-M cells with mesenchymal phenotype showed more resistance to cisplatin and EGFR-TKI (erlotinib) compared to A549 cells:

Epithelial-to-mesenchymal transition (EMT) phenotypic cancer cells have been shown to acquire drug resistance (Bao et al. 2011;Gomes et al. 2011;Hotz, Hotz, and Buhr 2011, 448-454;Nicolini et al. 2011, 1486-1499). Our data showed that A549 with mesenchymal phenotype (A549-M) acquire invasiveness *in vitro* as will as *in vivo* (Maitah et al. 2011, e16068), and thus I investigated whether A549 cells with mesenchymal phenotype (A549-M) has indeed acquired the drug resistance phenotype associated with the acquisition of mesenchymal phenotype. A549 or A549-M cells were treated with cisplatin or erlotinib, and then measured cell viability by colorimetric assays (MTT). A549-M indeed showed greater viability compared to A549 cells, suggesting that A549-M cells are more resistant to cisplatin or erlotinib, consistent with the EMT phenotype (Figure. 22).

Hedgehog inhibitor GDC-0449 sensitizes mesenchymal phenotypic cells to cisplatin and EGFR-TKI (erlotinib):

Next, I evaluated whether Hedgehog inhibitor GDC-0449, can sensitize A549-M cells to cisplatin or erlotinib, and thus A549-M cells were treated with GDC-0449 (GDC) for three days, subsequently these cells where treated with cisplatin or erlotinib at different concentration, and the cell viability was assessed after 72 hrs of treatment. A549-M cells treated with GDC showed less cell viability (Table. 4), suggesting that Hh inhibitor GDC sensitizes mesenchymal phenotype cells to standard therapy. In our previous data TGF-β1 up-regulated Shh, and Shh was required to induce EMT by TGF-β1, and also found that the inhibition of Shh abrogated A549-M cell's migration and invasion ability. Therefore to further confirm the role of TGF-β1-induced up-regulation of Shh, Shh was inhibited by knock-down and tested whether the knock-down of Shh will sensitize A549-M cells siliar to treatment with GDC. A549-M cells were transfected with Shh specific siRNA, control cells were transfected with scrambeled siRNA, then the cells were treated with cisplatin or erlotinib. A549-M cells with Shh knock-down showed significant reduction in cell viability (Fig. 23). These data clearly suggests that Hh inhibition is a good therapeutic strategy to sensitize drug resistant cells with EMT phenotype to standard therapy.

Next, for confirming this conclusion, another NSCLC cell line with mesenchymal phenotype was used. H1299 cells reported to have mesenchymal

phenotype with resistance characterestics to cisplatin and erlotinib (Ceppi et al. 2010, 1207-1216;Takeyama et al. 2010, 216-224;Thomson et al. 2005, 9455-9462). Thus, H1299 cells were treated with GDC for 72 hrs prior to treatments with cisplatin or erlotinib whereas control cells were treated with cisplatin or erlotinib only, and after 72hrs of cisplatin or erlotinib treatments, the cellular viability were determined. H1299 cells treated with GDC showed lesser cell viability compared to H1299 cells untreated with GDC, and these results are consistent with the results obtained from A549-M cells (Fig. 24). I also confirmed the specific role of the ligand Shh in H1299 cells in drug-sensitivity after treatment with hedgehog inhibitor (GDC) and by using Shh siRNA knock-down prior to treatment with cisplatin or erlotinib. H1299 cells with Shh knock-down showed decreased cell viability, confirming the involvement of Shh in restoring sensitivity to standard therapy. Together, these results suggests that treatment of patients with hedgehog inhibitor prior to or concurrent with standard therapy could improve therapeutic outcome in patients whose tumor show EMT features.

Hedgehog inhibitor down-regulates epithelial to mesenchymal marker in non-small lung cancer cells:

In order to gain further insight on reversing drug sensitivity to conventional therapeutics by hedgehog inhibitor, further experiments were conducted. Hedgehog signaling pathway has been implicated in EMT regulation (Chen et al. 2011;Choi et al. 2010, 36551-36560;Maitah et al. 2011,

e16068;Omenetti et al. 2011, 1246-1258;Sarkar et al. 2010, 383-394;Takebe, Warren, and Ivy 2011, 211;Zheng et al. 2010, 965-970). Also, inhibition of hedgehog pathway showed down-regulation of EMT markers (Feldmann et al. 2007, 2187-2196;Merchant and Matsui 2010, 3130-3140;Xu, Ma, and Wang 2009, 119-124). I evaluated whether hedgehog inhibitor could down-regulate EMT markers in NSCLC cells, A549-M, H1299 and H1650 cells where treated with GDC, then EMT markers fibronectin and ZEB1 were examined together with assessment of epithelial marker E-cadherin. Data showed that mesenchymal markers fibronectin and ZEB1 were down-regulated after treatment with GDC whereas epithelial marker E-cadherin was up-regulated (Fig. 25), suggesting that the reversal of drug resistance is in part mediated through the reversal of EMT phenotype. Together, these data clearly suggest that hedgehog inhibitor will restore drug sensitivity by reversing the EMT phenotype, and thus Hh inhibitor may play a critical role in sensitizing EMT phenotypic NSCLC cells to standard therapies.

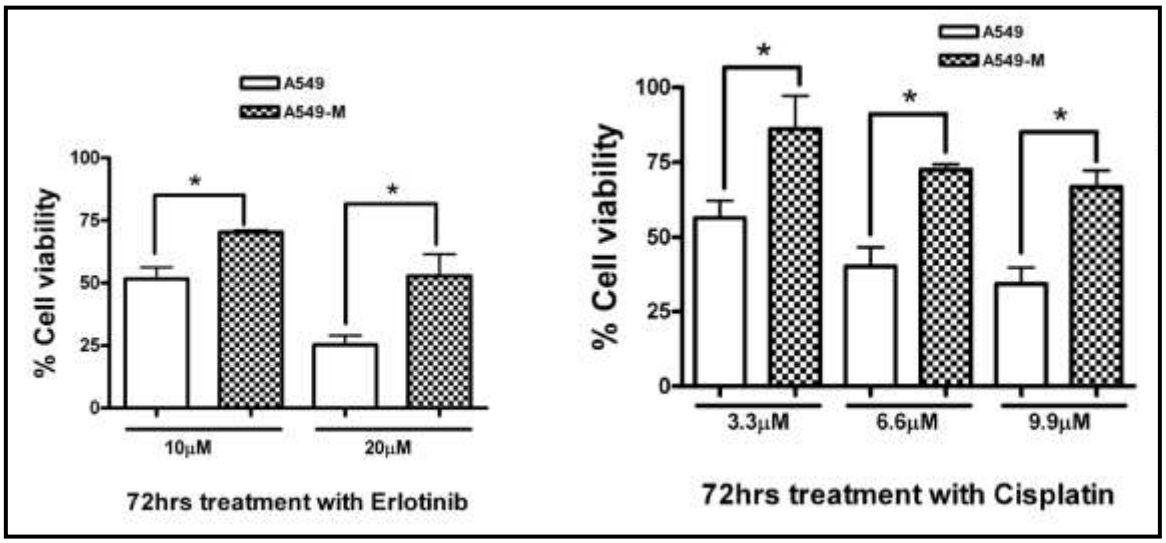

Figure 22. A549-M cells acquired drug resistance phenotype: Left panel, A549-M cells showed greater cell viability, after treatment with erlotinib compared to A549 cells. Right panel A549-M cells showed greater cell viability, after treatment with cisplatin compared to A549 cells.

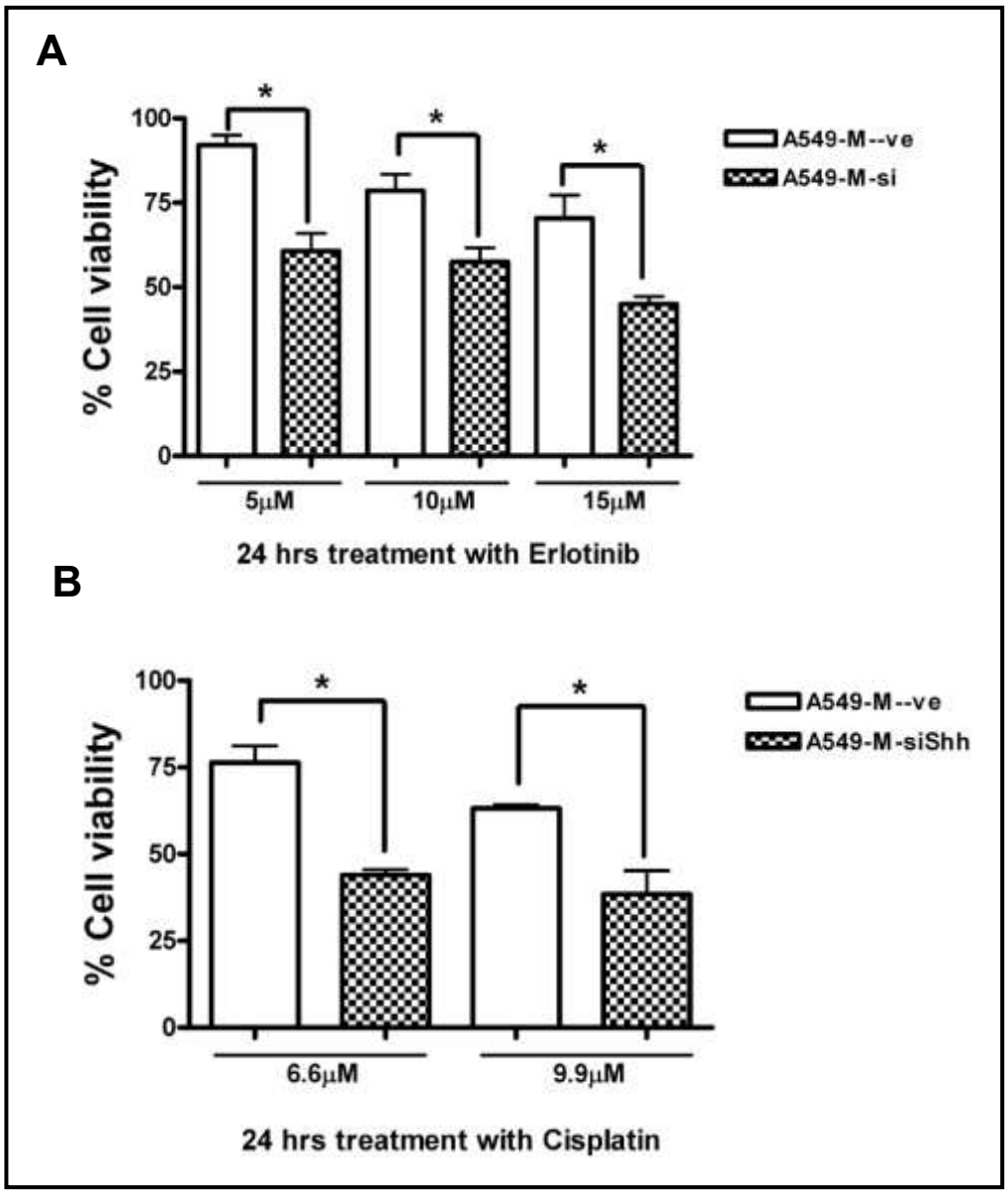

Figure 23. Knock-down of Shh sensitizes A549-M cells to standard therapies: (A) Reduction in cell viability of A549-M cells treated with erlotinib following Shh knock-down, (B) A549-M treated with cisplatin following Shh knock-down showed reduced cell viability.

