ABSTRACT

Wnt signaling pathway has recently been recognized as a critical pathway in lung carcinogenesis. Some of the cancers that the Wnt pathway is involved in include colorectal, and breast cancers. However, the roles of Wnt pathway components are contradictory as well. These ambiguities are a result of multiple branch paths that it is associated with. In particular, the Wnt pathway is branched off into the canonical and non-canonical subpaths, of which, each subpath crosstalks with other pathways. Many studies have been done in an effort to decipher the behaviour of Wnt components in lung cancer through gene silencing techniques and knock-out models of a particular gene however a coordinated expression of Wnt components in the context of defining the involvement of the canonical and non-canonical pathways have not been explored.

We investigated the role of the Wnt pathway in lung squamous cell carcinoma (SCC) by first establishing normal expression patterns of Wnt components in normal lung and deducing the aberrant expression of key components of the canonical and non-canonical pathway in tumor. In this study, 19 Wnt pathway genes representative of both canonical and non-canonical pathway were chosen for expression analysis via relative RT-PCR. The data suggested that both the canonical and non-canonical pathways are active in the normal lungs. In lung tumor, the expression of β-catenin/canonical pathway is surprisingly down-regulated in 50% of the samples while the non-canonical pathway components are up-regulated. These findings provide evidence that not all lung tumors are affected by the canonical Wnt pathway and that the non-canonical pathway may serve to be just as important in driving the proliferation of lung cancer cells.
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ABBREVIATIONS

BAC: Bacterial Artificial Chromosomes
cDNA: Complimentary DNA
CGH: Comparative Genomic Hybridization
CT: Computed Tomography
dATP: Deoxyadenosine Triphosphate
dCTP: Deoxycytosine Triphosphate
DEPC: Diethyl Pyrocarbonate
dGTP: Deoxyguanisine Triphosphate
dH2O: distilled H2O
DNA: Deoxyribonucleic Acid
dNTP: Deoxynucleotide Triphosphate
dTTP: Deoxythymidine Triphosphate
GOF: Gain of Function
LOF: Loss of Function
MTC (panels): Multiple Tissue cDNA
NaOAc: Sodium Acetate
NSCLC: Non-Small Cell Lung Cancer
PCP: Planar Cell Polarity
PCR: Polymerase Chain Reaction
PET: Positron Emission Tomography
RDD: Trade name for proprietary solution from Qiagen
RNA: Ribonucleic Acid
RPE: Trade name proprietary solution from Qiagen
RT-PCR: Reverse-Transcription-PCR
SCC: Squamous Cell Carcinoma
SCLC: Small Cell Lung Cancer
SMRT: Sub-megabase Resolution Tiling (Set/Array)
TBE: Tris/Borate/EDTA buffer solution
TNM (staging): Tumor, Lymph Nodes, Metastasis staging
TSG: Tumor Suppressor Gene

Gene name formats for DNA, RNA, and Gene Product:
Wnt: Gene Name/family
Wnt: DNA/RNA
WNT: Gene Product (Protein)
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CHAPTER 1: INTRODUCTION

1.1 Origin of the Wnt gene

The first Wnt gene has been discovered over 20 years ago. The term Wnt is a fusion between the names of two orthologous genes found in the mouse and drosophila. Int-1 (for integration) is a frequent site of viral integration in mouse mammary tumor virus (MMTV)-induced mammary tumors (Nusse and Varmus 1982). The gene product of Int-1 was later confirmed to have a causative role in mammary tumorigenesis. Sequence analysis of the Int-1 gene revealed that it was orthologous to the Drosophila segment polarity gene known as Wingless (Wg). These two terms were then combined to form the name Wnt for the mammalian Int-1 gene and its paralogs (Rijsewijk et al. 1987; Nusse et al. 1991; Ilyas 2005).

1.2 Classification of the Wnt pathway

The Wnt gene family has grown significantly since the original discovery in the mouse. A total of 19 members have been added to the family of Wnt genes. The Wnt proteins are small (39-46 kD) lipid-modified secreted glycoproteins (Willert et al. 2003) which play key roles in both embryogenesis and mature tissues. During embryogenesis, controlled Wnt signaling is crucial for proper patterning of cells and stem cell fate. The result of abnormal Wnt signaling in humans due to germline mutations lead to congenital defects (Jordan et al. 2001; Rodova et al. 2002; Niemann et al. 2004). In mature tissues,
there are reports that the Wnt pathway is involved in normal tissue maintenance by way of regulating the self-renewal of stem cells (Ross et al. 2000; Reya et al. 2003; Willert et al. 2003; He et al. 2004).

Although a lot of components that have been identified to date show contributions in various ways to the Wnt pathway, the implications of its roles remain to be elucidated in many types of organ systems. One of the difficulties in predicting the role or a phenotype for the Wnt pathway owes much credit to its genetic redundancy in signaling (Bhanot et al. 1999) – multiple gene members are found in each gene family of the Wnt pathway. Historically, the Wnt pathway is classified into two groups: the canonical and the non-canonical pathway. The canonical route stabilizes β-catenin and activates the transcription of TCF/LEF target genes (Papkoff et al. 1996) - this causes the activation of various pro-proliferative (Tetsu and McCormick 1999; Zhang et al. 2001) and potential metastatic genes (Agnihotri et al. 2001) in cancer. The non-canonical side activates the planar-cell-polarity (PCP)-like pathway that guides cell movements during gastrulation and the Wnt/Ca²⁺ pathway (Veeman et al. 2003).

1.3 The Wnt canonical/β-catenin pathway in the OFF state

The Wnt ligands are lipid modified in the cytoplasm and then packaged to be secreted. Wnt proteins are palmitoylated on a conserved cysteine residue that is conserved in all family members and lipid-modification is likely to occur to all Wnt proteins. Although secreted molecules are usually glycosylated and not acylated, removal of palmitate moiety or site-directed mutagenesis of the conserved cysteine
residue results in no secretion of Wnt and therefore lipid modulation is crucial for Wnt activity (Nusse 2003). Once outside the cell, they can activate signal transduction through an autocrine (Bafico et al. 2004) as well as in a paracrine (Jue et al. 1992) manner. Multiple Wnt ligands can bind to multiple Wnt receptors and vice versa. To control the activity of the Wnt pathway many players are involved to manage multiple checkpoints of the pathway. It is not certain why redundancies exist in the pathway. Since evolution had not selected against these redundancies from the gene pool perhaps there are potentially crucial branching pathways that have yet to be discovered that involve these alleged redundant components. Starting from the extracellular side, there are several secreted modulators of the Wnt pathway including the secreted frizzled-related protein family (sFRP) (Moon et al. 1997), Wnt-inhibitory factor-1 (WIF-1) (Hsieh et al. 1999), Cerberus (CER) (Piccolo et al. 1999), and Dickkopf (DKK) (Nusse 2001). The sFRPs, WIF-1, and CER proteins antagonize Wnt signaling by directly binding to Wnt ligands and preventing Wnt interactions with the cell surface receptors, the Frizzled proteins (Figure 1). However, controversies regarding the role of Wnt components always exist and therefore, there is evidence from one study that sFRPs can also interact with Frizzled receptors, which is thought to produce an inactive receptor complex (Bafico et al. 1999). The DKK protein inhibits Wnt signaling by interacting with the extracellular domain of the co-receptor low-density-lipoprotein-receptor-related proteins (LRPs) (Bafico et al. 2001; Semenov et al. 2001). This interaction causes the recruitment of a single pass transmembrane cell surface receptor, KREMEN 1/2 (Mao et al. 2001; Mao et al. 2002; Mao and Niehrs 2003), and together they promote the endocytosis of the LRP co-receptor and its eventual degradation (Mao et al. 2002). On the intracellular side, the
prevention of the Wnt ligand from binding onto its cell surface receptors promotes the
downstream degradation of the β-catenin protein. This is achieved through the tumor
suppressor AXIN which serves as a scaffolding protein that brings together protein
phosphatase 2A (PP2A) (Ikeda et al. 2000), glycogen synthase-3β (GSK-3β) (Yamamoto
et al. 1999), adenomatous polyposis coli (APC) (Kishida et al. 1998) and β-catenin. The
formation of this complex prompts GSK-3β to phosphorylate the β-catenin protein at four
amino-terminal residues (Behrens et al. 1998; Kishida et al. 1998; van Noort et al. 2002).
In order for GSK-3β to phosphorylate β-catenin, the motif for the GSK-3β recognition
site must be primed by phosphorylation of the first amino acid – serine 45 by casein kinase
I alpha (CK 1α). Thereafter, the remaining 3 residues: threonine-41, serine-37, and
serine-33, are sequentially phosphorylated by GSK-3β (Liu et al. 2002). Phosphorylated
β-catenin is then recognized by β-tansducin repeat containing protein (β-TrCP) as a
protein that is to be ubiquitylated. Ubiquitylated β-catenin is then destined to be
destroyed by 26S proteasome (Hart et al. 1999; Kitagawa et al. 1999; Liu et al. 1999).
Figure 1: Overview of the Wnt canonical/β-catenin signaling pathway in the OFF state. Extracellular inhibitors like Dickkopf (Dkk) and the secreted frizzled-related protein (sFRP) family regulate the activation of the Wnt pathway. Without Wnt ligand activation, β-catenin is phosphorylated by the degradation complex comprised of Axin, APC, GSK-3β, and PP2A. Phosphorylated β-catenin is targeted for ubiquitylation by the β-TrCP complex. Ubiquitylated β-catenin is then degraded by 26S proteasome.
1.4 The Wnt canonical/β-catenin pathway in the ON state

