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The Role of Spy1 in Mammary Morphogenesis

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by
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The Role of Spy1 in Mammary Morphogenesis

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ABSTRACT

Cell growth and development is regulated by the cell division cycle, which dictates how efficiently cells communicate with each other and is dependent on cellular morphology. Our lab focuses on a novel cell cycle regulator, Spy1A, which has been shown to enhance cellular proliferation and regulate mammary development. Elevated levels of Spy1A significantly increased cell invasion, coincident with an increase β-catenin transcriptional levels. We hypothesize that these characteristics may maintain cells in a more 'stem-like' state. Mammary stem cells are highly proliferative, they rely on adherence to surrounding cells for self renewal and invasion and migration into the mammary fat pad. We further demonstrate that Spy1A overexpression results in the formation of larger mammospheres, where mammosphere size has been correlative of stimulating stem cell self-renewal. Further resolving the roles of Spy1 in the developing mammary gland is essential to fully elucidate its roles in human disease.

DEDICATION

I dedicate this thesis to my parents, Teresa and Milan Malysa.

If it were not for their love, support and hard work, I would never have made it here.

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LIST OF ABBREVIATIONS

TEB………………………………………………………….....Terminal end buds

MMP…………………………………………………….....Matrix metalloproteinases

ECM…………………………………………………………..Extracellular matrix

BM……………………………………………………….......Basement membrane

ILC…………………………………………………………..Invasive lobular carcinoma

MSC…………………………………………………………..Mammary stem cells

MS……………………………………………………………..…..Mammospheres

Frz…………………………………………………………...…Frizzled receptor

GSK3…………………………………………………….............Glycogen synthase 3

APC……………………………………………………..……Adenomatous polyposis coli

LRP6…………………………………………….…..Low density lipoprotein related protein

TGF β…………………………………..…………………...Transforming growth factor β

Dsh……………………………………………………………..……Dishevelled

TCF/LEF…………………………………………..…T-cell factor/lymphoid enhancer factor

HSC……………………………………………..……………….Hematopoietic stem cells

EMT………………………………………..…………..Epithelial-mesenchymal transition

FGFR-1……………………………………..……………Fibroblast growth factor receptor 1

FGF………………………….………………………………....Fibroblast growth factor

CDK………………………………..……………………………..Cyclin dependent kinase

SAGE……………………………..…………….....Serial analysis of gene expression

I. INTRODUCTION

Cancer is a relentless disease that claims the lives of millions of people of any race, ethnicity or gender. It is a disease that knows no limits with which it targets, leaving many of the glands and tissues in the human body susceptible to its strike. Of the many targets, breast cancer remains as one of the most prevalent cancers to affect women worldwide (Nudelman et al., 2009). In 2004, the World Health Organization determined that cancer caused 13% of all deaths worldwide, where breast cancer was ranked the fifth most leading cancer to affect overall cancer mortality (WHO Fact Sheet, 2009). This disease has become so frequent that its incidence rates have increased by 40% as the population ages (Nudelman et al., 2009).

Breast cancer, like most cancers, is caused by a multitude of internal and/or external effects. It is through the interactions between genetic and environmental factors that cause a very complex, multi-stage process that encourages a normal mammary cell to become a tumorigenic. Abnormalities that occur within the tumorigenic cell can be attributed to carcinogens, which can damage the cell's genome, and random mutagenic events that disrupt DNA replication by targeting well-orchestrated cellular events such as proliferation and apoptosis leading to the formation of tumors (Rudel et al., 2007). Although there are a multitude of factors that play a role in the cancer process, this thesis will focus on how a novel protein affects cellular and molecular mechanisms in mammary gland development and how Spy1 contributes to breast cancer.

Implications of Novel Cell Cycle Regulator, Spy1A in Breast Cancer

Mammary gland development is a tightly regulated process where misregulation of key events, such as proliferation or apoptosis, could lead to mammary tumorigenesis.

This delicate equilibrium is highly regulated by the cell cycle. The cell division cycle is a series of phases and checkpoints that ensure a cell is able to replicate and divide properly. The primary coordinated events can be organized into 4 phases: G1, S, G2 and M (Schafer, 1998). During the cell cycle, there is a sequence of phosphorylation events that drives cells through the different phases and also includes various checkpoints that monitor the completion of critical events or delay cell cycle progression accordingly (Schafer, 1998). The coordinated phosphorylation events are governed by a kinase family called the cyclin dependent kinases (CDKs) (Malumbres, 2005). In order for CDKs to be activated they must be bound by a subunit, called cyclins that are transiently expressed. Together, CDKs and cyclins form an active complex that marks transitions in the cell cycle (Malumbres, 2005). A second type of cell cycle regulation is via checkpoint control, and this mechanism is especially important at times when problems are sensed during DNA replication and chromosome segregation (Vermeulen et al., 2003). These checkpoints are responsible for stopping the cell cycle, allowing DNA repair however if the damaged DNA is not reparable, then cellular apoptosis occurs and eliminates this damaged DNA (Vermeulen et al., 2003). Consequently, the association between cancer and the cell cycle is apparent, where the cell cycle program controls cellular proliferation and apoptosis and cancer is caused by disruption of these cellular events which leads to uncontrolled cell proliferation (Vermeulen et al., 2003). All types of cancers are characterized by uncontrolled cell numbers and these disproportionate cell numbers have been linked to a reduced sensitivity to signals that normally tell a cell to adhere, proliferate, differentiate or undergo apoptosis accurately.

Although there are a multitude of genes that are involved in initiating and promoting cancer, this thesis will be focusing on a novel cell cycle regulator, named Speedy or Spy1, which belongs to a family of proteins called the Speedy/RINGO proteins. *Xenopus* Speedy (XSpy1) was first identified in a screen for genes that conferred resistance to UV radiation (LeNormand et al., 1999). Consequently, a screen to identify genes that are involved in G2/M transition in Xenopus oocytes demonstrated the existence of another family member, called Xenopus RINGO (Rapid Inducer of G2/M Oocytes). Together, Spy1 and RINGO formed a protein family of novel cell cycle regulators. During this screen, it was shown that expression of XRINGO in G2 arrested oocytes could increase meiotic maturation when compared to control oocytes that were induced with Mos expression or progesterone (LeNormand et al., 1999). Similarly, Spy1A2, another family member, could also increase meiotic maturation more efficiently than its control (LeNormand et al., 1999). However, our lab is interested in studying Spy1A1 (herein referred to as Spy1A), a family member that shares 40% sequence identity with its *Xenopus* counterpart (Porter et al., 2002). LeNormand and colleagues discovered that Spy1, a protein that has no known homology to cyclins, was capable of overcoming G2 arrest and could induce rapid oocyte maturation (LeNormand et al, 1999). They also observed that in Spy1 injected oocytes that cdc2 kinase activity was stimulated, which lead to the discovery of Spy1 and its family members (LeNormand et al., 1999). This family of proteins contains a conserved region that is essential for binding to CDKs, called the Speedy/RINGO (S/R) box. It was later discovered that Speedy/RINGO protein could bind to and activate Cdk2, where certain family members could also bind and activate cdc2 (Porter et al., 2002) (LeNormand et al.,1999) It has also