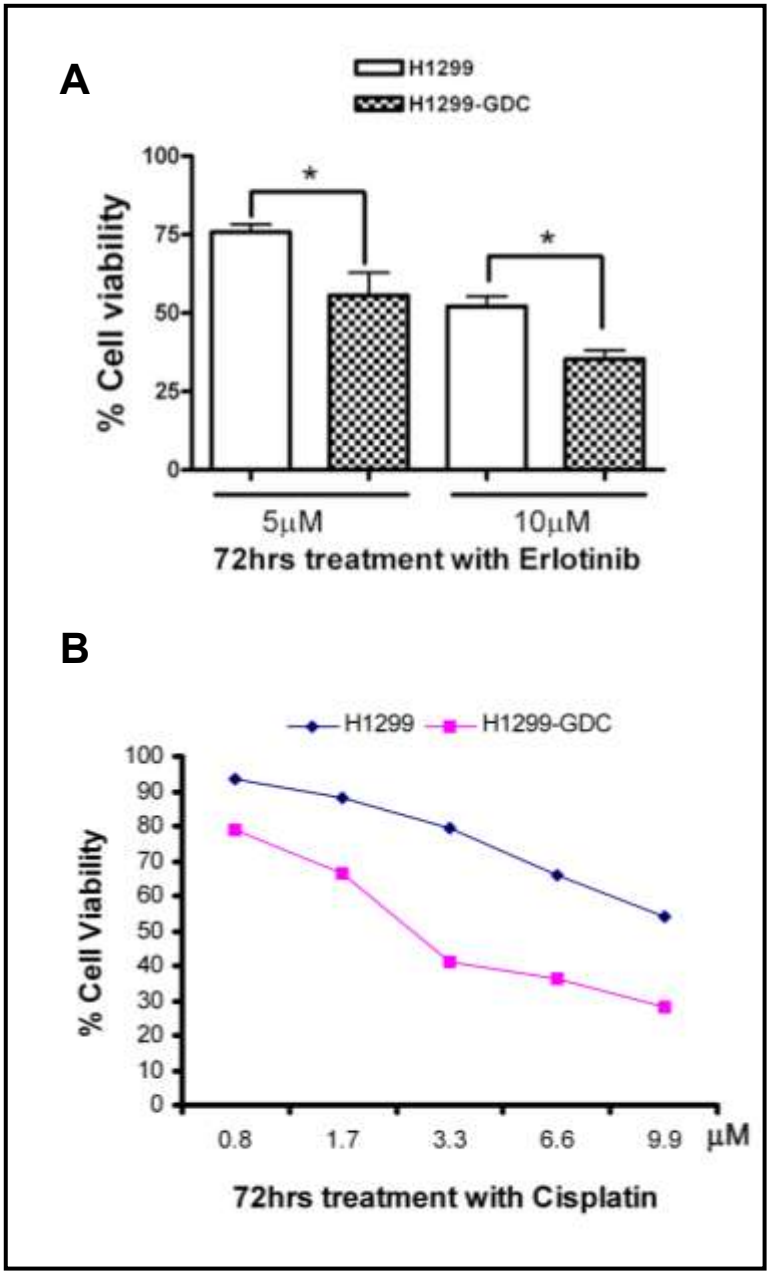

Figure 24. Hedgehog inhibitor (GDC) sensitizes H1299 cells to standard therapies: (A) Reduction in cell viability of H1299 cells treated with erlotinib following Hedgehog inhibitor (GDC), (B) H1299 treated with cisplatin following Hedgehog inhibitor (GDC) showed reduced cell viability.

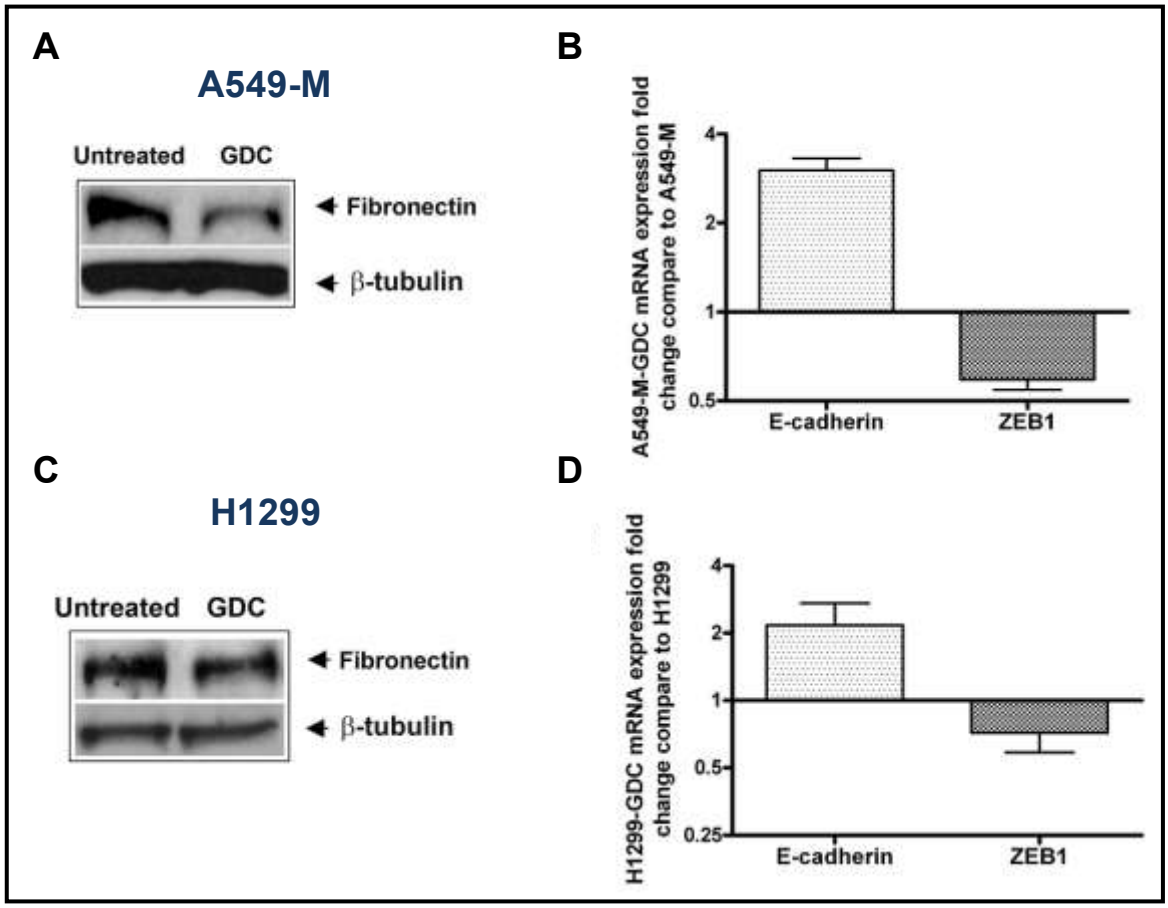

Figure 25. Hedgehog inhibitor down-regulates epithelial to mesenchymal marker in NSCLC: (A, C) western blot showing reduction in the expression of fibronectin, a mesenchymal marker, following treatment with Hedgehog inhibitor (GDC) in both A549-M and H1299 cells; (B, D) qRT-PCR showed down-regulation in the EMT marker ZEB1, and up-regulation of epithelial marker E-cadherin after treatment with Hedgehog inhibitor (GDC) in both A549-M and H1299 cells.

Cell line	Standard therapy	IC50 (μM)		% decrease in IC50 with GDC treatment
		Without GDC	With GDC	
A459-M	Erlotinib	43.64	15.76	63.89
	Cisplatin	36.16	9.641	73.34
H1299	Erlotinib	10.57	7.198	31.90
	Cisplatin	12.15	4.185	65.56

Tabel 4. Treatment of NSCLC cells with hedgehog inhibitor showed significant decrease the half-maximal inhibitory concentration (IC_{50}) of both erlotinib and cisplatin: The table shows changes in the IC_{50} of erlotinib and cisplatin with and without prior treatment with GDC; last column on the right shows the persantage decrease in the IC_{50} following GDC, in both A549-M and H1299 cells.

CHAPTER 6

Discussion

Previous studies have shown that the treatment of NSCLC cells (A549 cells) with TGF-β1 could induce EMT phenotype (Kasai et al. 2005, 56;Kim et al. 2007, 898-904;Saitoh et al. 2010, 263-270). EMT is a process that was originally reported to be involved in embryogenesis and gastrulation (Gunhaga, Jessell, and Edlund 2000, 3283-3293;Kang and Massague 2004, 277-279;Thiery 2003, 740-746;Thiery and Sleeman 2006, 131-142). The induction of EMT in cancer cells confers these cells with the ability to become more motile and invasive with increased tumorigenic potential (Kang and Massague 2004, 277-279;Larue and Bellacosa 2005, 7443-7454;Singh and Settleman 2010, 4741-4751;Thiery and Sleeman 2006, 131-142;Thomson et al. 2005, 9455-9462;Tsuji, Ibaragi, and Hu 2009, 7135-7139;Yauch et al. 2005, 8686-8698). Furthermore, the EMT phenotype appears to be involved in resistance to conventional therapeutics. Thus, reversal of EMT by novel approaches may provide a tool by which one could enhance the effects of conventional therapeutics with better treatment outcome in NSCLC patients.