Without intervening inhibitors, the activation of the canonical pathway occurs upon the binding of the Wnt ligand to the cell surface receptors, as shown in Figure 2. The Wnt receptor complex that helps activate the canonical pathway contains two components: a member of the frizzled family (a family of 10 serpentine receptors in the human) (Bhanot et al. 1996; Wang et al. 1996; He et al. 1997) and a member of the lipoprotein receptor-related proteins 5 and 6 (LRP5/6) (Wehrli et al. 2000). Activation of signal transduction is mediated through the interactions of the Wnt ligands with the Frizzled receptors and one of the LRP co-receptors (Pinson et al. 2000; Mao et al. 2001). After binding of the Wnt ligand to the receptor complex, the AXIN, PP2A, GSK-3β, APC degradation complex disassembles. First, the intracellular protein dishevelled (Dvl) is recruited to the cell membrane where it interacts with the intracellular domain of the frizzled receptor and also gets phosphorylated by casein kinase I epsilon (CK Iε) (Kishida et al. 2001). The phosphorylation of DVL allows it to form a complex with FRAT1 and GSK-3β, hence suppressing the action of GSK3β by dismembering it from the degradation complex (Lee et al. 2001). When this is happening, AXIN is also recruited to the cell membrane to initiate its own demise through an LRP5/6-mediated degradation (Mao et al. 2001). The goal of these processes lead to the destabilization of the degradation complex, which allows β-catenin to escape phosphorylation. Free β-catenin is able to translocate into the nucleus by unknown mechanisms where it acts as a co-
Figure 2: Overview of the Wnt canonical/β-catenin signaling pathway in the ON state. Activation of the Wnt pathway is initiated when the Wnt ligand binds onto its cell surface receptors that is comprised of the Frizzled family of serpentine receptors and the co-receptors lipoprotein-receptor-related proteins (LRP) 5/6. The interaction of the Wnt ligand with the cell surface receptors recruits cytoplasmic Dvl protein near the cell membrane where it gets phosphorylated. Phosphorylation of Dvl disassembles the degradation complex by pulling GSK-3β away from the complex via the help of a GSK-3β binding protein (FRAT1). Axin is pulled towards LRP5/6 where it is eventually degraded and β-catenin is set free in the cytoplasm. β-catenin is then able to translocate to the nucleus by unknown mechanism where it acts as a co-activator for the transcription of target genes.
activator to initiate the transcription of LEF/TCF target genes that have been implicated in many types of cancer (Eastman and Grosschedl 1999).

1.5 The Wnt non-canonical pathway

The non-canonical pathway is the less understood Wnt pathway (Weidinger and Moon 2003) especially in terms of cancer. The lack of knowledge is attributed to the scarceness of studies done in this particular area. So far, the non-canonical pathway is further sub-divided into several other pathways that include Wnt/Ca\(^{2+}\) pathway and the Wnt/PCP pathway. Within the Wnt/PCP pathway, it is further divided into the Wnt/C-Jun N-terminal kinase (JNK) pathway and the Wnt/Rho signaling pathway (Figure 3). The Wnt/PCP pathway is important for regulating the polarity of cells through the regulation of the actin cytoskeletal organization (Boutros and Mlodzik 1999; Adler and Lee 2001) and it is achieved through the activation of the Rho and the JNK pathway. Activation of the small GTPase Rho requires the dishevelled associated activator of morphogenesis protein (DAAM) which in turn leads to the activation of the Rho-associated kinase (ROCK) to mediate cytoskeletal re-organization (Habas et al. 2001; Veeman et al. 2003) in vertebrate gastrulation. Activation of the JNK pathway during development is also crucial for the correct cell movements during vertebrate gastrulation in the Xenopus (Tada et al. 2002). The Wnt/Ca\(^{2+}\) pathway causes an increase in the intracellular Ca\(^{2+}\) concentration and the activation of protein kinase C (PKC) and Ca\(^{2+}\)/calmodulin-dependent kinase II (CaMKII). In the developing chick wing, over-expression of CaMKII increases the number of slow muscle fibers (Anakwe et al. 2003).
Figure 3: Overview of the Wnt non-canonical pathways.
Amongst the large family of Wnt components, Wnt5a, Wnt11, and Fzd2 have been reported to be involved in the non-canonical pathways but the function of which remains to be explored (Liu et al. 1999; Liu et al. 1999; Sheldahl et al. 1999).

1.6 Wnt pathway in the normal lung

The role of the Wnt pathway has mainly been exemplified through developmental studies. The canonical pathway has been shown to play a critical role during the development of the lung. For example, significant $\beta$-catenin nuclear localization found in the fetal lung suggests active Wnt activity (Eberhart and Argani 2001). Knockout studies of $\beta$-catenin in mice has also revealed that it is crucial to the formation of the peripheral airways of the lungs, and conducting gas exchange (Mucenski et al. 2003). However, too much Wnt signaling can be hazardous as can be drawn from the constitutive activation of the canonical Wnt pathway using $\beta$-catenin-LEF1 fusion protein. The formation of peripheral airways was still functional, but the lung was reduced in size and lacked alveoli (Okubo and Hogan 2004). Therefore, strict regulation of the Wnt pathway is central to the normal development of the lung.

While organogenesis is no longer needed in the adult tissue, the canonical Wnt pathway is thought to be responsible for the maintenance of normal tissues through its involvement in the self-renewal of stem cells (Ross et al. 2000; Reya et al. 2003; Willert et al. 2003; He et al. 2004). For example, treatment of embryonic stem cells with an inhibitor of GSK-3$\beta$ activates the canonical Wnt pathway and sustains pluripotency and self-renewal (Sato et al. 2004). Generally, Wnt signaling retains cells in a low
differentiation state (Kleber and Sommer 2004) and if one assumes that the maintenance of adult organs is governed by stem cells and that stem cells require β-catenin activation, then the role of Wnt signaling would be important. Similar to the intestine (Pinto et al. 2003) and skin (Alonso and Fuchs 2003), stem cell niches in proximal and distal airways of the lungs exist as well (Borthwick et al. 2001). A recent report discovered that the canonical/β-catenin pathway plays a role in repair processes in adult bronchial epithelial cells (Steel et al. 2005) and that nuclear β-catenin immunoreactivity was still found in the adult lungs of 44 years of age reflecting that the Wnt pathway may in fact serve as a cell maintenance pathway (Eberhart and Argani 2001).

For the non-canonical Wnt pathway, Wnt2 is expressed in the mesenchyme of the developing lung (Levay-Young and Navre 1992). Another non-canonical pathway gene, Wnt11, is responsible for activating the PCP pathway and has been reported to be expressed in the mesenchyme of the mature lung (Lako et al. 1998) but its role is not known at this point. From the various studies shown, the non-canonical pathway may contribute to lung development just like the canonical pathway but their roles and mechanisms in the adult lung still need more investigation.

1.7 Wnt pathway in cancer

Many players of the Wnt pathway have been reported in human cancer. However, there is not one experiment that can pinpoint the exact role of the Wnt pathway in any of these cancers. Cancer is a muti-step process and with the ever increasing family of Wnt related genes, its involvement affects multiple pathways in cancer development.
Therefore, it is often hard to identify the genes and processes of the Wnt pathway that are important to the progression of a specific type of cancer. Ever since the discovery of the Wnt gene scientists have tried to find some sort of commonality in the disruption of the Wnt pathway in cancer. As explained earlier, the canonical Wnt signaling pathway is regulated at multiple checkpoints to control the activity of the transcriptional co-activator, β-catenin. Constitutive activation of β-catenin in human, a common fate for most malfunctioning Wnt signaling in cancer, will lead to the transcription of various genes that can promote cell growth or migration. Such target genes include cyclin D1, c-myc, c-Jun, Sox9, matrilysin (MMP-7) etc (He et al. 1998; Mann et al. 1999; Shtutman et al. 1999; Tetsu and McCormick 1999; Blache et al. 2004). Cyclin D1 and c-myc are well established oncogenes that regulate cell growth and proliferation. Deregulation of c-Jun expression can have positive influence on the development of malignant transformation (Schutte et al. 1989). The Sox genes have been reported in various types of tumors but current studies only suggest a correlative relationship with the progression of cancer (Roh et al. 2004). Matrix metalloproteinases (MMPs) participate in extracellular matrix remodeling and degradation - MMP7, MMP14, and MMP26 are all targets of the canonical/β-catenin pathway (Brabletz et al. 1999; Takahashi et al. 2002; Marchenko et al. 2004). Activation of MMPs may influence a variety of processes. They are proteolytic enzymes that allow cells to acquire invasive characteristics (Tan et al. 2005). MMPs can also contribute to angiogenesis in cancer through the activation of osteopontin (Paoni et al. 2003) and interaction with vascular endothelial growth factor (VEGF – a direct target β-catenin target) (Agnihotri et al. 2001; Xu et al. 2001) which is required for angiogenesis.
In cancer, most of the problems associated with the Wnt pathway have been attributed to mutations induced in several downstream members (downstream of β-catenin) of the pathway that render it constitutively active. Mutation of the β-catenin protein is an example where the change causes a gain of function mutation (GOF) that allows it to be constitutively active as a transcriptional co-factor. Such a mutation is evident in numerous reports including colorectal, prostate, gastric cancer and etc (Ilyas et al. 1997; Morin et al. 1997; Voeller et al. 1998; Park et al. 1999; Chesire et al. 2000; Clements et al. 2002). APC and AXIN are also affected by mutational changes as well. Under normal conditions, both APC and AXIN work to regulate the amount of free cytoplasmic β-catenin; mutations invoke a loss of function (LOF) for both members rendering them incapable of sequestering β-catenin (Rowan et al. 2000; Satoh et al. 2000). There have also been increasing reports of deregulated expression changes of upstream Wnt components in a growing range of tumors. These evidence suggest that other than mutational changes of downstream regulators of the Wnt pathway, expression changes involving upstream members (upstream of β-catenin) may also contribute to cancer as well.