been demonstrated that Spy1 interacts with cyclin dependent kinase inhibitor, p27, where Spy1 can overcome p27 induced G1 arrest *in vitro* (Porter et al., 2003). It is known that numerous cyclin/CDK complexes are disrupted in different types of cancers, however of the Speedy/RINGO protein, one family member, Spy1A, stands out as showing great potential in understanding the process of mammary carcinogenesis. It was shown that Spy1A is ubitiquously expressed in most human tissues with high expression seen in thymus, liver, heart and testes. It was also observed through overexpression studies that Spy1 was capable of enhancing cellular proliferation through shortening the G1/S phase and that its abilities to enhance proliferation is dependent on Cdk2 activity (Porter et al., 2002). Interestingly, overexpressed Spy1A has been correlated to breast cancer, where a serial analysis of gene expression (SAGE) on cells isolated from primary invasive ductal carcinoma showed that Spy1A was one of 50 upregulated genes expressed in this breast cancer subtype (Zucchi et al., 2004).

Evidence from our lab has demonstrated in more detail the role of Spy1A in breast cancer. Golipour et al determined that Spy1A levels, both RNA and protein, are elevated during puberty, early pregnancy and involution, which are highly proliferative events during mammary gland development in mice (Golipour et al., 2008). In addition, *in vivo* experiments demonstrated that Spy1A overexpression in Balb-c mouse mammary glands increased the rate of ductal morphogenesis, ductal side branching and also terminal end (TEB) bud elongation, where gland development was accelerated (Golipour et al., 2008). Additionally, *in vitro* studies done in our lab showed that Spy1A overexpression can disrupt acini development in HC11 differentiation timecourse experiments (Golipour et al., 2008). Interestingly, a novel relationship between Spy1A

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and c-Myc, a well known oncogene, was discovered. It was determined that Spy1A follows the same expression patterns as c-Myc during normal mammary gland development (Golipour et al., 2008). Though complete understanding of the role of Spy1 in mammary gland tumorigenesis has yet to be achieved, this thesis will build upon previous work to further understand the contribution of Spy1 to many important aspects of mammary cell behavior which play a critical role in mammary tumorigenesis.

Structure of the Mammary Gland

The mammary gland is a dynamic organ that undergoes major changes in morphology during its development. The basic structure of the mammary gland consists of fatty tissue, called stroma, and a branching ductal system, which is referred to as the lobuloalveolar system (Richert et al., 2000). The lobuloalveolar system is composed of specialized structures, called the mammary acini (or also referred to as alveoli), that are vital for the functioning of the gland. Acini are pivotal in milk production, where their physical structure permits milk secretion (Richert et al., 2000). The mammary acini are spherical monolayers of epithelial cells that are composed of an inner lumen that is lined by a layer of milk producing cuboidal cells, which is surrounded by contractile, myoepithelial cells that are responsible for the expulsion of milk into the branching mammary ducts (Dontu et al., 2003). Moreover, the mammary acini unite together to form lobules wherein each lobule is connected to lactiferous ducts that empty into the nipple openings (VanHouten et al., 2003). Additionally, lobules join together to form lobes, where approximately 15-25 lobes are present within each breast (Watson et al., 2008). The full development of the mammary gland involves the gland phasing through various development stages in order to become functional (Watson et al., 2008).

Mammary gland development is a complex, extensive and well orchestrated process that is not only essential for the correct patterning of the gland but it is also vital for its proper functioning.

Mammary Gland Development

The mammary gland undergoes the majority of its development postnatally where a rudimentary epithelium is formed before birth. After birth, the rudimentary epithelium begins forming the branching network of ducts (Hennighausen et al., 2001). Throughout its development, the mammary gland goes through rounds of proliferation, apoptosis and differentiation which correspond to distinct developmental stages (Hennighausen et al., 2001). Once the rudimentary epithelial tree is formed, the mammary gland undergoes a stagnant phase until the onset of puberty (Fata et al., 2003). In the first stage of mammary gland development, ductal branching is regulated through paracrine interactions between adjacent mesenchymal and epithelial cells (VanHouten et al., 2003). During these interactions, epithelial cells have the ability to proliferate, which allows them to invade the fat pad and establishes the beginning of ductal branching (Ball, 1998). As the maturing ductal tree contacts the boundaries of the mammary fat pad, the tips of the TEBs bifurcate and sprout to form the secondary branching structure (Chu et al., 2004). The secondary branching is stimulated by ovarian hormones, in which progesterone inhibits ductal branching differentiation and estrogen promotes ductal branching (Fata et al., 2003). Subsequently, mesenchymal cells, with the help of secreting factors, are able to alter mammary mesenchyme to form the lymph and blood vessels that encompass the mammary ductal tree which aids in the completion the ductal architecture (Robinson, 2004). In the next mammary gland developmental stage, the period between puberty and

adulthood, the mammary gland forms to become fully functional but remains stagnant (Fata et al., 2003).

At pregnancy, the mammary gland undergoes changes that allow the gland to perform its most vital task, milk production. For the gland to be capable of producing milk, the ductal branches experience a rapid period of proliferation which is stimulated by pregnancy related hormones such as estrogen, progesterone, and prolactin (Welsch et al., 1977) (Saji et al 2000). These hormones stimulate additional ductal branching, more affluent blood flow as well as cause an increase in adipose tissue (Saji et al., 2000). This rapid proliferation of the ductal branches leads to the formation of spherical epithelial structures, which will become the mammary acini (Fata et al., 2003). During mid to late pregnancy, the spherical epithelial structures begin to organize themselves where the innermost cells undergo apoptosis to aid in the creation of the acinar lumen (Debnath et al., 2008). This apoptotic event establishes the inner luminal epithelial layer where at the end of pregnancy, these luminal epithelial cells differentiate into alveolar cells, which are capable of synthesizing milk proteins (Bissell et al., 1989). After birth, the alveoli begin producing an abundance of milk due to the drop in progesterone levels and an increase in prolactin, a hormone that is responsible for regulating milk protein secretion and acini formation (Saji et al., 2000). The myoepithelial cells, that line that basal portion of the acini, play a vital role of directing milk secretion by providing the contractile force to expel milk out of the mammary acini. The contraction of the myoepithelial cells occurs through binding between prolactin receptor to essential extracellular matrix proteins, laminin and collagen (Bissell et al., 2005). With the coordination of systemic hormones

along with the differentiated mammary acini, the mammary gland serves its purpose for milk secretion during this crucial developmental stage (Stein et al., 2007).