In this study, NSCLC cell lines (A549 and H2030) with EMT phenotypic changes (A549-M and H2030-M cells) after chronic exposure to TGF-β1 was employed, which showed consistent results with decreased expression of

epithelial markers concomitant with increased expression of mesenchymal markers (Fig. 5A–C; Fig. 9A). To investigate the aggressivness of A549-M cells, I assessed the ability of A549-M cells compared to A549 parental cells for cell migration, invasion and tumorigenic potential *in vitro* as will as *in vivo.* Our data showed increased ability of A549-M cells for cell migration, invasion and tumorigenic potential compared to parental A549 cells (Fig. 6A–C, and Fig. 7). Interestingly, we also found that A549-M and H2030 cells showed high expression of Shh both at the mRNA and protein levels compared to parental cells (undetectable levels of Shh expression) (Fig. 8A–B; Fig. 9A). The up-regulation of Shh expression in A549-M cells is the first such report. Shh up-regulation was also consistent with increased expression of GLI1 transcription factor, a downstream target gene of Hh signaling pathway (Fig. 8C–D) although the basal level of GLI1 expression was found to be high in the parental A549 cells, suggesting that Hh signaling could be active through noncanonical pathway (ligand-independence) in these cells. This novel finding is very interesting, because it connects two very important molecules in the developmental pathway, TGF-β1 and Shh, with tumor aggressiveness (Bethea et al. 2009, 94-108;Hogan 1999, 225-233). Also this finding is consistent with published reports showing the role of EMT in tumor aggressiveness and metastasis (Katoh and Katoh 2008, 271-275;Sanchez-Tillo et al. 2010, 3490-3500;Thayer et al. 2003, 851-856;Zhao et al. 2009, 776-779). However, no studies have shown direct up-regulation of Hh ligand Shh mRNA and protein by TGF-β1 as documented in this report although

Shh has been reported to activate TGF-β family signaling through the ALK5-Smad 3 pathway in gastric cancer cells (Yoo et al. 2008, 480-490). Moreover, it has been reported that TGF-β1 can induce GLI2 activation through Smad3 in pancreatic adenocarcinoma cell lines (Dennler et al. 2007, 6981-6986), and these results suggest that there may be a feedback loop connecting TGF-β1 with Shh activation. Our finding also suggest that Hh signaling pathway reactivation in cancer epithelial cells within the tumor microenvironment could lead to the acquisition of aggressive phenotype of cancer cells within a tumor. Also, our data clearly suggest that the up-regulation of Shh by TGF-β1 leads to increased tumor cell migration, invasion and tumorigenic potential of A549-M cells as documented by our mechanistic experiments using knock-down approach and by using inhibitors of Hh-signaling pathway (Fig. 10 and Fig. 11).

Our results also suggest that the maintenance of EMT phenotype in A549-M cells may be related to the sustained activation of Hh. These results are also consistent with two other NSCLC cell lines that were derived from patients metastasis (H1650, H1299), and these two cell lines showed high basal levels of Shh expression, suggesting that lung metastatic cells have the ability to undergo EMT consistent with higher expression of Shh *in vivo*. Interestingly, the inhibition of TGF-β1-induced Shh signaling by pharmacological inhibitors or by siRNA, decreased the ability of A549-M cells to migrate, invade and their colony-forming ability, and these results are consistent with previous reports showing that the activation of Shh signaling could increase invasion and metastasis (Bailey,

Singh, and Hollingsworth 2007, 829-839;Feldmann et al. 2007, 2187-2196;Fukaya et al. 2006, 14-29;Teglund and Toftgard 2010, 181-208;Watkins et al. 2003, 313-317;Yoo et al. 2008, 480-490). We have also shown that NIH 3T3 cells cultured in the presence of conditioned medium collected from A549-M cells showed increased activation of hedgehog signaling, which suggests that TGF-β1-induced EMT can be mediated by the activation of Shh through both autocrine, paracrine or juxtacrine mechanisms. More importantly, our data show for the first time that TGF-β1 induced EMT is mediated through up-regulation of Shh because knock-down of Shh by siRNA significantly attenuated EMT induction by TGF-β1 treatment (Fig. 9B, C and D).

Next, I investigated the mechanism by which TGF-β1 up-regulates Shh gene. Since, understanding of this process would lead to specific therapeutic treatments, at least, in a subset of NSCLC patients, thus, I investigated the molecular mechanisms involving up-regulation of Shh gene expression in NSCLC following TGF-β1 treatment. I have characterized human Shh promoter and its activation as well. Using combination of electrophoretic mobility shift assay (EMSA), site-directed mutagenesis, luciferase reporter transient transfection, and chromatin immunoprecipitation assays, I found that NF-κB, was activated in A549-M cells following TGF-β1 treatment, which activated the expression of Shh gene (Fig. 16, 17, 18). Furthermore, I investigated the posttranscriptional regulation of Shh by microRNAs (miRNA), which are negative

regulators of gene expression at the posttranscriptional level, and which are involved in tumorigenesis (Cho 2007, 60;Krutovskikh and Herceg 2010, 894-904;Mocellin, Pasquali, and Pilati 2009, 70-80). To address this question, I performed miRNA microarray using total RNA extrcated from A549 and A549-M cells. Two miRNAs, miR-15a and miR-16, were found to be down-regulated in A549-M cells compared to A549 cells. Both, miR-15a and miR-16 are located at chromosome 13q14, and have been implicated in cell cycle control and apoptosis. Both are frequently deleted or down-regulated in squamous cell carcinomas and adenocarcinomas of the lung (Bandi et al. 2009, 5553-5559;Palamarchuk et al. 2010, 3916-3922). Interestingly, computational analysis of miRNA profiles and their targets, using UCSC, TargetScan and PITA to predict potential target genes of miR15a and miR-16, Shh 3' untranslated regions (3' UTRs) was identified as a potential target. This is the first time documenting an interesting correlation between miR15a and miR-16 and Shh 3' untranslated regions (3' UTRs). Combining miRNA microarray data, and miRNA target genes data, we found a good correlation between Shh gene up-regulation and the loss of miR-15a and miR-16, suggesting that Shh up-regulation in A549-M can be partly mediated by the loss of miR-15a and miR-16.

Interestingly, our data shows that phospho-Smad is constitutively activated in A549-M cells even in the absence of TGF-β1, suggesting a positive feedback mechanism. Considering the reports that Shh treatment leads to activation of Smads (Chow et al. 2010, G275-G282), our results suggest a

visious cycle interconnecting TFG-β1, NF-κB, Shh, pSMAD and miRNAs. Thus, it is our contention that initially TGF-β1 activates NF-kB, which up-regulates Shh expression, and in turn, Shh further activates TGF-β signaling. These findings would be helpful in the development of newer therapeutic strategies by targeting not only Shh but also finding avenues by which one could re-express the lost miRNAs to achieve reversal of therapeutic resistance to conventional therapeutics, which would results in better treatment outcome.

Collectively, all of the above mentioned studies clearly suggest that Hh signaling is a legitimate and novel target for the development of innovative therapeutic strategies for the sensitization of invasive and metastatic cancer with EMT or cancer stem-like cell characteristics to standard therapies in NSCLC. To address this possibilty, A549-M and H1299, mesenchymal phenotypic cell lines, were further treated with Hh inhibitor GDC-0449 (table. 4), for three days. Next, cells were treated with cisplatin (Cis) or Erlotinib (Erlo), the epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI), and then cell viability was assessed. Furthermore, Shh was knock-down by siRNA, then A549-M or H1299 were treated with Cis or Erlo, and cell viability was determined with the change in the half-maximal inhibitory concentration (IC_{50}). Data showed a significant decrease in IC_{50} for both Cis and Erlo (Fig. 23, 24, 25), suggesting that Hh inhibitor can be useful to sensitize resistant NSCLC cell lines with mesenchymal phenotype to standard therapies. Next, I showed that Hedgehog inhibitor can down-regulate EMT markers and up-regulate epithelial markers, in mesenchymal

phenotype NSCLC cell lines, suggesting the possibility of EMT reversal by hedgehog inhibitors and therefore the sensitization of these drug resistant cells to conventional therapeutics could be clinically envisaged.

Based on existing evidence in the literature and based on my current data, I propose a model where initially epithelial tumor cells chronically exposed to TGF-β1, excreted by either stromal cells, immune cells or the tumor cells within the tumor microenvironment *in vivo*. This chronic TGF-β1 exposure results in the up-regulation of Shh both at the mRNA and at the protein levels, through Smad-NF-κB activation and down-regulation of specific miRNAs that targets Shh, specificaly miR15a/16. Consequently, the activation of Hh signaling along with other molecules, leads to the acquisition of EMT phenotype, which is responsible for tumor cell aggressiveness, metastasis and drug resistance (Fig. 27). Furthermore, I propose that activated hedgehog-signaling acts as feedback loop, and activate Smad. Therefore, the inhibition of Shh signaling could be a useful approach for reducing tumor aggressiveness in NSCLC, and as such, the reversal of EMT could also be useful for re-sensitization of drug-resistant NSCLC to conventional therapeutics, which would likely contribute to the improved survival of patients who rightfully deserve better treatment outcomes.