Some of the deregulated upstream components will be discussed here. Aberrant Wnt signaling due to components upstream of β-catenin has been exemplified in colorectal cancer cell lines, which demonstrated that epigenetic inactivation of sFRP genes allow constitutive Wnt signaling (Suzuki et al. 2004). As mentioned before sFRP proteins are extracellular regulators that block Wnt signaling by binding to Wnt ligands. WIF1 is another extracellular Wnt inhibitory protein that was discovered to be down-regulated in prostate, breast, lung and bladder cancer (Wissmann et al. 2003). Another
group of scientists later found that WIF1 is also silenced by promoter hypermethylation in human lung cancer. As for Wnt ligands themselves, Wnt2 is up-regulated in primary gastric, colorectal cancer (Katoh 2001), and breast cancer (Huguet et al. 1994; Dale et al. 1996). Wnt2 is the second Wnt member found and is responsible for morphological transformation of C57MG cells through stabilization of β-catenin/canonical pathway. At the cell membrane, components including the Frizzled family and LRP co-receptors have also been implicated in cancer. So far, LRP5 has been reported to be overexpressed in only osteosarcoma (Hoang et al. 2004) while the Frizzled family is overexpressed in prostate, ovarian cancer (Wissmann et al. 2003), head and neck squamous cell carcinoma (Rhee et al. 2002), colorectal cancer (Holcombe et al. 2002), and more.

The majority of studies are concerned with the effects of the canonical Wnt pathway in cancer. Recently, there is an increasing interest for the non-canonical Wnt signaling pathway and cancer. Whether the non-canonical pathway work in parallel or antagonizes the canonical pathway is still controversial and cell context dependent. For example, WNT5a has been reported to be able to activate Nemo-like kinase (NLK) through CamkII and TAK1 (Ishitani et al. 2003). NLK is then able to phosphorylate TCF/LEF transcription factors and consequently inhibit their ability to activate the transcription of target genes (Torres et al. 1996). WNT5a can also inhibit the canonical pathway by inducing the transcriptional up-regulation of Siah2, the gene product of which can then stimulate GSK-3β independent degradation of β-catenin (Topol et al. 2003). From the studies above, the non-canonical pathway seems to be a tumor suppressor pathway. However, WNT5a expression is also able to contribute to the motility and invasiveness of UACC1273 melanoma cells (Weeraratna et al. 2002). In
addition, \textit{Wnt5a} expression is up-regulated in many types of cancer including lung, breast, and prostate cancer (Iozzo et al. 1995). Therefore, previous studies suggest that one role does not fit all cell types and much investigation is needed to define the role of the non-canonical pathway in different disease type.

\textit{1.8 Lung Cancer}

Lung cancer is a highly aggressive disease and is still the leading cause of cancer deaths throughout the world (Minna et al. 2002; Smith and Khuri 2004). All lung cancer can be classified into one of the two categories: small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC). SCLC is a very aggressive disease that accounts for 18% to 20% of all lung cancer reported. Patients who are diagnosed with extensive SCLC have a survival window of about 7-10 months. NSCLC accounts for the rest of the primary lung tumors and 3 histological subtypes are associated with it: squamous cell carcinoma (SCC), adenocarcinoma and large cell carcinoma.

The most common symptoms of advanced NSCLC are dyspnea, cough, pain, loss of appetite, and hemoptysis. Weight loss is usually associated with these symptoms and during the final months of the patient, all the symptoms become more frequent, severe and worsen (Natale and Zaretsky 2002). To date, the most valuable prognostic factor for survival in NSCLC patients remains to be tumor staging. The reason to this is that early stage disease can be surgically resected (Singhal et al. 2005). A major advance in lung cancer staging is through the introduction of $^{18}$F-2-fluoro-2-deoxyglucose (FDG) positron emission tomography (PET). The increase of FDG in tumor cells compared to normal cells is how this technology identifies malignant lesions and it is 95% sensitive
(Pieterman et al. 2000) with a false-positive rate of approximately 25% (Gould et al. 2003). However, recurrence rates remain high even after the patient has been cured with surgery. The 5 year survival rates range from 70% for stage IA disease to 40% for stage IIB tumors (Singhal et al. 2005). Nodal invasion in TNM staging is a good indication that the tumor has spread microscopically at the time that it is diagnosed as an early stage disease. Therefore, the need to develop markers to predict survival before and after lung cancer surgery is also very important.

Squamous cell carcinoma of the lung is the second most common subtype out of the three subclasses of NSCLC; it accounts for about 30% of all lung cancers and is the most closely linked subtype to smoking (Alberg and Samet 2003). SCC of the lung arises mainly from the central airways, from one of the major bronchus (Figure 4A & B), rather than a peripheral location. A general gross description of SCC is the presence of pale white to tan coloured hard granular neoplasms while uninvolved lung may often show emphysema or other smoking related pathology (Figure 4B). On the microscopic scale, squamous cells are large and have irregular nuclei. Pathologists usually refer to the shape of squamous cells as ‘polygonal’ and they are usually arranged in sheets. Within the nucleus, they have coarse nuclear chromatin with large nucleoli (Figure 4C & D).
Figure 4: A: Pictorial representation of the human lung (www.adam.com). B: Squamous cell carcinoma of the lung arising centrally and obstructing the right main bronchus. C: Medium power microscopic appearance of squamous cell carcinoma after hematoxylin and eosin Y staining. There are nests of polygonal cells with pink cytoplasm. D: High magnification of squamous cell carcinoma. The nuclei are hyperchromatic and angular (individual pictures from WebPath, at the University of Utah: http://www.medlib.med.utah.edu/WebPath/webpath.html).
Many pathways that are being studied in the field of lung cancer affect cellular processes such as cycle regulation, apoptosis, angiogenesis, growth factors, DNA repair, cell motility, and coagulation. Oncogenes that contribute to the pathogenesis of lung cancer include \textit{c-myc}, mutated \textit{K-ras} and overexpressed \textit{EGFR, cyclin D1}, and \textit{BCL2}. Evidence to support cellular immortality in lung cancers is the expression of telomerase RNA (hTR) and the catalytic component (hTERT). Tumor suppressors genes (TSGs) that are frequently involved include \textit{p53}, and \textit{p16}. One of the major mode of inactivation of the expression of many TSGs is through promoter hypermethylation (Zochbauer-Muller et al. 2001). The cellular signaling pathways that are deregulated in lung cancer include the Rb/p16/cyclin D1, p53/MDM2/p19ARF, Wnt/\(\beta\)-catenin, \textit{EGFR/Ras} and telomerase pathways (Minna et al. 2002).

Recent advances in chemotherapy utilizes small molecules and monoclonal antibodies to target signal transduction pathways (Spicer and Harper 2005). Some of these include angiogenesis inhibitors, epidermal growth factor receptor (EGFR) inhibitors, \textit{HER2/neu} receptor inhibitors and other tyrosine kinase inhibitors, inhibitors of Ras activation and function, matrix metalloproteinase inhibitors, cyclin dependent kinase inhibitors and inhibitors of autocrine and paracrine growth factor loops (Minna et al. 2002). Although there have been improvements in the median survival of the disease, increasing efforts to target important growth factor receptors, oncogenes and tumor-suppressor genes known to be aberrant in lung cancer is the only way to enhance improvements in the treatment of this disease. By combining standard clinical variables (i.e., tumor size, differentiation, or stage), with genetic or biochemical characteristics of
the tumors (gene expression or protein levels of selected candidates), one can better predict the patient’s prognosis or responses to a certain therapy.

1.9 Wnt in lung cancer

Parallel to its roles in stem cell self-renewal, tissue regeneration, and lung development, the Wnt pathway is also closely involved in tumorigenesis. Deregulated expression of the components belonging to the Wnt pathway are evident in NSCLC (Ju et al. 2005). For example, transcriptional silencing of tumor suppressors such as sFRP1 (Fukui et al. 2005) and WIF1 (Mazieres et al. 2004) that regulate the Wnt pathway have been documented. Over-expression of oncogenes such as Wnt5a and Dvl3 are also found in NSCLC (Uematsu et al. 2003). Finally, Garnis et al. detected genetic alterations of chromosome 1p that contained homologs of Wnt pathway components (Garnis et al. 2005).

Recently there is increasing interests on how Wnt signaling in stem cells might play a role in NSCLC. The importance of the Wnt pathway has been demonstrated in stem cell maintenance (Korinek et al. 1998; Karhadkar et al. 2004), tissue repair, and regeneration (Shackel et al. 2001; Polesskaya et al. 2003) in various organs. Due to the role that the Wnt pathway takes in the above processes, it is possible that stem cell niches may harbor disrupted Wnt signaling and therefore prevent stem cells from returning to a normal quiescent state (He et al. 2005). In fact, there have been reports that disrupted maintenance and self-renewal activity of cells in the gut epithelium leads to epithelial cancers and LOF mutation in the APC gene is responsible (Harada et al. 1999). Although
it was previously mentioned that strict regulation of Wnt signaling is key in development, the mechanisms of regulating Wnt signaling is rather confusing as in the case of controlling stem cell fate. The canonical Wnts have the ability to fulfill diverse functions in stem cells. Stem cells from different locations tend to interpret Wnt signals in different ways (Kleber and Sommer 2004) which makes it hard to predict. Different stem cell types differentially respond to canonical Wnt signaling and undergo either self-renewal or lineage commitment. There is evidence to show that over time, stem cells acquire intrinsic differences that can have a great influence on how they interpret Wnt signals and eventually, determining their fate decisions (Kruger et al. 2002). The Wnt pathway activity has been associated with chronic tissue injury as well as carcinogenesis but the two processes have yet to be causally linked (Coussens and Werb 2002). Can constant tissue injury leading to intrinsic changes in the stem cells cause them to attain a state of constant renewal and eventually resulting in carcinogenesis? If this is the case, it would make sense that in the lung where it is constantly exposed to environmental insults, the chances of some of these cells shifting into a constant or uncontrollable renewal state are much higher.