Once lactation is no longer desired, the mammary gland goes through its next developmental stage, the process of involution. There are a multitude of proteins that are involved in regulating involution however of these proteins; the one worth mentioning is Transforming Growth Factor Beta (TGFβ). TGFβ is responsible for regulating apoptosis, matrix remodeling and is also involved in regulating branching morphogenesis (Nguyen et al., 2000). Specifically, overexpression of TGFβ1 prevented lobuloalveolar morphogenesis which resulted in defects in lactation (Kordon et al., 1995). Involution allows the lactating mammary gland to be restored into its virgin form (Stein et al., 2007). This developmental stage is distinguished by massive tissue remodeling that involves: the collapse of the mammary acini, reorganization of mammary tissue and adipose tissue modifications. In general, involution is considered a two stage process, where the first phase focuses on breaking down the mammary acini, wherein mammary gland architecture is unaffected and the second phase focuses on reordering mammary gland structure (Debnath et al., 2008). In the first stage of involution, mammary tissue remodeling is restrained and apoptosis commences in the alveoli, an event that is triggered by the withdrawal of prolactin and oxytocin (Stein et al., 2007). *In vivo* studies have shown that when involution was forced upon in mice, the first step in involution, apoptosis of the mammary acini, lasted approximately 48 hours and this phase was reversible (Lund et al., 1996). It was shown that when mouse pups were returned to their mothers, undergoing involution, that apoptosis was stopped and that lactation resumed (Lund et al., 1996). The second stage in involution involves remodeling of the nearby fat

pad and reorganization of adipocytes (Watson et al., 2006). During this phase, the acini collapse and the stromal cells fill in the voids left behind by the destroyed acini (Debnath et al., 2008). There are a variety of proteases that are involved in the reorganization of the extracellular matrix such as the matrix metalloprotease (MMP) and serine proteases, which are secreted by stromal cells (Folguerras et al., 2004). It has been shown that the elimination of extracellular matrix promotes apoptosis of the alveolar cells and that the MMPs are responsible for activating this apoptotic event in addition to mediating remodeling of the mammary gland (Folguerras et al., 2004). Once the mammary gland has successfully completed involution, the resulting mammary gland is phenotypically identical to a virgin gland, where the gland is free to cycle through the previously mentioned developmental stages again (Motyl et al., 2007).

Factors that Regulate Mammary Acini Development and Function

As previously mentioned, the mammary acini are the most vital structures within the mammary gland where emphasis relies on their bilayered structures. Experimentally, scientists have been able to culture analogous structures *in vitro* through the use of matrigel, which is a viscous protein concoction that is derived from Engelbreth-Holm-Swarm mouse sarcoma cells (Debnath et al., 2003). Matrigel permits cells to grow in a 3-dimensional (3D) environment that resembles *in vivo* conditions because of its diverse composition of laminin and collagen, important components of the extracellular matrix (ECM) (Gudjonsson et al. 2002). Specifically, the normal human mammary cell line, MCF10a, is an example of a cell line that is capable of forming acinar structures that are similar to those found in the mammary lobules (Debnath et al., 2005). In the recent years, a xenograft model for human breast cancer was derived from the MCF10a. Cell lines

from this model encompass the entire range of human breast cancer from normal tissue (MCF10a) to malignant, metastatic tumors (MCF10CA1a) (Rhee et al., 2008). From the three dimensional experiments that have been performed to look more in depth into structure of the mammary acini, researchers have determined possible causes for breast cancer, including the deregulation of signaling pathways and abnormalities in cellular mechanisms that potentially lead to mammary tumorigenesis. It is through studying normal mammary acini development and functioning that scientists are beginning to understand the possible causes for tumorigenesis with the mammary gland. There are a multitude of factors that contribute to the mammary acini structure however the ECM and cell-cell contacts play a major role in regulating acini morphogenesis (Bilder et al., 2003).

Extracellular Matrix

The extracellular matrix (ECM) consists of specific components that are involved in permitting the epithelial cells to maintain their shape and structure. The ECM is composed of two parts: an interstitial matrix and a basement membrane. The interstitial matrix is filled with gels of fibrous proteins and polysaccharides that provide a rigid cellular support whereas the basement membrane (BM) is a sheet like structure made from components of the ECM where epithelial cells attach (Borg, 2004). Specifically, the mammary ducts consist of an epithelial layer that is associated with a single layer of myoepithelial cells. A sheath of BM surrounds the ducts and the ducts are surrounded by outer ring of interstitial matrix. It is the myoepithelial cells that are connected to the BM, which are composed of laminin 5, laminin 1, collagen IV, fibronectin and other ECM-related proteins (Albrechtsen et al., 1981). In order for cells to survive, they must be anchored to the ECM. However, cancerous cells lose their ability to adhere to the ECM,

which is a feature that has been extensively linked to metastasis in cancer (Fata et al., 2003). It has been shown that if epithelial cells in the forming acini, are not attached to the BM, they will undergo apoptosis and not form acini (Debnath et al., 2008). The ECM is involved in relaying messages to the acini so that cellular events, like proliferation, apoptosis and cell polarization, function accurately to form the appropriate structures. Epithelial cells detect the ECM through basally located integrins and non-integrin ECM receptors (Bisanz et al., 2005). Specifically, integrins are receptors responsible for mediating attachment between cells or cells and the ECM. The $\alpha2$ integrins have been identified as being important for ductal morphogenesis, where $\alpha2$ integrin knockdown studies have shown to cause a decrease in ductal branching (Debnath et al., 2005). These receptors are also involved in regulating the cell cycle, migration and cellular shape. In addition, O'Brien et al discovered that by disrupting the expression of integrin receptor $\alpha2$-$\beta1$, cultured mammary acini could not properly form and underwent apoptosis since epithelial cells within the acini could not detect the ECM (O'Brien et al., 2002). In essence, the ECM must function properly in order for normal mamamary gland development to occur.

Cell-Cell Contacts

The structure of the mammary acini is highly dependent on cell-cell contacts that are regulated by a family of adhesion proteins, called the cadherin family (Runswick et al., 2001). For instance, the outer myoepithelial cells of the acini are connected to each other by P-cadherin cell-cell contacts, whereas the inner luminal cells are adhered to one another by E-cadherin (Bilder et al., 2003). Additionally, the myoepithelial cells adhere to the luminal cells by desmosomal cadherins, and it has been determined that

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desmosomes are vital for the formation of the acini through their roles in correcting abnormalities in epithelial polarity (Bilder et al., 2003). Since the cadherin family plays a major role in helping to establish and maintain acini structure, it is not surprising that misregulation of this protein family have been observed in literature. Studies have shown that either downregulation or upregulation of E-cadherin causes mammary cells to become cancerous (Cowin et al., 2005). It was observed that E-cadherin expression was lost in ~85% in certain breast cancer subtypes, like invasive lobular carcinomas (ILC) (Cowin et al., 2005). It was determined that ILC was caused by the epigenetic silencing of E-cadherin, whose absent expression has characterized these tumors as being non-invasive and properly differentiated (Droufakou et al., 2001). However, a small percentage of ILC's show increased expression of E-cadherin, causing an aberration in epithelial polarity thereby defining a less differentiated and more invasive tumor phenotype (Droufakou et al., 2001). Additionally, aberrant E-cadherin expression has been linked to higher staged tumors as well as tumor invasiveness and this misregulation of E-cadherin has been identified in not only breast cancer, but also lung cancer and colorectal cancer (Murtagh et al., 2004).