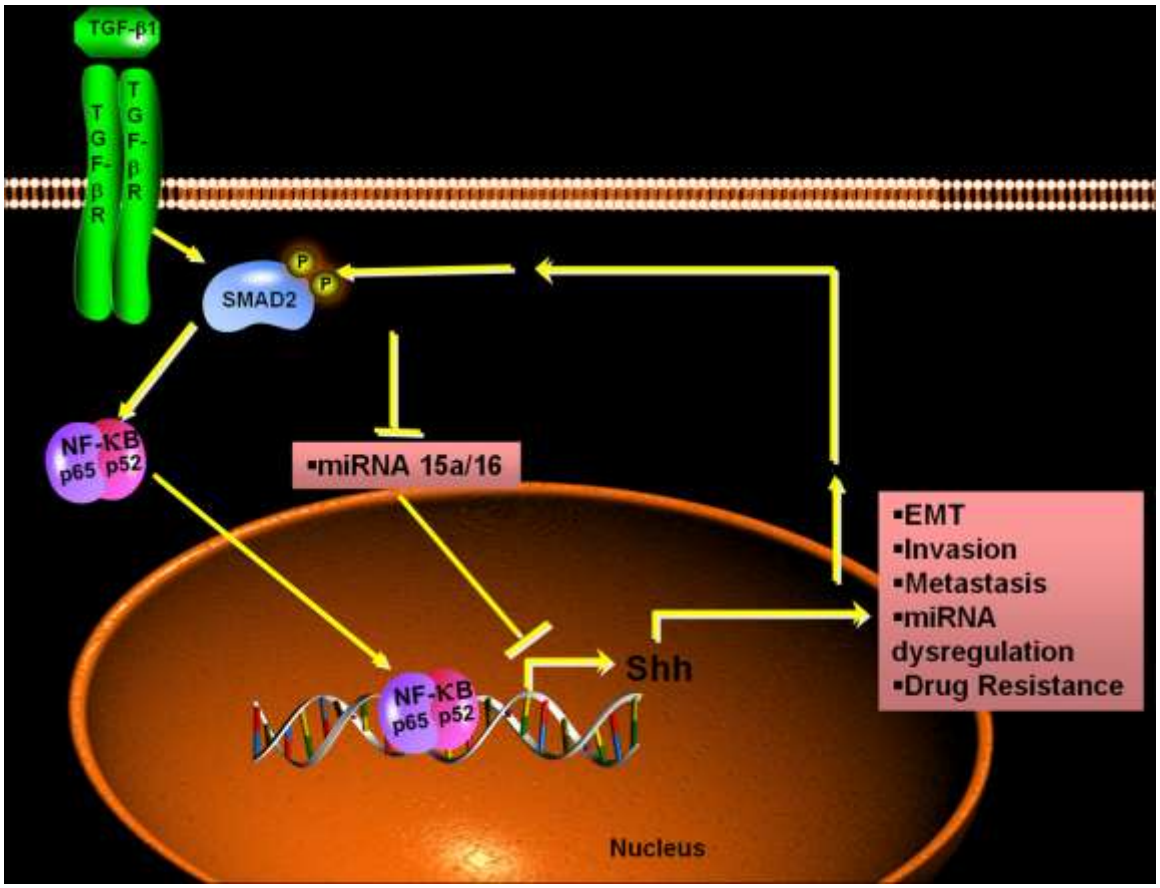

Figure 26. TGF-β1 treatment up-regulates sonic hedgehog through activation of Smad-NF-κB signaling pathway, and also by deregulation of miR-15a and miR-16. Shh up-regulation contributes to EMT, invasiveness, metastasis, and that the loss of miRNAs contributes to EMT and drug resistance. In addition, Shh up-regulation may contribute to constitutive activation of SMAD.

REFERENCES

1. Arsura, M., Panta, G. R., Bilyeu, J. D., Cavin, L. G., Sovak, M. A., Oliver, A. A., Factor, V., Heuchel, R., Mercurio, F., Thorgeirsson, S. S., & Sonenshein, G. E. 2003. Transient activation of NF-kappaB through a TAK1/IKK kinase pathway by TGF-beta1 inhibits AP-1/SMAD signaling and apoptosis: implications in liver tumor formation. *Oncogene*, 22(3): 412-425.

2. Ateeq, B., Unterberger, A., Szyf, M., & Rabbani, S. A. 2008. Pharmacological inhibition of DNA methylation induces proinvasive and prometastatic genes in vitro and in vivo. *Neoplasia.*, 10(3): 266-278.

3. Bailey, J. M., Singh, P. K., & Hollingsworth, M. A. 2007. Cancer metastasis facilitated by developmental pathways: Sonic hedgehog, Notch, and bone morphogenic proteins. *J.Cell Biochem.*, 102(4): 829-839.

4. Bandi, N., Zbinden, S., Gugger, M., Arnold, M., Kocher, V., Hasan, L., Kappeler, A., Brunner, T., & Vassella, E. 2009. miR-15a and miR-16 are implicated in cell cycle regulation in a Rb-dependent manner and are frequently deleted or down-regulated in non-small cell lung cancer. *Cancer Res.*, 69(13): 5553-5559.

5. Bao, B., Wang, Z., Ali, S., Kong, D., Banerjee, S., Ahmad, A., Li, Y., Azmi, A. S., Miele, L., & Sarkar, F. H. 2011. Over-expression of FoxM1 leads to epithelial-mesenchymal transition and cancer stem cell phenotype in pancreatic cancer cells. *J.Cell Biochem.*.

6. Basseres, D. S., Ebbs, A., Levantini, E., & Baldwin, A. S. 2010. Requirement of the NF-kappaB subunit p65/RelA for K-Ras-induced lung tumorigenesis. *Cancer Res.*, 70(9): 3537-3546.

7. Baud, V. & Karin, M. 2009. Is NF-kappaB a good target for cancer therapy? Hopes and pitfalls. *Nat.Rev.Drug Discov.*, 8(1): 33-40.

8. Baum, B., Settleman, J., & Quinlan, M. P. 2008. Transitions between epithelial and mesenchymal states in development and disease. *Semin.Cell Dev.Biol.*, 19(3): 294-308.

9. Bissell, M. J. & Radisky, D. 2001. Putting tumours in context. *Nat.Rev.Cancer*, 1(1): 46-54.

10. Buck, E., Eyzaguirre, A., Barr, S., Thompson, S., Sennello, R., Young, D., Iwata, K. K., Gibson, N. W., Cagnoni, P., & Haley, J. D. 2007. Loss of homotypic cell adhesion by epithelial-mesenchymal transition or mutation limits sensitivity to epidermal growth factor receptor inhibition. *Mol.Cancer Ther.*, 6(2): 532-541.

11. Campbell, K. J. & Perkins, N. D. 2006. Regulation of NF-kappaB function. *Biochem.Soc.Symp.*,(73): 165-180.

12. Ceppi, P., Mudduluru, G., Kumarswamy, R., Rapa, I., Scagliotti, G. V., Papotti, M., & Allgayer, H. 2010. Loss of miR-200c expression induces an aggressive, invasive, and chemoresistant phenotype in non-small cell lung cancer. *Mol.Cancer Res.*, 8(9): 1207-1216.

13. Chen, L. F. & Greene, W. C. 2004. Shaping the nuclear action of NF-kappaB. *Nat.Rev.Mol.Cell Biol.*, 5(5): 392-401.

14. Chen, L. M., Kuo, C. H., Lai, T. Y., Lin, Y. M., Su, C. C., Hsu, H. H., Tsai, F. J., Tsai, C. H., Huang, C. Y., & Tang, C. H. 2011. RANKL increases migration of human lung cancer cells through intercellular adhesion molecule-1 up-regulation. *J.Cell Biochem.*, 112(3): 933-941.

15. Chen, L., Fischle, W., Verdin, E., & Greene, W. C. 2001. Duration of nuclear NF-kappaB action regulated by reversible acetylation. *Science*, 293(5535): 1653-1657.

16. Chen, W., Li, Z., Bai, L., & Lin, Y. 2011b. NF-kappaB in lung cancer, a carcinogenesis mediator and a prevention and therapy target. *Front Biosci.*, 16: 1172-1185.

17. Chen, W., Li, Z., Bai, L., & Lin, Y. 2011a. NF-kappaB in lung cancer, a carcinogenesis mediator and a prevention and therapy target. *Front Biosci.*, 16: 1172-1185.

18. Chen, X., Lingala, S., Khoobyari, S., Nolta, J., Zern, M. A., & Wu, J. 2011. Epithelial mesenchymal transition and hedgehog signaling activation are associated with chemoresistance and invasion of hepatoma subpopulations. *J.Hepatol.*.

19. Cho, W. C. 2007. OncomiRs: the discovery and progress of microRNAs in cancers. *Mol.Cancer*, 6: 60.

20. Choi, S. S., Syn, W. K., Karaca, G. F., Omenetti, A., Moylan, C. A., Witek, R. P., Agboola, K. M., Jung, Y., Michelotti, G. A., & Diehl, A. M. 2010. Leptin promotes the myofibroblastic phenotype in hepatic stellate cells by activating the hedgehog pathway. *J.Biol.Chem.*, 285(47): 36551-36560.

21. Chow, J. Y., Ban, M., Wu, H. L., Nguyen, F., Huang, M., Chung, H., Dong, H., & Carethers, J. M. 2010. TGF-beta downregulates PTEN via activation of NF-kappaB in pancreatic cancer cells. *Am.J.Physiol Gastrointest.Liver Physiol*, 298(2): G275-G282.

22. Cui, W., Wang, L. H., Wen, Y. Y., Song, M., Li, B. L., Chen, X. L., Xu, M., An, S. X., Zhao, J., Lu, Y. Y., Mi, X. Y., & Wang, E. H. 2010. Expression

and regulation mechanisms of Sonic Hedgehog in breast cancer. *Cancer Sci.*, 101(4): 927-933.

23. Dolgin, E. 2011. Companies race to develop first Hedgehog inhibitor cancer drug. *Nat.Med.*, 17(5): 523.

24. Feldmann, G., Dhara, S., Fendrich, V., Bedja, D., Beaty, R., Mullendore, M., Karikari, C., Alvarez, H., Iacobuzio-Donahue, C., Jimeno, A., Gabrielson, K. L., Matsui, W., & Maitra, A. 2007. Blockade of hedgehog signaling inhibits pancreatic cancer invasion and metastases: a new paradigm for combination therapy in solid cancers. *Cancer Res.*, 67(5): 2187-2196.

25. Frederick, B. A., Helfrich, B. A., Coldren, C. D., Zheng, D., Chan, D., Bunn, P. A., Jr., & Raben, D. 2007. Epithelial to mesenchymal transition predicts gefitinib resistance in cell lines of head and neck squamous cell carcinoma and non-small cell lung carcinoma. *Mol.Cancer Ther.*, 6(6): 1683-1691.