Despite the accumulation of reports demonstrating the involvement of the Wnt pathway in various human cancers, relatively little is known about the pathway in lung cancer. Mutational events of the destruction complex are common in other types of cancer but rarely found in lung cancer. To date, aberrant signaling of the Wnt pathway in lung cancer is mostly due to expressional changes. For example, \textit{Wnt1} is overexpressed in NSCLC cell lines and primary tumor tissues (He et al. 2004). \textit{Wnt2} was also demonstrated to be overexpressed in non-small cell lung cancer (You et al. 2004).
1.10 Whole Genome Tiling Resolution BAC Array CGH

Whole genome tiling resolution BAC array CGH allows genome-wide analysis of tumor genomes. This technology allows the identification of genetic alterations that may be specific to a certain type of cancer (Aarts et al. 2006; Garnis et al. 2006) or a particular stage in carcinogenesis (Duesberg et al. 2005). The gains and/or losses of gene regions may contain genes that are differentially expressed as a result of the genetic alterations (Garnis et al. 2005). However, not all genetic alterations will be reflected at the expression level and not all expression changes will be the result of altered genetic copy number changes. Therefore, the application of this technology with expression analysis allows the validation of observed genetic changes and also gives insight as to what happens downstream.

1.11 Hypotheses & Objectives

The hypothesis for this thesis:
The Wnt pathway, which includes the canonical and non-canonical pathway, is disrupted in non-small-cell lung carcinoma (NSCLC). If the hypothesis is true then the expression pattern of genes in both pathways will be disrupted.
Three objectives have been designed in order to test the hypothesis:

The first objective of this thesis is to identify Wnt genes in the lung by data-mining various databases and literature to look for components of the Wnt pathway that are implicated in lung cancer.

The second objective is to establish gene expression profile of Wnt components in the normal lung. To do this, 20 frozen NSCLC samples with matched normal lung samples were acquired from St. Paul's Hospital.

The third objective is to identify Wnt genes that are differentially expressed between normal and tumor lung. Expression of components from the Wnt pathway in tumor lung was compared to that of their matched normals for all 20 patients.
2.1 Frozen Tissue Samples, Dissection & DNA/RNA Extraction

Frozen lung SCC tumor and matched lung normal samples from the same patient were obtained from St. Paul's Hospital. Tumor samples were stored at -80°C and were OCT embedded just prior to sectioning. A total of 18 sections were obtained from each sample at 10 microns in thickness. Hematoxylin and Eosin Y staining was performed on the first slide of each sample and evaluated by a lung pathologist, Dr. Julia Flint at Vancouver General Hospital, to identify preferred areas for dissection.

Frozen normal lung tissue samples were crushed in the presence of liquid nitrogen and then homogenized in Trizol lysis buffer (Invitrogen, Burlington, ON, CAN). The RNA was subsequently extracted with a standard acidic phenol-chloroform protocol followed by precipitation with ethanol.

For frozen lung tumors, sectioned slides were fixed in diethyl pyrocarbonate (DEPC)-treated 70% ethanol and stored dried at -80°C until RNA extraction. Marked sites of tumor cells were dissected, and homogenized in a guanidine thiocyanate and β-mercaptopethanol containing proprietary lysis buffer from RNeasy Mini Kit (Qiagen, Mississauga, ON, CAN). The homogenate was then further homogenized using a QIAshredder spin column (Qiagen). One volume of 70% ethanol was added to the homogenized lysate, mixed and applied to the RNeasy mini column where it was spun down for 15s at ≥10,000 rpm. The flow-through was discarded and the column was washed with a 70% ethanol containing buffer (RW1 buffer, Qiagen). The column was
then subjected to 27.2 Kunitz units of DNase I in 70 μl of RDD buffer mixture (Qiagen) to get rid of any traces of DNA. The DNase I reaction was incubated at room temperature for 15 min, after which, the column was washed once with RW1 buffer (Qiagen) and twice with RPE buffer (Qiagen). Total RNA was eluted with 30 μl of RNase-free dH₂O. To ensure there are no trace sources of contamination, the eluted RNA was further cleaned with one round of standard acidic phenol-chloroform extraction and then precipitated with 100% ethanol and DEPC-treated NaOAc. The RNA pellet was washed with DEPC-treated 70% ethanol and then resuspended in DEPC-treated dH₂O.

2.2 Whole Genome SMRT Array Hybridization

All 20 lung SCC samples with matched normals were assayed for genetic alterations using the whole-genome tiling path BAC array in comparative genomic hybridization experiments. The submegabase-resolution tiling set (SMRT) array contains 32,433 overlapping BAC-derived DNA segments that provide tiling coverage over the human physical genome map (Ishkanian et al. 2004). For detailed description of the SMRT array refer to Watson et al. (Watson et al. 2004).

2.3 cDNA Synthesis & Semi Quantitative PCR

For cDNA synthesis, 50 ng of total RNA was used in a 20 μl reaction including 0.5 μg of poly(dT) primers (5’-TTTTTTTTTTTTTTTTTTTTTT-3’), 4 μl of 5X First Strand buffer (250 mM Tris-Cl, pH 8.3, 375 mM KCl, 15 mM MgCl₂), 2 μl of 0.1 M DTT, 1 μl
of 10 mM dNTP (i.e. 10 mM each dATP, dGTP, dCTP, and dTTP at neutral pH), and 1μl of 200 units of SUPERSCRIPT II (Invitrogen, Burlington, ON, CAN). The reaction was incubated for 50 min at 42°C and then heat inactivated at 70°C for 15 min.

Semi-quantitative reverse transcription-PCR (RT-PCR) was used to analyze the amount of cDNA products. To confirm the absence of contaminating DNA, a set of β-actin primers was specifically designed to span an intron sequence of β-actin (5'-TCGTGCCTGACATTAAAGGAG-3'/5'-AGTACTTGCCTCAGGAGGA-3') yielding a 597 bp fragment for genomic DNA and approximately a 400 bp fragment for cDNA amplification. To assess candidate genes 1μl of cDNA from each sample was PCR amplified in a 20 μl reaction using gene specific primers (Table 1). Positive control was done using cDNA template from Clontech Human Multiple Tissue cDNA (MTC) Panels 1 and 2 (BD Biosciences Clontech, Mississauga, ON, Canada). Another set of β-actin primers were used as a reference gene to normalize the expression of the candidate genes. The primers for this β-actin gene anneals to within an exon segment of the β-actin sequence (5'-GATGTGGATCAGCAAGCA-3'/5'-GAAAGGGTGTAACGCAACT-3'). A 20 μl PCR reaction volume containing 2 μl of 10X PCR buffer, 0.4-0.8 μl 50 mM MgCl₂, 0.5 μl 10 mM dNTPs, 0.6 μl 10 μmol/μl primers (forward and reverse combined) and 0.2 μl Taq (Invitrogen) DNA polymerase (5U/μl) was used to amplify 1 μl of cDNA. The PCR program used is shown in Table 2.
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<th>Gene Name</th>
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<th>Cycles</th>
<th>Tm (°C)</th>
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<td>5'-gagccctggaagaagaggttg-3'</td>
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</tr>
<tr>
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<td>52</td>
</tr>
<tr>
<td></td>
<td>5'-gtgccgtaacaggctacac-3'</td>
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<td></td>
</tr>
<tr>
<td>Fzd3</td>
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<td>5'-gaacggcaggctcaggct-3'</td>
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<td>5'-gtgccagacactgctcaggtt-3'</td>
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<td>Wnt11</td>
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<td>31</td>
<td>64</td>
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<td>E-cadherin</td>
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<td>50</td>
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<td>5'-ccagagggctgtggccacctct-3'</td>
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<tr>
<td></td>
<td>5'-ggcgatcgcagctggtgaagaa-3'</td>
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<td>β-catenin</td>
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<tr>
<td></td>
<td>5'-agcggaggtgtgctgcttgg-3'</td>
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Table 2: PCR conditions

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<th>Steps</th>
<th>Conditions</th>
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<tr>
<td>1</td>
<td>95°C for 1 min</td>
</tr>
<tr>
<td>2</td>
<td>95°C for 30 sec</td>
</tr>
<tr>
<td>3</td>
<td>64°C for 30 sec ($\beta$-actin spanning intron)/55°C for 30 sec ($\beta$-actin without intron span)$^1$</td>
</tr>
<tr>
<td>4</td>
<td>72°C for 30 sec</td>
</tr>
<tr>
<td>5</td>
<td>Repeat steps 2 to 4 for 34 times$^1$</td>
</tr>
<tr>
<td>6</td>
<td>72°C for 10 min</td>
</tr>
</tbody>
</table>

$^1$Genes of interest were amplified with similar conditions but with variable cycles and annealing temperature, depending on the nature of the primer – see Table 1 for primer specific conditions.
Aliquots of 1 μl of β-actin PCR product for each sample was loaded onto a 10% polyacrylamide gel (37.5 acrylamide: 1 bis-acrylamide) and ran for 1.15 hours in 1X TBE at 400V. Aliquots of 1 μl of amplified PCR product with one of the above gene primers was loaded into the corresponding sample well and separated for an additional 1.15 hours at 400V. A third gene-specific amplified product of the same amount was added again into the same corresponding sample well and ran for 1 more hour at 400V (gel configuration shown in Figure 5). The gel was then stained in SYBR-Green (Roche, Laval, Quebec, Canada)/1X TBE for 20 min and scanned using a Storm phosphoimager (Molecular Dynamics).

2.4 Data Analysis

Image generated from the Storm phosphoimager was transferred to ImageQuant where (Molecular Dynamics, Piscataway, NJ, USA) band intensities were analyzed for relative gene expression comparison. Band intensities were acquired by placing rectangular objects over each band. Background is set by averaging five spots on the gel that gives the best representation of the overall background for the gel. The averaged background intensity value was subtracted from each sample’s band intensity value. For each gene, the volume intensity of each gene of interest was divided by the β-actin band intensity to obtain the intensity ratio of each gene.