Structure of the Mammary Acini

Mammary acini are composed of two types of epithelial cells, which are a cell type that undergo cellular polarization. Cell polarization is a process where cells organize their intracellular structures along apical, lateral and basal axes which transduce signals to help organize cells intercellularly (Debnath et al., 2008). In the literature, mammary acini, grown in 3D, deposit BM components such as collagen IV and laminin 5 on their basal surface whereby epithelial cells are connected to each other through their lateral

membranes (Debnath et al., 2008). Subsequently, the apical and basal domains of the epithelial cell contain a distinct protein constitution that provides the axes specific properties that permits them to transport necessary molecules across the epithelial sheet (BM) (Zhan et al., 2008). Interestingly, all epithelial cells express E-cadherin, which is localized at the junctions between the lateral and apical axes (Runswick et al., 2001). Epithelial cells bind to each other by their extracellular domains and their intracellular domains primarily bind to the actin cytoskeleton via adaptor proteins, specifically E-cadherin's interaction with β and α–catenin (Zhan et al., 2008). These adherens junctions are vital for reinforcing epithelial cell shape as well as changes during tissue and gland development (Cowin et al., 2005). With respect to the mammary acini, apical-basolateral polarization occurs through the attachment of $\alpha6$-$\beta4$ integrin to the basement membrane (BM) (Schreider et al., 2001). It has been observed that mammary acini, whether derived from primary cells or immortalized MCF10a's, express high amounts of $\alpha6$-$\beta4$ integrins in dense structures, called hemidesmosomes (Underwood et al., 2006). Hemidesmosomes are responsible for allowing the myoepithelial layer of the mammary acini to connect with the ECM through utilizing integrin proteins to promote adherence. Consequently, cell-cell adhesion between luminal and myoepithelial cells is through desomosomes that are structures that utilize the cadherin family for promoting cell-cell adhesion in the formation of the acini (Underwood et al., 2006).

Existence of Mammary Stem Cells in the Mammary Gland

Mammary gland development is reliant on a variety of cellular events and cellular factors, like the cell-ECM and cell-cell contacts, that govern its proper formation and functioning. Conversely, the mammary gland is under another plane of regulation, which

is at the level of cell fate decisions. The mammary gland is a prime example of tissues that contain adult stem cells not unlike the bone marrow, salivary glands and pancreas (Lombaert et al., 2008). With respect to the mammary gland, it is during the developmental stage of involution that the mammary gland is very reliant on the stem cell pool (Filimore et al., 2008). Studies have proven the existence of these mammary stem cells (MSCs) through utilizing serial fat pad transplantation techniques. In these experiments, it has been demonstrated that samples taken from any part of the mammary gland can give rise to mammary epithelial outgrowths that have total development capabilities no matter the developmental stage or age (Smith et al., 1988). With the discovery of MSCs, studies have shown that these cells are able to accumulate mutations because of their prolonged and quiescent nature (Woodward et al., 2005). To be able to study MSC, putative markers were identified, using flow cytometry, to detect cell surface markers that could be used to identify a definitive stem cell population. Surface markers that were denoted MSC populations were CD24$^-$/CD44$^+$ (Bissell et al., 2008). Studies done by Al-Hajj et al demonstrated that CD24$^-$/CD44$^+$ tumor cells showed an increased ability to form tumors when injected into NOD/SCID mice fat pads (Al-Hajj et al., 2003). They observed that as few as 100 CD24$^-$/CD44$^+$ cells were capable of forming tumors, whereas 10,000 tumors cells from the same population with phenotype CD24$^+$/CD44$^-$ were not able to form tumors showing that this particular subpopulation of cells possess stem cell characteristics (Al-Hajj et al., 2003). Unfortunately, it remains relatively unclear what the relationship between CD24 or CD44 expression, as well as other MSC surface markers, and how this could relate to these markers being potential drug targets (Bissell et al., 2008). There is a great interest in these cell surface markers because of

their potential in understanding molecular mechanisms however there is still much debate into whether it is possible to isolate a pure population of stem cells from the normal mammary gland.

Consequently, the most definitive evidence that MSCs exist is through culturing structures *in vitro* called mammospheres. Mammospheres (MS) are solid, spherical structures that are grown in anchorage independent and undifferentiated conditions (Grimshaw et al., 2008). Studies have shown that when MS are grown in adherent conditions, cells from these structures can differentiate into multiple lineages such as myoepithelial, luminal and alveolar. Additionally, when MS are grown in matrigel, cells from these structures can differentiate and form branching structure analogous to those seen in the mammary gland (Liao et al., 2007). In the literature, it has been shown that MS composition consists of small population of stem cells, progenitor cells, differentiated cells as well as senescing cells (Dey et al., 2009). It has been demonstrated that with subsequent MS passaging, it is possible to enrich the stem cell pool within the MS (Grimshaw et al., 2008). Dey et al demonstrated that $CD24^{-}/CD44^{+}$ cells were capable of forming MS as opposed to subpopulations of cells that exhibited alternative phenotypes (Dey et al., 2009). They also postulated that the composition of MS from passage to passage varied because of the way the stem cell niche was responding to the culturing environment (Dey et al, 2009). Several factors are involved in regulating the stem cell niche such as: cell-cell interactions between stem cells and interactions between stem cells and ECM; each are capable of directing self-renewing abilities (Dontu et al., 2003). In another study, Iorns et al suggest that during MS passaging the stem cell niche may become undesirable for stem cells (Iorns et al., 2009). They hypothesize that the

undesirable environment promotes an increasing population of senescing cells which could be forcing stem cells to differentiate thereby shifting the balance between stem/progenitor and differentiated cells (Iorns et al., 2009). In summary, the culturing of MS provides a method to study properties of mammary stem cells and their niche as well as to determine how specific molecular mechanisms alter stem cell decisions in a manner that may contribute to mammary tumorigenesis.

Signaling Pathways Implicated in Mammary Development, Stemness and Breast Cancer

In order to understand how misregulation of stemness and cellular processes could contribute to tumorigenesis, it is imperative to study signaling pathways that are known to be involved in mammary tumorigenesis. There are a myriad of signaling pathways known to control self-renewal and preservation of stem cells but to also play a role in contributing to tumorigenesis. Some examples are TGFβ, Notch, Wnt and c-Myc (Dontu et al., 2003). One example of regulating stem cell self-renewal is through the Wnt pathway whose role is essential during development but also contributes to carcinogenesis (Neaves et al., 2006). The Wnt pathway participates in various developmental events including cellular polarization, proliferation, self-renewal and differentiation (Cadigan et al., 1997). In the canonical pathway the presence of Wnt activates the intracellular signaling protein Dishevelled (Dsh) which subsequently inactivates glycogen synthase kinase 3 (GSK3), a negative regulator of β−catenin. β−catenin is then able to accumulate in the cytoplasm and then translocate into the nucleus where it binds to transcription factor family, TCF/LEF which promotes the transcription of Wnt-responsive genes including, cyclin D1 and c-Myc (Polakis, 2000). In

the absence of Wnt, GSK3 and a group of other proteins [axin and adenotamous polyposis coli (APC)] form the "β−catenin degradation complex" where β−catenin gets phosphorylated by GSK3 and casein kinase 1 (CK1) and then is sent to the proteosome for degradation (Polakis, 2000). With respect to tumorigenesis, it has been observed that β−catenin overexpression contributes to over 90% of human breast cancers and has also been linked to poor prognosis in breast cancer (Michaelson et al., 2001).