26. Fukaya, M., Isohata, N., Ohta, H., Aoyagi, K., Ochiya, T., Saeki, N., Yanagihara, K., Nakanishi, Y., Taniguchi, H., Sakamoto, H., Shimoda, T., Nimura, Y., Yoshida, T., & Sasaki, H. 2006. Hedgehog signal activation in gastric pit cell and in diffuse-type gastric cancer. *Gastroenterology*, 131(1): 14-29.

27. Gillan, L., Matei, D., Fishman, D. A., Gerbin, C. S., Karlan, B. Y., & Chang, D. D. 2002. Periostin secreted by epithelial ovarian carcinoma is a ligand for alpha(V)beta(3) and alpha(V)beta(5) integrins and promotes cell motility. *Cancer Res.*, 62(18): 5358-5364.

28. Gingery, A., Bradley, E. W., Pederson, L., Ruan, M., Horwood, N. J., & Oursler, M. J. 2008. TGF-beta coordinately activates TAK1/MEK/AKT/NFkB and SMAD pathways to promote osteoclast survival. *Exp.Cell Res.*, 314(15): 2725-2738.

29. Gomes, L. R., Terra, L. F., Sogayar, M. C., & Labriola, L. 2011. Epithelial-Mesenchymal Transition: Implications in Cancer Progression and Metastasis. *Curr.Pharm.Biotechnol.*.

30. Gort, E. H., Groot, A. J., van der, W. E., van Diest, P. J., & Vooijs, M. A. 2008. Hypoxic regulation of metastasis via hypoxia-inducible factors. *Curr.Mol.Med.*, 8(1): 60-67.

31. Gregory, P. A., Bert, A. G., Paterson, E. L., Barry, S. C., Tsykin, A., Farshid, G., Vadas, M. A., Khew-Goodall, Y., & Goodall, G. J. 2008. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. *Nat.Cell Biol.*, 10(5): 593-601.

32. Gregory, P. A., Bracken, C. P., Bert, A. G., & Goodall, G. J. 2008. MicroRNAs as regulators of epithelial-mesenchymal transition. *Cell Cycle*, 7(20): 3112-3118.

33. Grilli, M., Chiu, J. J., & Lenardo, M. J. 1993. NF-kappa B and Rel: participants in a multiform transcriptional regulatory system. *Int.Rev.Cytol.*, 143: 1-62.

34. Guo, Y., Pakneshan, P., Gladu, J., Slack, A., Szyf, M., & Rabbani, S. A. 2002. Regulation of DNA methylation in human breast cancer. Effect on the urokinase-type plasminogen activator gene production and tumor invasion. *J.Biol.Chem.*, 277(44): 41571-41579.

35. Gupta, S. C., Kim, J. H., Prasad, S., & Aggarwal, B. B. 2010. Regulation of survival, proliferation, invasion, angiogenesis, and metastasis of tumor cells through modulation of inflammatory pathways by nutraceuticals. *Cancer Metastasis Rev.*, 29(3): 405-434.

36. Gupta, S. V., McGowen, R. M., Callewaert, D. M., Brown, T. R., Li, Y., & Sarkar, F. H. 2005. Quantitative chemiluminescent immunoassay for NF-kappaB-DNA binding activity. *J.Immunoassay Immunochem.*, 26(2): 125-143.

37. Hacker, H. & Karin, M. 2006. Regulation and function of IKK and IKK-related kinases. *Sci.STKE.*, 2006(357): re13.

38. Haefner, B. 2005. The transcription factor NF-kappaB as drug target. *Prog.Med.Chem.*, 43: 137-188.

39. Herbst, R. S., Ansari, R., Bustin, F., Flynn, P., Hart, L., Otterson, G. A., Vlahovic, G., Soh, C. H., O'Connor, P., & Hainsworth, J. 2011. Efficacy of bevacizumab plus erlotinib versus erlotinib alone in advanced non-small-cell lung cancer after failure of standard first-line chemotherapy (BeTa): a double-blind, placebo-controlled, phase 3 trial. *Lancet*, 377(9780): 1846-1854.

40. Herbst, R. S. & Sandler, A. 2008. Bevacizumab and erlotinib: a promising new approach to the treatment of advanced NSCLC. *Oncologist.*, 13(11): 1166-1176.

41. Hotz, H. G., Hotz, B., & Buhr, H. J. 2011. Genes associated with epithelial-mesenchymal transition: possible therapeutic targets in ductal pancreatic adenocarcinoma? *Anticancer Agents Med.Chem.*, 11(5): 448-454.

42. Hugo, H., Ackland, M. L., Blick, T., Lawrence, M. G., Clements, J. A., Williams, E. D., & Thompson, E. W. 2007. Epithelial--mesenchymal and mesenchymal--epithelial transitions in carcinoma progression. *J.Cell Physiol*, 213(2): 374-383.

43. Inman, G. J. 2011. Switching TGFbeta from a tumor suppressor to a tumor promoter. *Curr.Opin.Genet.Dev.*, 21(1): 93-99.

44. Iwano, M., Plieth, D., Danoff, T. M., Xue, C., Okada, H., & Neilson, E. G. 2002. Evidence that fibroblasts derive from epithelium during tissue fibrosis. *J.Clin.Invest*, 110(3): 341-350.

45. Jemal, A., Bray, F., Center, M. M., Ferlay, J., Ward, E., & Forman, D. 2011. Global cancer statistics. *CA Cancer J.Clin.*, 61(2): 69-90.

46. Jemal, A., Siegel, R., Ward, E., Hao, Y., Xu, J., Murray, T., & Thun, M. J. 2008. Cancer statistics, 2008. *CA Cancer J.Clin.*, 58(2): 71-96.

47. Jemal, A., Siegel, R., Ward, E., Hao, Y., Xu, J., & Thun, M. J. 2009. Cancer statistics, 2009. *CA Cancer J.Clin.*, 59(4): 225-249.

48. Johansson, D., Andersson, C., Moharer, J., Johansson, A., & Behnam-Motlagh, P. 2010. Cisplatin-induced expression of Gb3 enables verotoxin-1 treatment of cisplatin resistance in malignant pleural mesothelioma cells. *Br.J.Cancer*, 102(2): 383-391.

49. Kalluri, R. & Weinberg, R. A. 2009. The basics of epithelial-mesenchymal transition. *J.Clin.Invest*, 119(6): 1420-1428.

50. Kang, Y. & Massague, J. 2004. Epithelial-mesenchymal transitions: twist in development and metastasis. *Cell*, 118(3): 277-279.

51. Karin, M. & Greten, F. R. 2005. NF-kappaB: linking inflammation and immunity to cancer development and progression. *Nat.Rev.Immunol.*, 5(10): 749-759.

52. Kasperczyk, H., Baumann, B., Debatin, K. M., & Fulda, S. 2009. Characterization of sonic hedgehog as a novel NF-kappaB target gene that promotes NF-kappaB-mediated apoptosis resistance and tumor growth in vivo. *FASEB J.*, 23(1): 21-33.

53. Katoh, Y. & Katoh, M. 2008. Hedgehog signaling, epithelial-to-mesenchymal transition and miRNA (review). *Int.J.Mol.Med.*, 22(3): 271-275.

54. Kim, J. H., Jang, Y. S., Eom, K. S., Hwang, Y. I., Kang, H. R., Jang, S. H., Kim, C. H., Park, Y. B., Lee, M. G., Hyun, I. G., Jung, K. S., & Kim, D. G. 2007. Transforming growth factor beta1 induces epithelial-to-mesenchymal transition of A549 cells. *J.Korean Med.Sci.*, 22(5): 898-904.

55. Kodama, J., Hasengaowa, Kusumoto, T., Seki, N., Matsuo, T., Ojima, Y., Nakamura, K., Hongo, A., & Hiramatsu, Y. 2007. Prognostic significance of stromal versican expression in human endometrial cancer. *Ann.Oncol.*, 18(2): 269-274.

56. Kong, D., Li, Y., Wang, Z., Banerjee, S., Ahmad, A., Kim, H. R., & Sarkar, F. H. 2009. miR-200 regulates PDGF-D-mediated epithelial-mesenchymal

transition, adhesion, and invasion of prostate cancer cells. *Stem Cells*, 27(8): 1712-1721.

57. Krishnan, V., Pereira, F. A., Qiu, Y., Chen, C. H., Beachy, P. A., Tsai, S. Y., & Tsai, M. J. 1997. Mediation of Sonic hedgehog-induced expression of COUP-TFII by a protein phosphatase. *Science*, 278(5345): 1947-1950.

58. Krutovskikh, V. A. & Herceg, Z. 2010. Oncogenic microRNAs (OncomiRs) as a new class of cancer biomarkers. *Bioessays*, 32(10): 894-904.

59. Larue, L. & Bellacosa, A. 2005. Epithelial-mesenchymal transition in development and cancer: role of phosphatidylinositol 3' kinase/AKT pathways. *Oncogene*, 24(50): 7443-7454.

60. Leng, S., Bernauer, A. M., Zhai, R., Tellez, C. S., Su, L., Burki, E. A., Picchi, M. A., Stidley, C. A., Crowell, R. E., Christiani, D. C., & Belinsky, S. A. 2011. Discovery of common SNPs in the miR-205/200 family-regulated epithelial to mesenchymal transition pathway and their association with risk for non-small cell lung cancer. *Int.J.Mol.Epidemiol.Genet.*, 2(2): 145-155.

61. Libermann, T. A. & Baltimore, D. 1990. Activation of interleukin-6 gene expression through the NF-kappa B transcription factor. *Mol.Cell Biol.*, 10(5): 2327-2334.

62. Lin, Y., Bai, L., Chen, W., & Xu, S. 2010. The NF-kappaB activation pathways, emerging molecular targets for cancer prevention and therapy. *Expert.Opin.Ther.Targets.*, 14(1): 45-55.

63. Liotta, L. A. 1986. Tumor invasion and metastases--role of the extracellular matrix: Rhoads Memorial Award lecture. *Cancer Res.*, 46(1): 1-7.

64. Maitah, M. Y., Ali, S., Ahmad, A., Gadgeel, S., & Sarkar, F. H. 2011a. Up-regulation of sonic hedgehog contributes to TGF-beta1-induced epithelial to mesenchymal transition in NSCLC cells. *PLoS.One.*, 6(1): e16068.