To establish the gene expression pattern of the Wnt pathway components in normal lung tissue, a pairwise summation comparison of gene expression level was
Figure 5: Gel configuration
performed. For expression analysis in normal lungs, the intensity ratio of each gene was shifted by adding a constant to get rid of negative values. A trimmed mean was calculated (excluding the lower and upper 2% values) for all the genes expressed in the normal samples and a scaling factor was calculated as 500 divided by the trimmed mean. Each raw value was then multiplied by the scaling factor to create a new distribution centered at 500. The value displayed in Figure 7 is the log_{10} value of the scaled data.

For expression analysis in tumor lung, the intensity ratio of each gene in tumor was divided by the intensity ratio of genes in normals. A cutoff value of 2 fold was used as a determinant for a change in expression. Concordance data was calculated based on samples that only showed down-regulated or unchanged expression of \( \beta\)-catenin (17 samples). Raw data from the 17 samples were categorized into -1 for under-expressed genes, 0 for unchanged expression, and +1 for overexpressed genes. A percentage was then obtained based on the similarity of expression changes between two given genes.

For the Affymetrix data, scaled expression values that have been floored to 0 for negative expression indices were acquired from (Bhattacharjee et al. 2001). There were a total of 21 lung SCC samples and 17 non-matched normal lung samples. Expression of Wnt components were normalized with \( \beta\)-actin expression averaged across either tumor or normal lung samples. A t-test performed on Vassar Stats (http://faculty.vassar.edu/lowry/VassarStats.html) was used to compare the mean expression of each gene in the 21 tumor samples with the mean expression of each gene in the 17 normal samples.
CHAPTER 3: RESULTS

3.1 Data-mining of literature and databases related to Wnt pathway

Data-mining for components related to the Wnt pathway was first conducted in Pubmed literature database (www.pubmed.com). Evidence supporting the implication of Wnt pathway genes in various types of epithelial cancers include breast, colorectal, colon, prostate etc. Disruptions of the Wnt pathway in other cancers are diverse. GOF mutations of downstream components such as β-catenin and the APC gene are highly frequent events in colorectal cancer. From Table 3, no mutational changes of Wnt components have been documented so far rather, changes in expression levels are a lot more common. When comparing lung cancer studies to studies in other types of cancer there is relatively less literature on the Wnt pathway in lung cancer therefore, knowledge about it is still lacking. So far, studies have demonstrated that components upstream of β-catenin are involved in NSCLC. Specifically, loss of Wnt7a contributes to the progression of lung cancer. Deregulation of extracellular regulators, Wnt ligands and receptors have all been reported in lung cancer. A crude analysis on various databases were attempted to see if components related to the Wnt pathway showed any disruptions in lung cancer. Amongst these Affymetrix studies, the data provided by a research group at Harvard (Bhattacharjee et al. 2001) had the most complete gene descriptions to perform statistical tests on. A t-test (performed online at VassarStats) on Bhattacharjee et al.'s Affymetrix experiments showed that Wnt11, Fzd6, Dvl3, β-catenin, E-cadherin, and vimentin are disrupted in 21 lung squamous cell carcinoma samples when compared to 17
<table>
<thead>
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<th>Wnt Pathway Components</th>
<th>Change</th>
<th>Cancer Type</th>
<th>Reference</th>
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<td>Wnts</td>
<td>Overexpression</td>
<td>NSCLC</td>
<td>(You et al. 2004)</td>
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<td></td>
<td></td>
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<td>(Holcombe et al. 2002)</td>
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<td>Ovarian</td>
<td>(Ricken et al. 2002)</td>
</tr>
<tr>
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<td>Down-regulation</td>
<td>NSCLC</td>
<td>(Mazieres et al. 2004)</td>
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<td></td>
<td></td>
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<td>(Wissmann et al. 2003)</td>
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<tr>
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<td></td>
<td>Prostate</td>
<td>(Wissmann et al. 2003)</td>
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<td>Down-regulation</td>
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<td>(Fukui et al. 2005)</td>
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<td>(Klopopci et al. 2004); (Ugolini et al. 2001)</td>
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<td>Colorectal</td>
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<td>(Takada et al. 2004)</td>
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<tr>
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</tr>
<tr>
<td>Vimentin</td>
<td>&lt;0.0001</td>
<td>Significantly down</td>
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non-matched normal lung samples (Table 4). Of these, Wnt11 and β-catenin are up-regulated while Fzd6, Dvl3, E-cadherin, and vimentin are significantly down-regulated. Limited knowledge of the Wnt pathway in literature provided opportunity to select a large set of Wnt pathway components that are representative of both canonical and non-canonical pathways to be studied in lung SCC samples with matched normals.

3.2 Genetic Profiles of Wnt Loci in Squamous Cell Carcinoma

Whole genome BAC array profiles were done on 20 lung SCC samples and matched normals. The Wnt genes selected for this study are located at different loci of the genome. These loci were analyzed for genetic copy number changes between tumor and normal lung samples. Amongst these samples, 8 out of 20 show the hallmark 3p arm loss that is frequently found in NSCLC. The components, Wnt5a and β-catenin are located on the 3p arm. Other loci containing Wnt components did not display any genetic alterations.

3.3 Expression analysis of Wnt components

3.3.1 Choosing Wnt components for analysis

Increasing number of studies over the recent years have provided evidence that the canonical Wnt pathway is active in normal human lungs. However, the knowledge of the Wnt pathway and its associated subpaths in lung is still limited compared to what is known in other types of cancers. In this study, Wnt pathway components representing
different subpaths were selected for expression analysis using RT-PCR in 20 normal lung samples in an effort to reveal the various pathways that are active in normal lungs. Limited amount of samples prohibited the analysis of too many genes, therefore, genes that are known to activate multiple subpaths of the Wnt pathway were excluded from this study. The genes representing the canonical pathway in this study include \textit{Wnt1}, \textit{Wnt3a}, \textit{Fzd1}, \textit{LRP5}, \textit{LRP6}, and \textit{\beta}-\textit{catenin}. The non-canonical components chosen for this study include \textit{Wnt5a}, \textit{Wnt11}, \textit{Fzd2}, \textit{Fzd3}, and \textit{Fzd6}. In addition to the genes that are well established in their representative subpaths, a Dvl member and the sFRP family were also chosen for this analysis. Although the Dvl family has been demonstrated to activate both the canonical and non-canonical pathways in other model organisms such as drosophila, its role in the human lung is still a mystery. The most recent study catering to defining a role for the Dvl family in lung was attempted by (Uematsu et al. 2003) where they discovered that DVL3 was overexpressed in NSCLC. Such a study led us to believe that \textit{Dvl2}, another Dvl family member, may have a role in lung cancer as well. Similarly, the implication of an sFRP gene member in NSCLC has recently been revealed by (Fukui et al. 2005) and we have chosen 5 sFRP members for expression analysis in the normal and tumor lung as well.

\textit{3.3.2 Expression analysis of Wnt components in the normal lung}

The expression profile of the Wnt components in the 20 normal lung samples is shown in Figure 7. The coloured halos around the name of each gene indicate where they belong in the Wnt pathway, which is shown in Figure 6. The first column of the diagram
represents negative controls without cDNA template. Analysis of 19 genes in the normal lung samples show that both canonical and non-canonical components are present. In Figure 2, the canonical components include: Wnt1, Wnt3a, Fzd1, LRP5, LRP6, and β-catenin while they are expressed in the normal lungs with a frequency of 50%, 70%, 70%, 65%, 75%, and 75%, respectively. Although the canonical ligand, Wnt1, is shown to be expressed quite frequently in normal lungs its expression levels are actually very low. Vimentin is a transcriptional target of β-catenin and it is expressed in all 20 samples. As expected, the cell-cell adhesion protein, E-cadherin, is expressed in 80% of the normal lung samples. For the non-canonical pathway the components that are expressed in normal lungs include Wnt5a, Wnt11, Fzd2, Fzd6, and Dvl2. Wnt5a is expressed with a frequency of 80%, Wnt11-70%, Fzd2-70%, Fzd3-35%, Fzd6-95%, and Dvl2-75% in normal lungs. None of the non-canonical Wnt components have been reported to be expressed in the human adult lung before.