There has been mounting evidence of the Wnt signaling pathway being involved in regulating self-renewal (Nusse, 2008). Inhibition of β−catenin in mammary alveolar progenitors has been shown to inhibit pregnancy stimulated proliferation as well as mammary development, where β−catenin has been associated as a survival signal for stem cells in the mammary gland (Tepera et al., 2003). As well, Anton et al were able to show that murine embryonic stem cells are reliant on β−catenin expression because of β−catenin's ability to promote stemness by turning on the expression of stem cell associated genes such as *Oct4* and *Nanog* (Anton et al., 2007). Considering the crucial role that Wnt plays in regulating molecular mechanisms contributing to cancer, there is considerable crosstalk among pathways that makes elucidating specific molecular mechanisms in cancer extremely difficult. Of all the crosstalk that Wnt participates in, an interesting example is the crosstalk that occurs between the E-cadherin and Wnt pathways. At the center of these two well-established pathways is β−catenin, whose connections are very much present in both E-cadherin and Wnt pathways, where its role are in regulating adhesion and various developmental events respectively (Cowin et al., 2005). Serine/threonine phosphorylation of β−catenin or E-cadherin stabilizes the cadherin/catenin complex, increase cell-cell adhesion and depletes cytoplasmic β−catenin

(Daugherty et al., 2007). It has been observed that overexpression of cadherins reduces available β−catenin by sequestering it at the plasma membrane to promote cell-cell adhesion and actin cytoskeleton reorganization (Daugherty et al., 2007). Conversely, both Wnt and E-cadherin mediated cell-cell adhesion are essential for the organization and maintenance of stem cells. For instance, mammalian hematopoietic stem cells (HSC) have demonstrated reliance on Wnt signaling and nuclear β−catenin for permitting cellular proliferation and limiting their differentiation potential (Nemeth et al., 2007). In addition, HSC niche in bone marrow are dependent on HSC attachment to osteoblast through cell-cell adhesion (Nelson et al., 2004).

In order to understand how Spy1 could be affecting self-renewal, it is important to mention c-Myc, an oncogene whose expression Spy1A mimics during mammary gland development (Golipour et al., 2008). Studies have shown that c-Myc participates in many cellular functions, such as replication, growth, metabolism, differentiation and apoptosis, and has been associated with poor prognosis in breast cancer (Liao et al., 2000). Interestingly, studies have shown that c-Myc is capable of reprogramming mouse fibroblasts into embryonic stem-like cells, which are called induced pluripotent stem cells (Wernig et al., 2008). In addition, c-Myc has also shown its abilities to maintain the stem cell differentiation and self-renewal through the regulation of the hematopoietic niche (Wilson et al., 2004). Additionally, Cdk2, Spy1's binding partner in the cell cycle, has also shown links to affecting stem cell self-renewal. For instance, Jablonska and colleagues demonstrated that cdk2 -/- mice showed significant decreases in self-renewal capacity and an increase in neural progenitor cells, suggesting that Cdk2 plays an important role in regulating proliferation and self-renewing abilities of neuronal stem

cells (Jablonska et al., 2007). Interestingly, studies have shown that Cdk2 regulates c-Myc's ability to promote senescence, which may have affects with how stem cell self-renewal is regulated (Jablonska et al., 2007). As well, Spy1's other known interaction, p27 has some links with affecting progenitor cells but not stem cells. It was discovered that p27 plays no role in affecting stem cell number or self-renewal, but it is involved in maintaining progenitor cell pool size and proliferation in primary mouse mammary cells (Cheng et al., 2000). It is understood that alterations in cell fate, adhesion and migration are characteristic of cancer and that abrogated expression of Wnt and cadherin contribute to mammary tumorigenesis.

Epithelial-Mesenchymal Transition

Mammary tumorigenesis is a process that is defined by misregulated signaling pathways, cellular processes and changes in morphology. It is a process that can transform tumor cells so that they are able to metastasize into different parts of the body. If tumors become metastatic then this has been correlated in promoting more advanced stages in cancer. In metastasis, cancer cells can spread from the initial tumor site, invade into the surrounding tissue, enter the bloodstream, travel through the bloodstream and then colonize a new site where the cancer cells can begin forming secondary tumor mass (Zetter, 1998). The epithelial-mesenchymal transition (EMT) is an example of a process that has been correlated to cancer invasion and metastasis in breast, colon and lung (Mani et al., 2008). EMT is a process where cells undergo a developmental switch from a low motility epithelial phenotype to a highly mobile, mesenchymal phenotype (Perez-Pomares et al., 2002). EMT has been proven to be essential for certain developmental events such as formation of tissues and organs, such as the heart and the peripheral

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nervous system (Mani et al., 2008). Specifically, EMT has been able to promote metastasis through its abilities to disrupt the normal functioning of specific cellular events as well as downregulate important factors that prevent cancer cells from becoming metastatic (Perez-Pomares et al., 2002). Studies have characterized EMT as cells possessing a loss of E-cadherin expression, a gain of N-cadherin and increased cellular migration.

The induction of EMT has been under the control of the TGFβ pathway coordinating with the Ras pathway (Huber et al., 2005). Several additional pathways have been associated with EMT including Wnt, Notch and Hedgehog, and coincidentally these pathways have shown to be essential in stem cell function and early development as well as in cancer (Huber et al., 2005). However, for the scope of this thesis, only the Wnt signaling pathway will be highlighted with respect to its role in EMT. In the canonical Wnt pathway, β–catenin levels and stability to its TCF/LEF transcription partner are enhanced when E-cadherin is degraded or when GSK3 is inhibited by the PI3K pathway (Nelson et al., 2004). Researchers are becoming aware that misregulated E-cadherin is one of the key events in EMT (Huber et al., 2005). Numerous EMT inducing regulators repress E-cadherin transcription by means of interacting with specific E boxes in the E-cadherin promoter. Some common examples of transcriptional regulators of E-cadherin are Snail and Slug, where Snail has been identified as the more common E-cadherin repressor in mammary tumorigenesis (Cowin et al., 2005). *In vivo* studies show that mice that lack Snail expression are embryonic lethal because of a defective EMT and constant expression of E-cadherin (Batlle et al., 2000). Another example of an E-cadherin repressor is Twist, a helix-loop-helix transcription factor that regulates cellular events

during gastrulation (Yang et al., 2004). With respect to EMT, studies by Yang and colleagues demonstrated that when Twist was knocked down in the 4T1 cell line, cells were able to metastasize into the lungs during fat pad transplant experiments. As well, Twist overexpression, in human epithelial cell lines, induced EMT and transcriptionally repressed E-cadherin (Yang et al., 2004).