65. Maitah, M. Y., Ali, S., Ahmad, A., Gadgeel, S., & Sarkar, F. H. 2011b. Up-regulation of sonic hedgehog contributes to TGF-beta1-induced epithelial to mesenchymal transition in NSCLC cells. *PLoS.One.*, 6(1): e16068.

66. Mani, S. A., Guo, W., Liao, M. J., Eaton, E. N., Ayyanan, A., Zhou, A. Y., Brooks, M., Reinhard, F., Zhang, C. C., Shipitsin, M., Campbell, L. L., Polyak, K., Brisken, C., Yang, J., & Weinberg, R. A. 2008. The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell*, 133(4): 704-715.

67. Massague, J. 2008. TGFbeta in Cancer. *Cell*, 134(2): 215-230.

68. Merchant, A. A. & Matsui, W. 2010. Targeting Hedgehog--a cancer stem cell pathway. *Clin.Cancer Res.*, 16(12): 3130-3140.

69. Meylan, E., Dooley, A. L., Feldser, D. M., Shen, L., Turk, E., Ouyang, C., & Jacks, T. 2009. Requirement for NF-kappaB signalling in a mouse model of lung adenocarcinoma. *Nature*, 462(7269): 104-107.

70. Miyamoto, S. 2011a. Nuclear initiated NF-kappaB signaling: NEMO and ATM take center stage. *Cell Res.*, 21(1): 116-130.

71. Miyamoto, S. 2011b. Nuclear initiated NF-kappaB signaling: NEMO and ATM take center stage. *Cell Res.*, 21(1): 116-130.

72. Mocellin, S., Pasquali, S., & Pilati, P. 2009. Oncomirs: from tumor biology to molecularly targeted anticancer strategies. *Mini.Rev.Med.Chem.*, 9(1): 70-80.

73. Montesano, R., Matsumoto, K., Nakamura, T., & Orci, L. 1991. Identification of a fibroblast-derived epithelial morphogen as hepatocyte growth factor. *Cell*, 67(5): 901-908.

74. Morel, A. P., Lievre, M., Thomas, C., Hinkal, G., Ansieau, S., & Puisieux, A. 2008. Generation of breast cancer stem cells through epithelial-mesenchymal transition. *PLoS.One.*, 3(8): e2888.

75. Nakanishi, C. & Toi, M. 2005. Nuclear factor-kappaB inhibitors as sensitizers to anticancer drugs. *Nat.Rev.Cancer*, 5(4): 297-309.

76. Nakashima, H., Nakamura, M., Yamaguchi, H., Yamanaka, N., Akiyoshi, T., Koga, K., Yamaguchi, K., Tsuneyoshi, M., Tanaka, M., & Katano, M. 2006b. Nuclear factor-kappaB contributes to hedgehog signaling pathway activation through sonic hedgehog induction in pancreatic cancer. *Cancer Res.*, 66(14): 7041-7049.

77. Nakashima, H., Nakamura, M., Yamaguchi, H., Yamanaka, N., Akiyoshi, T., Koga, K., Yamaguchi, K., Tsuneyoshi, M., Tanaka, M., & Katano, M. 2006a. Nuclear factor-kappaB contributes to hedgehog signaling pathway activation through sonic hedgehog induction in pancreatic cancer. *Cancer Res.*, 66(14): 7041-7049.

78. Nicolini, A., Ferrari, P. P., Fini, M. M., Borsari, V. V., Fallahi, P. P., Antonelli, A. A., Carpi, A., & Miccoli, P. P. 2011. Cancer stem cells: perspectives of new therapeutical approaches for breast cancer. *Front Biosci.(Schol.Ed)*, 3: 1486-1499.

79. Ohta, H., Aoyagi, K., Fukaya, M., Danjoh, I., Ohta, A., Isohata, N., Saeki, N., Taniguchi, H., Sakamoto, H., Shimoda, T., Tani, T., Yoshida, T., & Sasaki, H. 2009. Cross talk between hedgehog and epithelial-

mesenchymal transition pathways in gastric pit cells and in diffuse-type gastric cancers. *Br.J.Cancer*, 100(2): 389-398.

80. Omenetti, A., Bass, L. M., Anders, R. A., Clemente, M. G., Francis, H., Guy, C. D., McCall, S., Choi, S. S., Alpini, G., Schwarz, K. B., Diehl, A. M., & Whitington, P. F. 2011. Hedgehog activity, epithelial-mesenchymal transitions, and biliary dysmorphogenesis in biliary atresia. *Hepatology*, 53(4): 1246-1258.

81. Ono, M., Hirata, A., Kometani, T., Miyagawa, M., Ueda, S., Kinoshita, H., Fujii, T., & Kuwano, M. 2004. Sensitivity to gefitinib (Iressa, ZD1839) in non-small cell lung cancer cell lines correlates with dependence on the epidermal growth factor (EGF) receptor/extracellular signal-regulated kinase 1/2 and EGF receptor/Akt pathway for proliferation. *Mol.Cancer Ther.*, 3(4): 465-472.

82. Palamarchuk, A., Efanov, A., Nazaryan, N., Santanam, U., Alder, H., Rassenti, L., Kipps, T., Croce, C. M., & Pekarsky, Y. 2010. 13q14 deletions in CLL involve cooperating tumor suppressors. *Blood*, 115(19): 3916-3922.

83. Park, S. M., Gaur, A. B., Lengyel, E., & Peter, M. E. 2008. The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2. *Genes Dev.*, 22(7): 894-907.

84. Perou, C. M., Sorlie, T., Eisen, M. B., van de, R. M., Jeffrey, S. S., Rees, C. A., Pollack, J. R., Ross, D. T., Johnsen, H., Akslen, L. A., Fluge, O., Pergamenschikov, A., Williams, C., Zhu, S. X., Lonning, P. E., Borresen-Dale, A. L., Brown, P. O., & Botstein, D. 2000. Molecular portraits of human breast tumours. *Nature*, 406(6797): 747-752.

85. Pikarsky, E. & Ben-Neriah, Y. 2006. NF-kappaB inhibition: a double-edged sword in cancer? *Eur.J.Cancer*, 42(6): 779-784.

86. Polyak, K. & Weinberg, R. A. 2009. Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. *Nat.Rev.Cancer*, 9(4): 265-273.

87. Prudkin, L., Liu, D. D., Ozburn, N. C., Sun, M., Behrens, C., Tang, X., Brown, K. C., Bekele, B. N., Moran, C., & Wistuba, I. I. 2009. Epithelial-to-mesenchymal transition in the development and progression of adenocarcinoma and squamous cell carcinoma of the lung. *Mod.Pathol.*, 22(5): 668-678.

88. Radisky, D. C., Levy, D. D., Littlepage, L. E., Liu, H., Nelson, C. M., Fata, J. E., Leake, D., Godden, E. L., Albertson, D. G., Nieto, M. A., Werb, Z., & Bissell, M. J. 2005. Rac1b and reactive oxygen species mediate MMP-3-induced EMT and genomic instability. *Nature*, 436(7047): 123-127.

89. Ramalingam, S. & Belani, C. 2008. Systemic chemotherapy for advanced non-small cell lung cancer: recent advances and future directions. *Oncologist.*, 13 Suppl 1: 5-13.

90. Rho, J. K., Choi, Y. J., Lee, J. K., Ryoo, B. Y., Na, I. I., Yang, S. H., Kim, C. H., & Lee, J. C. 2009. Epithelial to mesenchymal transition derived from repeated exposure to gefitinib determines the sensitivity to EGFR inhibitors in A549, a non-small cell lung cancer cell line. *Lung Cancer*, 63(2): 219-226.

91. Riethdorf, S. & Pantel, K. 2008. Disseminated tumor cells in bone marrow and circulating tumor cells in blood of breast cancer patients: current state of detection and characterization. *Pathobiology*, 75(2): 140-148.

92. Riethdorf, S., Wikman, H., & Pantel, K. 2008. Review: Biological relevance of disseminated tumor cells in cancer patients. *Int.J.Cancer*, 123(9): 1991-2006.

93. Robson, E. J., Khaled, W. T., Abell, K., & Watson, C. J. 2006. Epithelial-to-mesenchymal transition confers resistance to apoptosis in three murine mammary epithelial cell lines. *Differentiation*, 74(5): 254-264.

94. Sabbah, M., Emami, S., Redeuilh, G., Julien, S., Prevost, G., Zimber, A., Ouelaa, R., Bracke, M., De, W. O., & Gespach, C. 2008. Molecular

signature and therapeutic perspective of the epithelial-to-mesenchymal transitions in epithelial cancers. *Drug Resist.Updat.*, 11(4-5): 123-151.

95. Saitoh, Y., Martinez, B., V, Uota, S., Hasegawa, A., Yamamoto, N., Imoto, I., Inazawa, J., & Yamaoka, S. 2010. Overexpression of NF-kappaB inducing kinase underlies constitutive NF-kappaB activation in lung cancer cells. *Lung Cancer*, 70(3): 263-270.

96. Sanchez-Tillo, E., Lazaro, A., Torrent, R., Cuatrecasas, M., Vaquero, E. C., Castells, A., Engel, P., & Postigo, A. 2010. ZEB1 represses E-cadherin and induces an EMT by recruiting the SWI/SNF chromatin-remodeling protein BRG1. *Oncogene*, 29(24): 3490-3500.

97. Sarkar, F. H., Li, Y., Wang, Z., & Kong, D. 2010. The role of nutraceuticals in the regulation of Wnt and Hedgehog signaling in cancer. *Cancer Metastasis Rev.*, 29(3): 383-394.

98. Sasaki, H., Dai, M., Auclair, D., Fukai, I., Kiriyama, M., Yamakawa, Y., Fujii, Y., & Chen, L. B. 2001. Serum level of the periostin, a homologue of an insect cell adhesion molecule, as a prognostic marker in nonsmall cell lung carcinomas. *Cancer*, 92(4): 843-848.

99. Sato, N., Leopold, P. L., & Crystal, R. G. 1999. Induction of the hair growth phase in postnatal mice by localized transient expression of Sonic hedgehog. *J.Clin.Invest*, 104(7): 855-864.

100. Sethi, G., Sung, B., & Aggarwal, B. B. 2008. Nuclear factor-kappaB activation: from bench to bedside. *Exp.Biol.Med.(Maywood.)*, 233(1): 21-31.