In addition to seeing the expression of these potential oncogenes (canonical Wnt pathway genes) in normal lungs, the sFRP gene family, which is responsible for regulating the activity of the Wnt pathway, is also expressed in normal lungs. SFRP1 is expressed in normal lungs with a frequency of 80%, sFRP2-100%, sFRP3-65%, sFRP4-65%, and sFRP5-50%. Expression of sFRP2, sFRP3, sFRP4, and sFRP5 in the human adult lung are novel findings not previously reported.
Figure 6: Schematic representation of the canonical and non-canonical Wnt pathways. (a) Canonical Wnt pathway in its OFF state. (b) Canonical Wnt pathway in its ON state. (c) Non-canonical Wnt pathway. Color halos represent genes that were used in this study. Grey: sFRP1, sFRP2, sFRP3, sFRP4, sFRP5; Blue: Wnt1, Wnt3a; Purple: Fzd1; Yellow: LRP5, LRP6; Red: β-catenin; Orange: Wnt5a, Wnt11; Teal: Fzd2, Fzd3, Fzd6; Green: Dvl2.
Figure 7: Expression profiles of 19 genes in 20 normal lung samples. Raw data was shifted by adding a constant to get rid of negative values. A trimmed mean was calculated (excluding the lower and upper 2% values) and a scaling factor was calculated as 500 divided by the trimmed mean. Each raw value was then multiplied by the scaling factor to create a new distribution centered at 500. The value displayed is the log_{10} of the scaled data. * - represent expression of genes that have not been reported in normal lung in literature.
3.3.3 Expression Analysis of Wnt Components in Lung SCC

A total of 20 lung SCC frozen samples with matched normals were used to analyze the expression levels of the 19 genes from the Wnt pathway (see Appendix 1 for actual polyacrylamide gel profiles). Data generated were normalized with $\beta$-actin controls. The expression changes illustrated on Figure 8A-T, were obtained by dividing tumor intensity ratio ($\text{gene of interest}/\beta$-actin) by the intensity ratio of normal lungs. Each gene is organized into its corresponding Wnt pathways based on the information provided by the literature and their expression changes are attached to the name of the gene. The arrangements of the genes with respect to where they exist in the cell is an accurate representation of what is known in literature so far and therefore are subject to change as more is understood about the pathway. In addition, not all of the relationships illustrated in the figures apply to the human lung as some components have not been detected in human lungs before. To investigate which Wnt pathway components are disrupted in lung tumor, pairwise comparisons between normal and tumor lung samples were performed on the Wnt pathway genes. The different colours depicted in each figure are explained by a legend on the top right hand corner of each diagram. A condensed form of Wnt profiles is shown in Figure 9. The colours in the condensed profiles represent either overexpression (red) and under-expression (green). An intense red colour or a green colour represent fold changes that are 5 fold overexpressed or under-expressed, respectively. The lighter colours represent fold changes that are between 2 to 5 fold. No colour represents changes that are lower than 2 folds. In this study, only 3 samples show 2 fold increase of $\beta$-catenin expression in lung tumor. Furthermore, 50%
of the tumors show down-regulated expression of β-catenin that are 2 fold or greater and 7 other lung tumor samples do not show changes in β-catenin expression.

A coordinate pairwise comparison of the components in the canonical and non-canonical pathway also shows that the non-canonical pathway may be involved in a subset of tumor cases where canonical pathway is inactive. For example, patient 4 (Figure 8D) shows high level disruptions of all non-canonical components with highly overexpressed transcripts while there is minimal disruption for the canonical components. On the other hand, patient 12 (Figure 8L) shows high level under-expressed canonical components indicating that the canonical Wnt pathway is inactive. At the same time, there are minimal disruptions for the non-canonical components which suggest a basal level of activity in the non-canonical pathway still exists.
Patient 1

Figure 8: Gene expression profiles of Wnt components in tumor versus normal lung samples.
Patient 2
Patient 4
Patient 5
Patient 6
Patient 7
HsFRP1  sFRP2  sFRP3  sFRP4  sFRP5

Non-canonical pathway

Wnt5a  Wnt11

Canonical pathway

Fzd2  Fzd3  Fzd6  Fzd1  LRP5  LRP6  E-cadherin

G-protein  Dvl2  GSK-3β  Axin  APC

Wnt/Ca²⁺ pathway  Planar Cell Polarity

β-catenin

Intracellular

Nucleus

TCF/LEF

Cell proliferation  vimentin

Patient 8
Non-canonical pathway

Canonical pathway

Fold Changes

>10

>5

>2

0

< -2

<-5

<-10

Fzd2 Fzd3 Fzd6 Fzd1 LRP5 LRP6 E-cadherin

G-protein

Wnt/Ca²⁺ pathway

Dvl2

GSK-3β Axin APC

β-catenin

TCF/LEF

Cell proliferation

Patient 9
Patient 10
Non-canonical pathway

Canonical pathway

Intracellular

Nucleus

Patient 11
Non-canonical pathway

Canonical pathway

Fzd2  Fzd3  Fzd6  Fzd1  LRP5  LRP6  E-cadherin

G-protein

Dvl2

Wnt/Ca\(^{2+}\) pathway

Planar Cell Polarity

GSK-3b  Axin  APC

\(\beta\)-catenin

Intracellular

Nucleus

TCF/LEF

Cell proliferation  vimentin

Patient 12
Patient 13
Patient 15
Non-canonical pathway

Canonical pathway

Patient 16
Non-canonical pathway

Canonical pathway

Wnt5a
Wnt11

Wnt1
Wnt3a

Fzd2
Fzd3
Fzd6

Fzd1
LRP5
LRP6

Dvl2

GSK-3\(\beta\)
Axin
APC

\(\beta\)-catenin

TCF/LEF

Cell proliferation

vimentin

Patient 17
Patient 18
Patient 19
Patient 20
Figure 9: Expression data of the 19 genes in a pairwise comparison between lung tumors and their matched normals. Colored spots represent expression fold changes of genes by dividing tumor intensity ratio by the normal intensity ratio. Only 2 fold changes are displayed.
As a follow-up to the lowered expression of $\beta$-catenin, we next asked whether $\beta$-catenin activated genes would be down-regulated as well. *Vimentin* has been shown to be activated by $\beta$-catenin in human breast cancer cells (Gilles et al. 2003) and as anticipated, *vimentin* is down-regulated in 8 out of the 10 samples showing down-regulated expression of $\beta$-catenin. On the other hand, down-regulation of $\beta$-catenin does not correspond to *E-cadherin*’s expression patterns. Although $\beta$-catenin and E-cadherin are involved in the cell-cell adhesion complex with $\alpha$-catenin, the results in the current study actually shows up-regulation of *E-cadherin* in 9 tumor samples. Only 2 samples show a coordinate down-regulation of *E-cadherin* and $\beta$-catenin.

The expression patterns of 5 sFRP genes (*sFRP1*, *sFRP2*, *sFRP3*, *sFRP4*, and *sFRP5*) were analyzed in lung tumor (Figure 9). Sample 10 shows down-regulation of *sFRP1*, *sFRP2*, *sFRP3*, and *sFRP4*. The same 4 genes are up-regulated in sample 18 (Figure 8R). Samples 10, 13, and 17 show down-regulated expression of *sFRP1*, *sFRP2* and *sFRP4*. In this study, the frequency of down-regulation of the sFRP genes range from 15% to 45%. At the same time their frequency of up-regulation ranged from 10% to 45%. The expression of upstream Wnt ligand activators (*Wnt1*, *Wnt3a*, *Wnt5a*, and *Wnt11*) and $\beta$-catenin transcripts are either down or unchanged regardless of whether the expression levels of the sFRP genes are up- or down-regulated.

### 3.4 Concordance Analysis

Since not all tumors involve the canonical pathway, we next asked which particular non-canonical components are involved in the samples that show inactive or
minimal activity of β-catenin/canonical pathway. To pursue this, we focused on the 17 lung tumor samples that show decrease or no change in β-catenin expression. Only genes belonging to either the canonical and non-canonical pathway were used in this section of the study. The expression of each gene was categorized as +1 for up-regulation, -1 for down-regulation, and 0 for unchanged. The genes were paired and a percentage was calculated for each pair of genes based on the number of times they showed the same category of expression. In other words, the percentage is an indication of how similar a given set of genes are. The table of gene comparisons with the corresponding percentages is shown in Table 5. Eliminating gene pairs that were less than 50% similar still left an overwhelming amount of genes therefore, the stringency was increased to filter out gene pairs that are less than 65% similar and 3 pairs of genes were left, as shown in Table 5 (highlighted).
Table 5: Concordance data of gene-gene relationships between 17 tumor and matched normals. (%) – percentage of similarity between two genes in 17 samples where β-catenin is down-regulated.

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3.4.1 Identification of gene pairs with similar patterns of expression

The genes pairs that have passed the above mentioned filter criteria are: Fzd3 and Dvl2, LRP5 and sFRP4, and Wnt5a and Fzd2. For the first pair of genes, the non-canonical components, Fzd3 and Dvl2 are similar in 77% of the 17 tumor samples. We discovered that both the expression levels of Fzd3 and Dvl2 are up-regulated in 7 out of 17 tumor samples and unchanged in 6 tumor samples where the expression of β-catenin is down or unchanged. The second pair of genes showing high concordance value is LRP5 and sFRP4 and they are both down-regulated in 6 out of 17 tumor samples and unchanged in 4 out of 17 samples. Lastly, Wnt5a and Fzd2 is similar in 65% of the tumor samples. Fzd2 is coordinately up-regulated in 5 samples and down-regulated in 4 samples with Wnt5a. Pattern of expression of Fzd2 does not suggest that it has any effect on the expression of β-catenin.
CHAPTER 4: DISCUSSION

4.1 Summary of results

The focus of this study was to investigate the role of the Wnt pathway in lung squamous cell carcinoma. An exhaustive literature search reveal that information on the Wnt pathway in cancer is still scarce. Expression studies on single components in the Wnt pathway provide limited information on how the multi-branched pathway behaves as a whole and what its role is in lung cancer. Perhaps the multiple and seemingly redundant deregulated expression of these components may serve as nodes that branch off to different pathways. In this study, a pairwise (normal and matched tumor samples) coordinated comparison was performed to seek out expressional changes along the Wnt pathway. To pursue this, we established expression of Wnt components in normal lungs and then determined which genes are disrupted based on their expression patterns in tumor. Pairwise differential expression analysis resulted in the isolation of 17 samples that showed a down-regulated or unchanged expression level of \( \beta \)-catenin. Further analysis of these 17 samples showed that some non-canonical components are up-regulated suggesting a role for the non-canonical pathway in lung cancer.

4.2 Canonical pathway and non-canonical pathways in normal lungs

A total of 20 normal lung samples were investigated for the expression of Wnt components. Various components belonging to the canonical Wnt pathway have
previously been reported to be expressed in the normal lung. As expected, the presence of canonical pathway transcripts in this study confirms that it is active in the normal lungs. However, active non-canonical components in clinical human lungs have not been reported in literature before. The focus of many studies on lung cancer has always been the canonical genes. This is the first report to show that the non-canonical pathway genes are expressed in normal human clinical lung samples. As mentioned before, the non-canonical pathway has only been known to play roles in lung development. However, their roles in the adult lungs remain to be a mystery. In the next section, descriptions of Wnt components that are found to be expressed in the normal lung for the first time are presented.