Although E-cadherin regulation represents only part of the understanding behind EMT, other candidate genes, such as c-Myc and N-cadherin, have been proving themselves as worthy targets for EMT. Studies have shown that c-Myc when ovexpressed has been shown to decrease cellular migration and invasion (Alfano et al., 2010). In addition, c-Myc has also been shown to cooperate with TGFβ to induce EMT through activation of Snail (Smith et al., 2008). Cowling and colleagues were able to show that c-Myc overexpression lead to changes in mammary epithelial cell morphology from a cuboidal to a fibroblastic phenotype (Cowling et al, 2007). This research group also showed that overexpressing c-Myc mammary epithelial cells exhibited less contact inhibition than their controls, which suggests that c-Myc could be influencing EMT (Cowling et al., 2007). Alternatively, another study showed that c-Myc overexpression enhanced invasion in highly metastatic breast cancer cells (Coma et al., 2010).

N-cadherin is a pro-migratory cell adhesion protein and has been directly linked to metastasis (Hazan et al., 2000). Early studies have found that increased N-cadherin expression disrupted migration, invasion and was later shown to promote metastasis in immortalized cancerous mammary cell lines (Niemana et al., 1999). Hazan et al demonstrated that N-cadherin overexpression lead to increased invasive and migratory abilites in weakly metastatic cells, MCF7 as well they were able to demonstrate N-

cadherin's abilities to promote metastasis in mice (Hazan et al., 2000). Derycke et al determined that N-cadherin's ability to promote metastasis results from a synergistic interaction with fibroblast growth factor receptor (FGFR) via N-cadherin's extracellular domain, which stabilizes FGFR and sustains matrix metalloproteinase-9 production (Derycke et al., 2004). While the relationship between E-cadherin and N-cadherin in EMT remains elusive, the fact that EMT plays a role in metastasis cannot be ignored for it adds yet another level of complexity in the pursuit of clarifying mammary carcinogenesis.

EMT acts as an important mechanism for metastasis, where cellular events such as increases in migration, decreases in adhesion and increases in invasive abilities prompt tumor cells to metastasize to different parts in the body. Grzesiak and colleagues found that pancreatic cancer cells showed decreases in adhesive abilities as well as increases in migratory abilities through misregulation of E-cadherin and β-catenin (Grzesiak et al., 2005). In addition, Cheng and colleagues determined that Akt downregulation, a protein whose overexpression has been correlated to highly aggressive breast cancers, leads to a reduction in migration as well as invasion (Cheng et al., 2007). However metastatic cancers are not all created equal, and there are cases where typical characteristics were not observed in metastatic tumors. For instance, Sander and colleagues demonstrated that overexpression of T-lymphoma invasion and metastatic gene 1 (Tiam-1), which regulates cell invasiveness, could decrease migration and invasion but enhance cell proliferation (Uhlenbrock et al., 2004). Yakshibaeva and colleagues showed that metastatic lung carcinoma cells had lower migratory rates than their controls from transplantation experiments (Yaskshibaeva et al., 2002). Gilles and colleagues showed that cervical

carcinomas upregulate vimentin which caused lymph node metastasis, however when the metastases were collected, the tumors had lost vimentin expression (Gilles et al., 1996). It should be emphasized that primary tumors are heterogeneous, which makes it possible for tumors to exhibit classic signs of metastasis but not become metastatic (Fiddler et al., 2003) (Thompson et al., 2005). With this being said, it is essential to maintain an unbiased perspective for understanding cancer progression, since this is a research field that does not follow the expression, "one size fits all".

Since the role of Spy1A in promoting mammary tumorigenesis is largely unknown, this thesis will explore the multifaceted role of Spy1A as a novel target for mammary tumorigenesis. Some questions that will be asked are: How does Spy1 manipulation affect the development of the mammary acini? What role does Spy1 play in affecting stem cells within the mammary gland? How are cellular events like migration, adhesion and invasion affected by Spy1 manipulation? The following data to be presented in this thesis further builds upon previous work and continues to shed more light into the role of Spy1A in mammary tumorigenesis.

Hypothesis

Spy1 affects various cellular events in mammary gland development, and misregulation of Spy1 contributes to mammary tumorigenesis.

Objectives

1) To elucidate the role of Spy1 in cellular events regulated by the Wnt pathway.

2) To assess the role of Spy1 in stem cell self-renewal.

II. DESIGN AND METHODOLOGY

Cell lines Utilized

Mammary cell lines: MCF10a, MCF7, MCF7 pLXSN, MCF7 pLXSN Spy1, MDA-MB 231 pLKO, MDA-MB 231 pLKO Spy1, MCF7 pLKO, MCF7 pLKO Spy1, MDA-MB 231 pEIZ, MDA-MB 231 pEIZ Spy1. Mouse Mammary cell lines: HCll, HCll pEIZ, HC11 pEIZ Flag Spy1 Other cell lines: Human Embryonic Kidney cells (HEK) 293. Cell counts were performed utilizing Trypan blue exclusion.

HC11 wildtype and stable cell lines

HC11 wildtype and stable cell lines were grown in RPMI 1640 (Sigma#: R5886), 10% newborn calf serum (Sigma#: N4637), 10 μl/mL insulin, 5 mL Pen/Strep (per 500 mL media), and 20 ng/mL human Epidermal growth factor (Gibco).

MCF7 wildtype and stable cell lines

MCF7, MCF7 pLXSN, MCF7 pLXSN Spy1, MCF7 pLKO, and MCF7 pLKO Spy1 stable cell lines were grown in RPMI 1640 (Sigma), 10% Calf serum (Sigma#: C9676), and 5 mL Pen/Strep (per 500 mL media) (Gibco).

MDA-MB-231 Stable cell lines

MDA-MB-231 pEIZ, 231 pEIZ Spy1, 231 pLKO, and 231 pLKO Spy1 were grown in DMEM (Hyclone#: SH30022), 10% Fetal bovine serum (FBS) (Hyclone#: SH30070), and 5 ml Pen/Strep (per 500 mL media) (Gibco).

HEK 293 cell line

HEK 293 cell line was grown in DMEM (Hyclone#: SH30022), 10% fetal bovine serum (FBS) (Hyclone#: SH30070), and 5 ml Pen/Strep (per 500 mL media) (Gibco).