101. Sheng, W., Wang, G., La Pierre, D. P., Wen, J., Deng, Z., Wong, C. K., Lee, D. Y., & Yang, B. B. 2006. Versican mediates mesenchymal-epithelial transition. *Mol.Biol.Cell*, 17(4): 2009-2020.

102. Sheridan, C., Kishimoto, H., Fuchs, R. K., Mehrotra, S., Bhat-Nakshatri, P., Turner, C. H., Goulet, R., Jr., Badve, S., & Nakshatri, H. 2006. CD44+/. *Breast Cancer Res.*, 8(5): R59.

103. Shih, J. Y. & Yang, P. C. 2011. The EMT Regulator Slug and Lung Carcinogenesis. *Carcinogenesis*.

104. Shipitsin, M., Campbell, L. L., Argani, P., Weremowicz, S., Bloushtain-Qimron, N., Yao, J., Nikolskaya, T., Serebryiskaya, T., Beroukhim, R., Hu, M., Halushka, M. K., Sukumar, S., Parker, L. M., Anderson, K. S., Harris, L. N., Garber, J. E., Richardson, A. L., Schnitt, S. J., Nikolsky, Y., Gelman, R. S., & Polyak, K. 2007. Molecular definition of breast tumor heterogeneity. *Cancer Cell*, 11(3): 259-273.

105. Shrader, M., Pino, M. S., Brown, G., Black, P., Adam, L., Bar-Eli, M., Dinney, C. P., & McConkey, D. J. 2007. Molecular correlates of

gefitinib responsiveness in human bladder cancer cells. *Mol.Cancer Ther.*, 6(1): 277-285.

106. Singh, A. & Settleman, J. 2010. EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer. *Oncogene*, 29(34): 4741-4751.

107. Slade, M. J., Payne, R., Riethdorf, S., Ward, B., Zaidi, S. A., Stebbing, J., Palmieri, C., Sinnett, H. D., Kulinskaya, E., Pitfield, T., McCormack, R. T., Pantel, K., & Coombes, R. C. 2009. Comparison of bone marrow, disseminated tumour cells and blood-circulating tumour cells in breast cancer patients after primary treatment. *Br.J.Cancer*, 100(1): 160-166.

108. Sorlie, T., Perou, C. M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., Hastie, T., Eisen, M. B., van de, R. M., Jeffrey, S. S., Thorsen, T., Quist, H., Matese, J. C., Brown, P. O., Botstein, D., Eystein, L. P., & Borresen-Dale, A. L. 2001. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc.Natl.Acad.Sci.U.S.A*, 98(19): 10869-10874.

109. Sun, S. C. 2011c. Non-canonical NF-kappaB signaling pathway. *Cell Res.*, 21(1): 71-85.

110. Sun, S. C. 2011a. Non-canonical NF-kappaB signaling pathway. *Cell Res.*, 21(1): 71-85.

111. Sun, S. C. 2011b. Non-canonical NF-kappaB signaling pathway. *Cell Res.*, 21(1): 71-85.

112. Syn, W. K., Jung, Y., Omenetti, A., Abdelmalek, M., Guy, C. D., Yang, L., Wang, J., Witek, R. P., Fearing, C. M., Pereira, T. A., Teaberry, V., Choi, S. S., Conde-Vancells, J., Karaca, G. F., & Diehl, A. M. 2009. Hedgehog-mediated epithelial-to-mesenchymal transition and fibrogenic repair in nonalcoholic fatty liver disease. *Gastroenterology*, 137(4): 1478-1488.

113. Takebe, N., Warren, R. Q., & Ivy, S. P. 2011. Breast cancer growth and metastasis: interplay between cancer stem cells, embryonic signaling pathways and epithelial-to-mesenchymal transition. *Breast Cancer Res.*, 13(3): 211.

114. Takeyama, Y., Sato, M., Horio, M., Hase, T., Yoshida, K., Yokoyama, T., Nakashima, H., Hashimoto, N., Sekido, Y., Gazdar, A. F., Minna, J. D., Kondo, M., & Hasegawa, Y. 2010. Knockdown of ZEB1, a master epithelial-to-mesenchymal transition (EMT) gene, suppresses anchorage-independent cell growth of lung cancer cells. *Cancer Lett.*, 296(2): 216-224.

115. Teh, M. T., Wong, S. T., Neill, G. W., Ghali, L. R., Philpott, M. P., & Quinn, A. G. 2002. FOXM1 is a downstream target of Gli1 in basal cell carcinomas. *Cancer Res.*, 62(16): 4773-4780.

116. Teraishi, F., Zhang, L., Guo, W., Dong, F., Davis, J. J., Lin, A., & Fang, B. 2005. Activation of c-Jun NH2-terminal kinase is required for gemcitabine's cytotoxic effect in human lung cancer H1299 cells. *FEBS Lett.*, 579(29): 6681-6687.

117. Thayer, S. P., di Magliano, M. P., Heiser, P. W., Nielsen, C. M., Roberts, D. J., Lauwers, G. Y., Qi, Y. P., Gysin, S., Fernandez-del, C. C., Yajnik, V., Antoniu, B., McMahon, M., Warshaw, A. L., & Hebrok, M. 2003. Hedgehog is an early and late mediator of pancreatic cancer tumorigenesis. *Nature*, 425(6960): 851-856.

118. Thiery, J. P. 2003. Epithelial-mesenchymal transitions in development and pathologies. *Curr.Opin.Cell Biol.*, 15(6): 740-746.

119. Thiery, J. P. & Sleeman, J. P. 2006. Complex networks orchestrate epithelial-mesenchymal transitions. *Nat.Rev.Mol.Cell Biol.*, 7(2): 131-142.

120. Thomson, S., Buck, E., Petti, F., Griffin, G., Brown, E., Ramnarine, N., Iwata, K. K., Gibson, N., & Haley, J. D. 2005. Epithelial to mesenchymal transition is a determinant of sensitivity of non-small-cell

lung carcinoma cell lines and xenografts to epidermal growth factor receptor inhibition. *Cancer Res.*, 65(20): 9455-9462.

121. Tsuji, T., Ibaragi, S., & Hu, G. F. 2009. Epithelial-mesenchymal transition and cell cooperativity in metastasis. *Cancer Res.*, 69(18): 7135-7139.

122. Turley, E. A., Veiseh, M., Radisky, D. C., & Bissell, M. J. 2008. Mechanisms of disease: epithelial-mesenchymal transition--does cellular plasticity fuel neoplastic progression? *Nat.Clin.Pract.Oncol.*, 5(5): 280-290.

123. Umeda, H., Ozaki, N., Mizutani, N., Fukuyama, T., Nagasaki, H., Arima, H., & Oiso, Y. 2010. Protective effect of hedgehog signaling on cytokine-induced cytotoxicity in pancreatic beta-cells. *Exp.Clin.Endocrinol.Diabetes*, 118(10): 692-698.

124. Vallabhapurapu, S. & Karin, M. 2009. Regulation and function of NF-kappaB transcription factors in the immune system. *Annu.Rev.Immunol.*, 27: 693-733.

125. van Den Brink, G. R., de Santa, B. P., & Roberts, D. J. 2001. Development. Epithelial cell differentiation--a Mather of choice. *Science*, 294(5549): 2115-2116.

126. Vincan, E. & Barker, N. 2008. The upstream components of the Wnt signalling pathway in the dynamic EMT and MET associated with colorectal cancer progression. *Clin.Exp.Metastasis*, 25(6): 657-663.

127. Wajant, H. & Scheurich, P. 2011a. TNFR1-induced activation of the classical NF-kappaB pathway. *FEBS J.*, 278(6): 862-876.

128. Wajant, H. & Scheurich, P. 2011b. TNFR1-induced activation of the classical NF-kappaB pathway. *FEBS J.*, 278(6): 862-876.

129. Wang, Z., Banerjee, S., Li, Y., Rahman, K. M., Zhang, Y., & Sarkar, F. H. 2006. Down-regulation of notch-1 inhibits invasion by inactivation of nuclear factor-kappaB, vascular endothelial growth factor, and matrix metalloproteinase-9 in pancreatic cancer cells. *Cancer Res.*, 66(5): 2778-2784.

130. Watkins, D. N., Berman, D. M., Burkholder, S. G., Wang, B., Beachy, P. A., & Baylin, S. B. 2003. Hedgehog signalling within airway epithelial progenitors and in small-cell lung cancer. *Nature*, 422(6929): 313-317.

131. Witta, S. E., Gemmill, R. M., Hirsch, F. R., Coldren, C. D., Hedman, K., Ravdel, L., Helfrich, B., Dziadziuszko, R., Chan, D. C., Sugita, M., Chan, Z., Baron, A., Franklin, W., Drabkin, H. A., Girard, L., Gazdar, A. F., Minna, J. D., & Bunn, P. A., Jr. 2006. Restoring E-cadherin expression

increases sensitivity to epidermal growth factor receptor inhibitors in lung cancer cell lines. *Cancer Res.*, 66(2): 944-950.

132. Xu, F. G., Ma, Q. Y., & Wang, Z. 2009. Blockade of hedgehog signaling pathway as a therapeutic strategy for pancreatic cancer. *Cancer Lett.*, 283(2): 119-124.

133. Yamasaki, A., Kameda, C., Xu, R., Tanaka, H., Tasaka, T., Chikazawa, N., Suzuki, H., Morisaki, T., Kubo, M., Onishi, H., Tanaka, M., & Katano, M. 2010a. Nuclear factor kappaB-activated monocytes contribute to pancreatic cancer progression through the production of Shh. *Cancer Immunol.Immunother.*, 59(5): 675-686.

134. Yamasaki, A., Kameda, C., Xu, R., Tanaka, H., Tasaka, T., Chikazawa, N., Suzuki, H., Morisaki, T., Kubo, M., Onishi, H., Tanaka, M., & Katano, M. 2010b. Nuclear factor kappaB-activated monocytes contribute to pancreatic cancer progression through the production of Shh. *Cancer Immunol.Immunother.*, 59(5): 675-686.