4.2.1 Descriptions of the Wnt components expressed in normal lungs

Wnt5a: Wnt5a is a non-canonical Wnt specific ligand and has been demonstrated to be responsible for transmitting Wnt/PCP signals in a variety of cell models. The closest evidence that support a role for Wnt5a in the lung is in developmental studies of transgenic mice (Li et al. 2002). Wnt5a regulates sonic hedgehog (SHH) and fibroblast growth factor 10 (FGF10) signaling during lung development (Li et al. 2005).

Wnt11: Wnt11 is another non-canonical Wnt specific ligand which is also responsible for transmitting Wnt/PCP signals. Wnt11 expression has been reported in the skeleton, lung mesenchyme, adrenal cortex and urogenital system of the human embryo. In the normal mouse intestinal epithelium, Wnt11 stimulates proliferation, migration, cytoskeletal
rearrangement, and contact-independent growth (Ouko et al. 2004). Whether Wnt11 plays a similar role in the lung epithelium warrants further investigations.

Fzd3: Fzd3 is involved in PCP signaling through its control of neural tube closure and the orientation of a subset of auditory and vestibular sensory cells in mice (Wang et al. 2006). However, one study shows that Fzd3 is able to activate the canonical pathway without ligand activation in a xenopus model (Umbhauer et al. 2000).

Fzd6: Similar to Wnt5a, Fzd6 is able to negatively regulate the canonical pathway through the activation of TAK1-NLK pathway (Ishitani et al. 2003; Golan et al. 2004). As mentioned already, activation of NLK inhibits the TCF/LEF transcription factors. The function of TAK1-NLK pathway has never been documented in human lung models. So far, there is evidence that TAK1-NLK cascade also functions in the TGF-b family of signal transduction pathway (Ohkawara et al. 2004) and is also essential for early zebrafish development (Thorpe and Moon 2004).

Dvl2: From figure 1, Dvl proteins link Wnt signals from the cell membrane to the cytoplasm. It is able to transmit canonical as well as non-canonical signals. Therefore, Dvl proteins cannot be classified exclusively as one or the other Wnt subpaths. The role of Dvl in the normal adult lung is not known at this point but overexpression of its paralog, Dvl3, in NSCLC contributes to tumorigenesis. The Dvl2 protein has mainly been reported to be involved with non-canonical pathway signaling through activation of small GTPases such as RhoA (Habas et al. 2001). Experiments have demonstrated that
DVL2 forms an active complex with DAAM1 and RhoA. The Rho family is responsible for regulating actin cytoskeletal changes and gene transcription to promote various changes in cell behaviour (Dale et al. 1996; Kaibuchi et al. 1999). Further investigation is needed to find out the exact role of Dvl2 in the normal lung.

sFRPs: The sFRP genes that are found to be expressed in the normal adult lungs for the first time are sFRP2, sFRP3, sFRP4, and sFRP5. The sFRP family is responsible for regulating Wnt signals but the mechanism of inhibition still controversial at this point. It has been hypothesized that the sFRP proteins disrupt the interaction of the Wnt ligand and receptor complex through competitively binding onto Wnt ligands or the receptor complex (Bafico et al. 1999). Only sFRP1 has been implicated in NSCLC through methylation studies. Whether, the sFRPs function in the normal maintenance of the lung to control the activity of Wnt signaling must be further investigated.

4.3 Disruption of canonical Wnt pathway in lung SCC

Expression of β-catenin is commonly found to be up-regulated in many types of cancer including colon cancer, (Feng Han et al. 2006; Roca et al. 2006), ovarian cancer (Wang et al. 2006), and breast cancer (Ozaki et al. 2005). Increased β-catenin expression contributes to the proliferation of cancer cells through its activation of pro-growth transcripts (He et al. 1998; Mann et al. 1999; Blache et al. 2004).

This is not the first report to show the reduction of β-catenin expression in lung cancer (Retera et al. 1998; Hommura et al. 2002; Topol et al. 2003) and most
intriguingly, it has also been linked to clinical outcome in lung cancer. Down-regulation of $\beta$-catenin is associated with unfavourable prognosis in lung cancer (Hommura et al. 2002; Lee et al. 2002) and in one study with increased lymph node metastasis as well (Retera et al. 1998). Therefore, down-regulated expression of $\beta$-catenin/canonical pathway in a number lung tumor cases in this study indicate that the canonical pathway may not always contribute to cancer cell proliferation. A large proportion of the tumor samples also show that the expression level of $\beta$-catenin is unchanged (7 samples), which further suggests that $\beta$-catenin is either inactive or might have a basal level of activity, which may contribute very little to carcinogenesis. Furthermore, Le Floch et al. (Le Floch et al. 2005) demonstrated that pro-invasive activity can be initiated via a $\beta$-catenin independent pathway by WNT2, which agrees with our observation that the canonical/$\beta$-catenin pathway is not always active in lung cancer.

4.4 Disruption of Wnt regulators in lung SCC

Lowered expression of sFRP genes due to promoter hypermethylation is a common event in various types of cancers. For example, $sFRP1$, 4, and 5 are frequently methylated in human malignant pleural mesothelioma (Lee et al. 2004) and all five sFRP members are hypermethylated in bladder cancer (Urakami et al. 2006). In lung cancer, only $sFRP1$ has been demonstrated to be hypermethylated in NSCLC. Down-regulated expression of $sFRP1$ due to hypermethylation occurs 48% of the time in NSCLC (Fukui et al. 2005) and the frequency of down-regulated expression of sFRP genes in this study ranged from 15%-40%, with $sFRP1$ down-regulated in 30% of the samples, which is
close to the former study. These results suggest that the sFRP genes may serve as potential tumor suppressors in a subset of lung tumors but at the same time a subset of lung tumors show up-regulation of sFRP genes. As will be discussed in the next section, sFRP genes may not always serve as Wnt antagonists. In lung cancer, perhaps the sFRP genes behave differently in a subset of lung tumors.

4.5 Deregulation of E-cadherin in lung SCC

Epithelial cells are joined by adherens junctions that are composed of E-cadherin, β- and α-catenin (Gates and Peifer 2005). Although a lost of E-cadherin is frequently associated with lung cancer (Bremnes et al. 2002; Suzuki et al. 2004; Kato et al. 2005), expression of E-cadherin is actually up‐regulated in as many as 45% of the lung tumors in this study. However, this is not the first study to observe an increase in E-cadherin expression in NSCLC. A study involving the comparison of E-cadherin expression between human bronchial epithelium and NSCLC discovered that E-cadherin was actually up-regulated (Smythe et al. 1999) in tumors with N1 or N2 nodal status. Similarly, the nodal status of the lung tumors used in this study was also N1 or N2. Two other studies also discovered that down-regulated expression of E-cadherin only occurs in 33.8% (Xu et al. 2001) and 20% (Lee et al. 2002) of stage II NSCLC. Similarly, a majority of the samples used in this study are stage II tumors and they also show down-regulated expression of E-cadherin in 15% of the samples. Therefore, up-regulated expression of E-cadherin may be commonly detected in earlier stages of lung cancer.
4.6 Concordance analysis

As mentioned before, no studies have investigated how the non-canonical components are involved in lung cancer. In this study, we have identified 17 lung tumors with minimal activity to inactivated canonical pathway through the expression patterns of β-catenin. By looking at coordinated expression of non-canonical components in these 17 samples, we have isolated interesting gene relationships that might have a role in explaining the proliferation of lung cancer cells. Below are the descriptions of some of these gene relationships and their potential roles in cancer.

4.6.1 LRP5 and sFRP4 and their potential roles in cancer

The relationship between LRPs and sFRPs have not be reported in literature before. In this study LRP5 and sFRP4 show a high frequency of similar expressional changes. As mentioned earlier, LRP5 is a single transmembrane protein that forms an active complex with the Frizzled protein and an incoming Wnt ligand to activate the canonical Wnt signaling pathway. So far, only the Dkk family has been shown to inhibit the canonical pathway by binding onto the LRP co-receptor but not the sFRP family. Specifically, Dkk-1 binds directly to LRP5 to antagonize the Wnt signaling pathway (Mao et al. 2002; Kawano and Kypta 2003). As for sFRP4, although this protein exhibits the same domain architecture as other sFRP family members, its role in the Wnt pathway has never been directly studied. In contrast to the other sFRP members, sFRP4 is actually shown to be up-regulated in several types of cancer and may in fact be the only
sFRP member to become a potential oncogene. A study involving human colorectal carcinoma revealed that $sFRP4$ is up-regulated where there is positive expression of $\beta$-catenin (Feng Han et al. 2006). In rat studies, it has been demonstrated that sFRP4 can increase phosphorylated $\beta$-catenin concentration in renal tissue (Berndt et al. 2003). Lastly, in vitro studies show that overexpression of sFRP4 does not lead to decreased expression of $\beta$-catenin (Suzuki et al. 2004). If sFRP4 is able to activate the canonical pathway then the high frequency of similarity between LRP5 and sFRP4 in this study becomes more interesting. Although the mechanisms behind the activation of the canonical pathway by sFRP4 in these studies still need more investigations, previous and current evidence suggests that the sFRP genes may have more complex roles in addition to their predefined roles as Wnt antagonists.