Establishment of Stable Cell lines

Knockdown Spy1 stable cell lines (pLKO/pLKO Spy1) for MDA-MB-231 and MCF7 were established in the following manner. Eighty thousand cells were seeded onto a 24 well plate and grown overnight in 500 μl of growth media that contained 10% serum and no penicillin/streptomycin. The next day and one hour before infection, media was changed to 500 μl serum free and antibiotic free media. Multiplicity of infection (MOI) for these infections was 10 and the virus titer for both control and shSpy1 was 10^7 titer units (TU). To the serum and antibiotics free media, 10 μg/ml polybrene was added then virus was added. Plates were rocked back and forth and side to side twice and left to incubate for 6 hours. After 6 hours, media was aspirated and fresh 500 ul of growth media containing 10% serum and no antibiotics was added. Since the control and knockdown vector had puromycin selection, 1 μg/ml puromycin was added to begin selection and media with puromycin was changed every 2 days. Control cells for both MCF7 and 231 pLKO died after 3 days while, 231 pLKO Spy1 control cells died after 5 days.

Overexpressing Spy1 stable cell lines (pEIZ and pEIZ Spy1) for MDA-MB-231 was established in the following manner. The day before infection, 20,000 cells were seeded onto a 24 well plate with 500 ul of growth media that contain 10% serum and no antibiotics. The following day, growth media was changed to 500 ul serum and antibiotics free media containing 10 μg/ml polybrene. MOI for this infection was also 10 and the virus titer for both pEIZ and pEIZ Spy1 was 10^7. Virus was left on cells to incubate for 6 hours and after 6 hours, media was changed to 10% serum with no

antibiotics. Since pEIZ has no selectable marker but instead has fluorescence marker, it took approximately 7 days for pEIZ and 12 days for pEIZ Spy1 fluorescence to be robust.

Plasmid Construction

Lentiviral vector pEIZ Spy1 was constructed by cutting Spy1, using restriction sites *Eco*RI and *Xba*I, from pJT013 Spy1+NdeI expression vector, and ligating it into the pEIZ backbone using the same restriction sites (gift from Dr. Bryan Welm).

Transfection Method

HEK 293 were grown to 50-60% confluency using media that contained 10% FBS (Hyclone), 5 mL Pen/Strep antibiotic (Gibco) prior to transfection. On the day of transfection, growth media was changed four hours prior to transfection to complete growth media. Cells were transfected with 5 μl PEI (10 mg/mL stock) and 50 μl of 150 mM NaCl. Cells were lysed 16 hours post transfection. Cells were lysed with 0.1% NP40 Lysis buffer, 1 μl/mL Leupeptin (Sigma#:L2884), 0.5 μl/mL Aprotinin (Sigma#: A6103) and 10 μl/mL PMSF (Sigma#:P7626) and were lysed for 60 minutes on ice. Protein concentrations were determined using Bradford assay where 2 uL of protein sample was diluted in 198 μl of Bradford reagent and concentrations were calculated using Victor Plate Reader spectrophotometer. Western blot analysis was used to verify transfection efficiency. After transfer, PVDF membranes were blocked in 1% milk (1g skim milk powder, 100 mL TBST) and then probed with various primary antibodies (c-Myc: 1:1000; β-catenin: 1:1000; actin: 1:1000; flag mouse: 1:2000). E-cadherin, N-cadherin and Novus Spy1 primary antibodies were blotted at a ratio of 1:1000 in 1% Bovine Serum Albumin (BSA). Corresponding horseradish peroxidase secondary antibody (1:10000) was utilized to detect protein expression. Exposure times for antibodies were

as followed: Actin (1 minute), β-catenin (15 minutes), c-Myc (9 minutes), E-cadherin (6 minutes), N-cadherin (6 minutes), Spy1 (8 minutes) and Flag mouse (11 minutes). For chemiluminescence, equal parts of luminol and 1% hydrogen peroxide were utilized to visualize protein expression.

Three Dimensional (3D) Culture

Three dimensional cultures were utilized to grow mammary acini using non-tumorigenic MCF10a. To construct 3D cultures, Cultrex (Trevigen, catalog#: 3223-001-01) basement membrane extract (BME) was used in an overlay format. In the overlay method of 3D culturing, 3 or 4 glass coverslips (Fisherbrand microscope cover glass circles, catalog#: 12-545-80) were placed in a 35 mm tissue culture plate. Coverslips were then coated with 30 μl of undiluted Cultrex, and incubated for 15 minutes at 37°C to allow for solidification of Cultrex. After solidification, 1500 cells in 30 μl complete media were seeded onto the coated coverslips. The complete media used for MCF10a was DMEM-F12 containing 2% heat inactivated horse serum (Sigma, catalog), 10 μg/ml insulin (Sigma), 5 ng/mL human recombinant EGF (Gibco), and 0.5 μg/ml hydrocortisone.

Mammosphere Culture

MCF7 and 231 stable cell lines were utilized for mammosphere (MS) culture. Mammosphere (MS) culture for MCF7 and 231 stable cell line utilized MEGM Bullet kit (Clonetics, catalog #: CC-3150), 10 ng/mL human epidermal growth factor (Gibco), and 20 ng/mL human basic fibroblast growth factor (Invitrogen, catalog#: 13256-029; stock 25 μg/mL; dissolved in 20 mM Tris, pH 7.5), and 4 μg/mL heparin (Sigma#: H0777, stock: 140 units; dissolved in 50 mg/ml sterile distilled water). For all stable cell lines,

mammospheres were seeded 50,000 cells/ per well using low attachment plates (Corning, catalog#: 3471) grown for 7 days and then passaged. Mammospheres were fed with 10 ng/mL epidermal growth factor and 20 ng/mL fibroblast growth factor with 1 mL of fresh MEGM every 4 days. To dissociate mammospheres for passaging, 0.25% Trypsin (Hyclone) was added to MS culture and allowed to trypsinize for 8 minutes at 37°C. Dissociated mammosphere cultures were collected in 15 mL conical tubes and 1 mL of FBS was added and then cultures were spun down for 5 minutes at 800 rpm. Dissociated mammospheres was subjected to trypan blue exclusion assay to ensure equal numbers of cells were seeded in the low attachment plates.

Clonal Assay

To assess sphering ability, MCF7 and 231 stable cell lines were seeded onto a 96 well ultra low attachment plate (Corning, catalog#: 3474). Using serial dilution, 10 cells were seeded per well in 100 μl of MEGM (Clonetics) containing 10 ng/mL human epidermal growth factor (Gibco), and 20 ng/mL human basic fibroblast growth factor (Invitrogen) and were incubated at 37°C for 7 days. During this time, cells formed spheroids structures. To calculate the percentage sphering within a well, the following equation was used:

$$\textbf{\% Sphering} = \frac{\text{\# of spheres formed}}{10 \text{ cells}} \times 100\%$$

Migration Assay

Migration assay used was adapted from Cell Biolabs protocol. Migration assays were was performed in 12 well plates using 8 μm cell inserts (BD biosciences, catalog#: 353093). Cells were treated with 20 mM NaCl (control) and 20 mM LiCl to see effects of the presence and absence of GSK3. Cells were seeded, transfected and the day after

transfection, cells were utilized in the migration assay. In a 12 well plate, 1 ml of complete growth media was placed into each well, then cell inserts were placed one hour prior to assay. Cells were counted using trypan blue exclusion and making a cell suspension that contained 500,000 cells per mL of serum and antibiotic free media was prepared. 300 μl of cell suspension was seeded into each insert and cells were allowed to migrate at 37°C for the various timepoints as indicated. Upon collection at each timepoint, cell inserts were removed and the interior of the insert was washed with a distilled water dampened Q-tip, and then stained with 400 μl of crystal violet stain (Cell Biolabs, catalog#: CBA-100) for 10 minutes at room temperature. Cell inserts were gently washed with distilled water and allowed to dry for 10 minutes. Pictures of the stained cell inserts were taken at this point. Cell inserts were transferred to an empty well that contained 200 μl of cell extraction solution (Cell Biolabs, catalog#: CBA-100) and was incubated for 10 minutes. 100 μl of each sample was transferred to a 96 well plate and the optical density (OD) was read at 590 nm.