135. Yang, J. & Weinberg, R. A. 2008. Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. *Dev.Cell*, 14(6): 818-829.

136. Yauch, R. L., Januario, T., Eberhard, D. A., Cavet, G., Zhu, W., Fu, L., Pham, T. Q., Soriano, R., Stinson, J., Seshagiri, S., Modrusan, Z., Lin,

C. Y., O'Neill, V., & Amler, L. C. 2005. Epithelial versus mesenchymal phenotype determines in vitro sensitivity and predicts clinical activity of erlotinib in lung cancer patients. *Clin.Cancer Res.*, 11(24 Pt 1): 8686-8698.

137. Yilmaz, M., Christofori, G., & Lehembre, F. 2007. Distinct mechanisms of tumor invasion and metastasis. *Trends Mol.Med.*, 13(12): 535-541.

138. Yoo, Y. A., Kang, M. H., Kim, J. S., & Oh, S. C. 2008. Sonic hedgehog signaling promotes motility and invasiveness of gastric cancer cells through TGF-beta-mediated activation of the ALK5-Smad 3 pathway. *Carcinogenesis*, 29(3): 480-490.

139. Yoshino, I., Kometani, T., Shoji, F., Osoegawa, A., Ohba, T., Kouso, H., Takenaka, T., Yohena, T., & Maehara, Y. 2007. Induction of epithelial-mesenchymal transition-related genes by benzo[a]pyrene in lung cancer cells. *Cancer*, 110(2): 369-374.

140. Yu, F., Yao, H., Zhu, P., Zhang, X., Pan, Q., Gong, C., Huang, Y., Hu, X., Su, F., Lieberman, J., & Song, E. 2007. let-7 regulates self renewal and tumorigenicity of breast cancer cells. *Cell*, 131(6): 1109-1123.

141. Yu, L., Lin, Q., Liao, H., Feng, J., Dong, X., & Ye, J. 2010. TGF-beta1 induces podocyte injury through Smad3-ERK-NF-kappaB pathway

and Fyn-dependent TRPC6 phosphorylation. *Cell Physiol Biochem.*, 26(6): 869-878.

142. Yuan, Z., Goetz, J. A., Singh, S., Ogden, S. K., Petty, W. J., Black, C. C., Memoli, V. A., Dmitrovsky, E., & Robbins, D. J. 2007. Frequent requirement of hedgehog signaling in non-small cell lung carcinoma. *Oncogene*, 26(7): 1046-1055.

143. Zhang, H. J., Wang, H. Y., Zhang, H. T., Su, J. M., Zhu, J., Wang, H. B., Zhou, W. Y., Zhang, H., Zhao, M. C., Zhang, L., & Chen, X. F. 2011. Transforming growth factor-beta1 promotes lung adenocarcinoma invasion and metastasis by epithelial-to-mesenchymal transition. *Mol.Cell Biochem.*.

144. Zhao, C., Chen, A., Jamieson, C. H., Fereshteh, M., Abrahamsson, A., Blum, J., Kwon, H. Y., Kim, J., Chute, J. P., Rizzieri, D., Munchhof, M., VanArsdale, T., Beachy, P. A., & Reya, T. 2009. Hedgehog signalling is essential for maintenance of cancer stem cells in myeloid leukaemia. *Nature*, 458(7239): 776-779.

145. Zhao, X., Laver, T., Hong, S. W., Twitty, G. B., Jr., Devos, A., Devos, M., Benveniste, E. N., & Nozell, S. E. 2011. An NF-kappaB p65-cIAP2 link is necessary for mediating resistance to TNF-alpha induced cell death in gliomas. *J.Neurooncol.*, 102(3): 367-381.

146. Zheng, X., Yao, Y., Xu, Q., Tu, K., & Liu, Q. 2010. Evaluation of glioma-associated oncogene 1 expression and its correlation with the expression of sonic hedgehog, E-cadherin and S100a4 in human hepatocellular carcinoma. *Mol.Med.Report.*, 3(6): 965-970.

ABSTRACT

HEDGEHOG SIGNALING: A POTENTIAL THERAPEUTIC TARGET FOR NON-SMALL LUNG CANCER

by

MA'IN MAITAH

August 2011

Advisor: Dr. Fazlul H. Sarkar

Major: Pathology

Degree: Doctor of Philosophy

The American Cancer Society estimated that 222,520 Americans were diagnosed with lung cancer and 157,300 died of lung cancer in 2010 (Jemal et al. 2009, 225-249;Jemal et al. 2011, 69-90). The clinical outcome of patients diagnosed with non-small cell lung cancer (NSCLC), the major lung cancer sub-types, is very poor, which calls for innovative research for finding novel therapeutic targets and agents for better treatment outcome.

Emerging evidences have suggested that a phenomenon called Epithelial-to-Mesenchymal Transition (EMT), which shares similar molecular characteristics with cancer stem-like cells, contributes to lung cancer treatment failure. In view of the fact that EMT process has been implicated in the two important biological processes that are accountable for cancer-related deaths; the progression of cancer cells to a distant organ, and the acquisition of resistance to conventional cancer therapeutics (Leng et al. 2011, 145-155;Shih and Yang 2011;Zhang et al.

2011), needs further in-depth investigation. Therefore, further mechanistic understanding of the role of EMT in lung cancer is very important, which was the focus of my investigation.

In this study, I found for the first time that the induction of EMT by chronic exposure of A549 NSCLC cells to TGF-β1 (A549-M cells) led to the up-regulation of sonic hedgehog (Shh) both at the mRNA and protein levels causing activation of hedgehog signaling. These results were also reproduced in another NSCLC cell line (H2030), and these results were further associated with the induction of EMT phenotype. Induction of EMT was found to be consistent with aggressive characteristics such as increased clonogenic growth, cell motility and invasion. The aggressiveness of these cells was attenuated by the treatment of A549-M cells with pharmacological inhibitors of Hh signaling in addition to Shh knock-down by siRNA. The inhibition of Hh signaling by pharmacological inhibitors led to the reversal of EMT phenotype as confirmed by the reduction of mesenchymal markers such as ZEB1 and Fibronectin, and induction of epithelial marker E-cadherin. In addition, knock-down of Shh by siRNA significantly attenuated EMT induction by TGF-β1.

Next, I examined the involvement of NF-κB, as downstream of TGF-β-receptor signaling, and in the up-regulation of Shh. I tested whether NF-κB activity could indeed be induced by TGF-β1 in our model cell culture system. To gain further mechanistic insight, I also tested whether the active NF-κB could

bind to consensus sequences on Shh promoter in cell free system. Additionally, I determined the binding of the active NF-κB to Shh promoter in the cell by CHIP assay. Finally, I evaluated whether NF-κB can activate Shh expression directly using Shh promoter-luciferase reporter assays. The results clearly showed that TGF-β1 induced NF-κB activity in NSCLC cell line A549. In addition, active NF-κB bound to its consensus sequences in the Shh promoter. Likewise, on CHIP assay, I found that active NF-κB bound to Shh promoter. Also, by utilizing promoter-luceferase reporter assays, I confirmed that upon TGF-β1 treatment NF-κB was physically bound to Shh promoter, and activated its transcription and expression. Furthermore, I identified Shh gene as a target for miR15a and miR-16, the two miRNAs that has been reported to be deleted or down-regulated in NSCLCs.

To verify whether pharmacological inhibitors of Hh signaling pathway can sensitize mesenchymal tumors with resistant phenotype to standard therapy, and whether the sensitization of this tumor is a consequence of EMT reversal caused by the inhibition of hh signaling. For such studies, I treated NSCLCs A549-M, H1299, and H1650 cells with Hh inhibitors GDC-0449 for three days, and then further treated these cells with either cisplatin or erlotinib for different time points. My data showed an increase in NSCLCs sensitivity towards cisplatin and erlotinib, suggesting that Hh inhibitors sensitized drug resistant

mesenchymal phenotypic NSCLC tumor cells to standard therapy by reverting EMT phenotype.

From these results, I conclude that chronic exposure of cancer cell *in vivo* to TGF-β1 leads to the acquisition of EMT phenotype as documented by this in vitro study using NSCLC cells. The induction of EMT mediated by TGF-β1 was in part due to transcriptional activation of Shh which was due in part resulting from the activation of NF-κB and SMAD signaling in NSCLC cells, and causing tumor cell aggressiveness *in vitro* and in animal model *in vivo*. The acquisition of EMT resulted in therapeutic resistance and the treatment of these cells with Hh signaling inhibitors (either Shh siRNA or synthetic inhibitor, GDC-0449) caused reversal of EMT and sensitized cells to conventional therapeutics. Together, these results suggest that reversal of EMT by Hh inhibitors would be useful for achieving better treatment outcome in patients diagnosed with NSCLC.

AUTOBIOGRAPHICAL STATEMENT

MA'IN MAITAH

EDUCATION

2011 Ph.D. in Pathology, Wayne State University, Detroit, USA

2001 M.S. in Health science, Wayne state University, Detroit, USA

1996 B.Pharm. Jordan University of Science and Technology, Irbid, Jordan

PUBLICATIONS:

- **Maitah MY**, Shadan A, Ahmad A, Gadgeel S, Sarkar FH (2011) Up-regulation of sonic hedgehog contributes to TGF-β1-induced epithelial to mesenchymal transition in NSCLC cells. PLoS One. 2011 Jan 13;6(1):e16068.

- Qazi A, Pal J, **Maitah M**, Fulciniti M, Pelluru D, et al. (2010) Anticancer activity of a broccoli derivative, sulforaphane, in barrett adenocarcinoma: potential use in chemoprevention and as adjuvant in chemotherapy. Transl Oncol 3: 389-399.

- Ahmad A, Wang Z, Ali R, **Maitah MY**, Kong D, et al. (2010) Apoptosis-inducing effect of garcinol is mediated by NF-kappaB signaling in breast cancer cells. J Cell Biochem 109: 1134-1141.

- **Maitah MY**, Shadan A, Gadgeel S, Sarkar FH (2011) The role of GDC-0449, a hedgehog (Hh) pathway inhibitor, on epithelial–mesenchymal transition (EMT) in non-small cell lung cancer (NSCLC) cells lines and its effect on erlotinib and cisplatin. Abstract will be presented at The ASCO Annual Meeting, Chicago, Illinois.

- **Maitah MY**, Shadan A, Ahmad A, Gadgeel S, Sarkar FH (2011) TGF-β1-induced EMT is mediated by activation of sonic hedgehog signaling in NSCLC cells. Abstract presented at The 102nd AACR Annual Meeting, Orlando, Florida.