4.6.2 Multiple roles of $Wnt5a$ and its potential role in cancer

The relationship between $Wnt5a$ and $Fzd2$ genes is a novel association in lung. However, this association has been demonstrated in other animal models. Studies on zebrafish models suggest that RFZ2 induces intracellular Ca$^{2+}$ via WNT5a activation. Intracellular release of Ca$^{2+}$ involves the activation of the phosphatidylinositol pathway in a G-protein-dependent manner (Slusarski et al. 1997; Sheldahl et al. 1999; Kuhl et al. 2000) which in turn activates CamKII and PKC. CamKII is activated by calmodulin (CaM), which is a major receptor of Ca$^{2+}$. Studies in mammalian cells have demonstrated that manipulation of CaM expression affects the proliferative capacity of cells. Overexpression of CaM causes accelerated cell proliferation mostly due to a
shortening of G1 phase of the cell cycle. In addition, inhibiting CaM activity has prevented proliferation and colony formation of breast cancer cell lines (Kahl and Means 2003). Ectopic expression of WNT5a and RFZ2 also results in translocation of PKC to the plasma membrane and stimulates PKC kinase activity in vitro (Kohn and Moon 2005). The downstream effects of WNT5a mediated PKC activation is not known in cancer. However, the implications of PKCs have actually been reported in various types of cancer. Human SCLC cell line has shown to exhibit rapid growth due to overexpression of PKCe. Breast cancer cells, MCF-7, displayed enhanced proliferative rate due to PKCa transfection (Hofmann 2004). With respect to the Wnt pathway, WNT5a has been considered an inhibitor of canonical Wnt signaling and therefore assumes a role as a TSG. Intriguingly, a recent study by Mikels et. al (Mikels and Nusse 2006) has demonstrated that Wnt5a is able to activate both the canonical and non-canonical pathway. In their study, Wnt5a was able to activate the canonical pathway in the presence of LRP5 and FZD4. However, LRP5 and Wnt5a did not pass the concordance criteria in this study, showing similar changes in expression in 48% of the samples. Despite the controversy on the role of Wnt5a, current observations indicate that β-catenin expression is not disturbed by the effects of Wnt5a. However, there is overlapping expression of all three genes, Wnt5a, Fzd2, and LRP5 in 5 out of the 20 tumor samples in this study which involves up- and down-regulated expression changes. Perhaps there is a more complex mechanism behind the function of Wnt5a, after all, current and past studies suggest that the Wnt5a ligand is a more complex activator than what it was initially thought to be. Therefore, further investigation is needed to find out the functional involvement of Wnt5a in lung tumor.
4.6.3 Potential roles of Fzd3 and Dvl2 in cancer

*Fzd3* and *Dvl2* have independently been reported to be involved in the non-canonical pathway but have not been linked to Wnt5a. Expression patterns in this study suggest that there is either a basal or heightened expression of both *Fzd3* and *Dvl2* when *β-catenin* is down-regulated or unchanged. Patterns of expression of Fzd3 and Dvl2 does not seem to affect the expression levels of *β-catenin*. Only 3 samples show coordinated up-regulation of both *Fzd3* and *Dvl2* when *β-catenin* is down-regulated. Although Dvl has been shown to be able to activate the canonical and non-canonical pathway, *Dvl2* alone does not display a high frequency of coordinated expression changes with *β-catenin* in this study. Likewise, Fzd3 alone does not seem to affect the expression of *β-catenin* as well, which agrees the majority of studies done on Fzd3.

In cancer, previous implication of Fzd3 and Dvl2 is virtually non-existent. In fact, there is currently no report citing the implication of *Fzd3* in cancer. However, there have been studies that link Fzd3 with the susceptibility to schizophrenia. (Katsu et al. 2003; Yang et al. 2003). As mentioned in the previous sections, Dvl is known to function downstream of the frizzled receptors and has been shown to activate the PCP signaling pathway. Although Dvl2 has never been directly linked to cancer, its associations with Rho GTPases (Figure 10) have been reported and are among the most studied signaling molecules. Some of the essential cellular processes that the Rho family is responsible for include cell growth, lipid metabolism, cytoskeleton architecture, membrane trafficking, transcriptional regulation, and apoptosis (Aznar and Lacal 2001). The Rho family is also very important in cancer, affecting the process of tumorigenesis at multiple levels. RhoA
is able to inhibit specific components that are required for proper cell cycle progression. They can upregulate cyclin D1 expression, a common hallmark of many cancer types. RhoA can also activate AKT to promote cell growth and epithelial to mesenchymal transition. Besides promoting cell growth, the Rho GTPases can also modulate actin cytoskeleton, cell adhesion and motility and integrity of the extracellular matrix causing deregulated epithelial cell polarity. Tissue invasion and metastasis is a major hallmark of cancer and all of the above processes provide opportunity for cells to metastasize during cancer (Aznar et al. 2004). DVL2 has also specifically been shown to form a complex with DAAM1 and RhoA in human embryonic kidney cell lines (Habas et al. 2001). RhoA specifically activates the ROCK family which in turns have been intimately involved in tumor metastasis (Genda et al. 1999; Somlyo et al. 2000). Maybe activation of DVL2 is an early event for pre-metastatic processes in cancer and further investigation is needed to find out the downstream effects in human lung cancer.
Figure 10: Schematic representation of the non-canonical pathway involving Dvl2 and its potential role in cancer. The above diagram is composed from the information provided by the following studies: (Habas et al. 2001; Genda et al. 1999; Somlyo et al. 2000; Boutros and Mlodzik 1999; Adler and Lee 2001)
4.7 Significance

Limited knowledge known about the Wnt pathway in lung cancer prevents investigators to confidently choose candidate components to do their assays. Therefore, single gene experiments are not always conclusive and pose a definite problem in predicting the outcome of the Wnt pathway. Not to mention, the multiple branching characteristics of the Wnt pathway adds another variable to establishing a generalized fate for the pathway. Such a problem warrants larger scale experiments to first define normal functions of the Wnt pathway in normal lungs. Revealing relationships of the components related to the Wnt pathway in normal lungs will greatly facilitate analysis done in the tumor.

It is important to acknowledge that the data analysis used in this study does not attempt to answer existing questions but rather, generates more questions to widen the narrow views of the Wnt pathway depicted in lung cancer so far. The canonical pathway has always been the focus of many cancer studies. Rather than being confined within the established realms of the Wnt community, a coordinate analysis of gene expressions that are representative of the canonical and non-canonical pathway between tumor and matched normal lung will allow the identification of potential gene relationships that dogmas may oversee. For example, the current study shows that some of the non-canonical components are up-regulated in lung tumor. Their roles in regulating cell proliferation and movements during development may have applicable effects on cancer cell progression. Identifying these new candidates may help settle/explain controversies about the roles of certain Wnt components. Lastly, this approach is more complementary
rather than a replacement to existing techniques to unlock the biological involvement of the Wnt pathway or even any pathway in cancer.

4.8 Future directions

This thesis identified potential non-canonical pathway genes that may provide an alternate explanation to the proliferation of lung cancer cells. However, confirmation of the observed deregulated expression changes at the protein level will be necessary. We will be obtaining diagnostic paraffin embedded blocks for some of these samples to do immunohistochemistry (IHC). Post-translational modification of some genes may alter expected outcomes and are not detected at the mRNA level. Some of these post-translational modifications can be activating or deactivating depending on the context. Common modifications include glycosylation, phosphorylation, palmitoylation, peptide cleavages and etc. Furthermore, we will also acquire another set of SCC samples with matched normals and perform real-time RT-PCR on the non-canonical components that are up-regulated in our current study.

Analysis of epigenetic alterations on candidate genes in this study was not done. Investigating the methylation status of some of these Wnt related genes may provide an alternate explanation to some of the disruptions seen in this study. As with other studies, methylation is a common event for genes that regulate the activation of the Wnt pathways. For example, epigenetic silencing of sFRP genes have been recorded in colorectal cancer (Suzuki et al. 2004), ovarian cancer (Takada et al. 2004) and NSCLC (Fukui et al. 2005). Lastly, WIF1 is also silenced through promoter hypermethylation in
human lung cancer (Mazieres et al. 2004). Therefore, epigenetic alterations definitely carry a high level of precedence in some of the disruptions seen in this study.

A drawback in this study is that not all samples came with complete clinical staging information. Future analysis will require a larger sample size with clinical history recorded for each sample. A larger sample size will increase the fidelity of the study and the clinical information can help distinguish differences in patterns of gene expression at a particular stage of cancer progression. Altogether this will help aid in the understanding of the Wnt pathway in the progression of lung cancer.

Following the validation of key components in the Wnt pathway that is important in lung cancer, functional analysis of these genes is the next big step to further understand the mechanisms of these genes in tumorigenesis. Questions on what is causing the deregulation of certain genes can be answered. Cell models such as RNA interference or tetracycline inducible systems can be used to turn off (or on with TET) prospective oncogenic or tumor suppressive genes. Alternatively, transgenic mouse models in which genes can be “knocked out” or “knocked in” can be used to see the effects of these genes in an in vivo system. The unraveling of the functional aspects of the genes is a crucial step to the development of novel drugs in the treatment of lung cancer.
CHAPTER 5: CONCLUSIONS

The data generated from this thesis can be summarized in the following points:

1) Whole genome array CGH profiles of the 20 lung SCC samples with matched normals showed genetic losses of the 3p arm in 8 samples which contains β-catenin and Wnt5a.

2) The presence of expression of genes belonging to the Wnt pathway in normal lung suggests that the Wnt pathway is active this includes both canonical and non-canonical pathway.

3) Some genes representing the non-canonical pathway and Wnt pathway regulators have not been seen to be expressed in the normal lungs before. These genes include Wnt5a, Wnt11, Fzd3, Fzd6, sFRP2, sFRP3, sFRP4, and sFRP5.

4) Expression analysis of components belonging to the Wnt pathway in tumor versus normal indicates that not all lung tumor can be explained by the canonical/β-catenin pathway.

5) Non-canonical pathway is active in tumor samples where the canonical Wnt pathway is inactive or have very low level of activity. Therefore, non-canonical components may provide an alternative explanation to the proliferation of lung cancer cells.
REFERENCES


APPENDIX I: Gel profiles of the components analyzed in this study

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