Adhesion Assay

The adhesion assay was designed by Dr. Lisa Porter. Collagen and fibronectin plates were coated prior to the assay. To prepare collagen solution, 500 μl of collagen IV derived from rat (Sigma, catalog#: C7661) was dissolved in 14.5 ml of sterile 2N acetic acid. To coat plates, 100 μl was used to coat the well of a 24 well plate. Collagen was added to the well and then was swirled around to coat the entire surface area of well and the remainder was used to coat the next well. Plates were allowed to dry for 20 minutes at room temperature and subsequently were stored at 4°C. To prepare fibronectin solution (Disclaimer: do not agitate or vortex fibronectin), add 10 μl of 1 mg/ml fibronectin

solution (Sigma, catalog#: F2006) in 9990 μl of sterile 1X PBS. 100 μl was used to coat wells and wells were allowed to dry for 20 minutes. Coated plates were stored at 4°C. Prior to adhesion assay, cells utilized in this assay were either transiently transfected or were stable cell lines. On the day of the assay, cells were counted using trypan blue exclusion and 20,000 cells were seeded per well for various timepoints as indicated in growth media containing 10% serum and antibiotics. Upon collection at each timepoint, the growth media was aspirated and wells were washed with 1 ml of 1x PBS and then aspirated again. Two drops of Coomassie blue stain (~2 μl) were added to each well and pictures were taken immediately. Then, 200 μl of lysis buffer (1% NP40) was added to each well and then 100 μl of each sample was transferred to a 96 well plate, and optical density (OD) was read at 590 nm.

Invasion Assay

Invasion assay was performed in 24 well plates using using 8 um cell inserts (BD Biosciences). Each insert was coated with 100 μl of undiluted Cultrex (Trevigen) and allowed to solidify for 15 minutes at 37°C. Transiently transfected cells as well as stable cell lines were utilized in this assay. Cells were counted using trypan blue exclusion and a cell suspension containing 500,000 cells per mL in serum free and antibiotic free media. In a 12 well plate, 1 ml of growth media was added to each well and the cell insert was placed ½ hour prior to assay. 300 μl of cell suspension was added to each insert and allowed to invade for 24 hours at 37°C. The next day, cell inserts were cleaned with a distilled water dampened Q-Tip and stained with 400 ul of crystal violet stain (Cell biolabs) for 10 minutes at room temperature. Cell inserts were gently washed with distilled water, were allowed to dry for 10 minutes and pictures of stained cell inserts

were taken. Then, cell inserts were placed into a well that contained 200 µl of cell extraction solution (Cell biolabs) and were incubated for 10 minutes. 100 µl of cell lysates was transferred into a 96 well plate, where the optical density (OD) was read at 590 nm.

β-catenin Reporter Assay

Plasmids utilized in this assay were: M50 Super 8X TCF/LEF TOPFLASH reporter plasmid (Addgene) and M51 Super 8X TCF/LEF FOPFLASH mutant reporter plasmid (gift from Dr. R.T. Moon, University of Washington). HEK 293 cells were transfected with various combinations of 10 µg of pCS3, myc Spy1 pCS3, pLKO, pLKO with M51 and M50. 293 cells were transfected on 6 cm plates, containing 5 ml of growth media, and 5 µl of 10 mg/mL PEI, with 50 µl of 150 mM NaCl. Once PEI, NaCl and DNA were added together, the mixture was vortexed, incubated at room temperature for 10 minutes and in a dropwise manner the DNA mixture was added to plates. HEK 293 were transfected for 16 hours. After 16 hours, cells were collected without trypsin and were centrifuged for 3 minutes at 800 rpm and the growth media was aspirated off. Cells were re-centrifuged to remove any remaining growth media. 50 µl of growth media was added to each pellet as well as 50 µl of Bright GLO Luciferase reagent (Promega). Then, 50 µl of the luciferase mixture were added to a 96 well plate where luminescence (counts per second, cps) was measured at 440 nm.

QRT-PCR

Quantitative real time PCR was performed utilizing SYBR green master mix (Applied Biosystems) to determine the relative expression of Spy1, CK18, CD24 and CD44 across monolayer and mammosphere passages. QRT PCR primers were designed

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along exon-exon junctions and detected all isoforms of the target genes studied. Prior to QRT PCR, QRT PCR primers were verified by absolute quantification in order to verify that the gene of interest was only being amplified as well as proper conditions for QRT primers. For normalization of data, ΔCT values were calculated by subtracting CT values from the endogenous control (GAPDH) and the target. pEIZ monolayer was considered the calibrator for all QRT PCR reactions and all ΔCT values were compared against its ΔCT values to give the ΔΔCT value. Then, the equation $2^{-\Delta\Delta CT}$ was utilized to calculate the relative quantification for each target gene studied, where log RQ represents the fold change between the samples and the calibrator. The average of log RQ, log min RQ and log max RQ were calculated for each sample and was subsequently graphed. Standard error was calculated from the log RQ, log min RQ and log max RQ.

RNA was extracted from mammospheres and mammary acini utilizing RNeasy mini-prep kit (Qiagen). Following RNA extraction, cDNA synthesis was carried out in the following method: Amount of cDNA synthesize was dependent on RNA concentration. The range of cDNA synthesized was between 100 ng-3000 ng. For instance, 100 ng of total RNA and water were added to make final volume of 8 μl. Then, 1 μl of 10 mM dNTP, 1 μl random primers, and 1 μl oligo(dT). Then the RNA mixture is incubated at 65°C for 5 minutes. After 5 minutes, RNA mixture is put directly on ice. Then, 4 μl of 5X FS Buffer and 2 μl of DTT were added to the mixture and incubated at 42°C for 1 minute. Then 0.5 μl of SuperScript II Reverse transcriptase was added to each tube and incubate at 42°C for 60 minutes. To inactive SuperScript II reverse transcriptase, tubes were incubated at 70°C for 15 minutes. Since mammosphere and

acini yield such little RNA and cDNA, the number of cycles utilized in every Q-PCR run was always 65 cycles.

Statistics

Statistics were performed using non-paired two sample t-test. Standard error was calculated as standard deviation (SD)/number of replicates (n).

III. RESULTS

To further understand the role of Spy1 in mammary development, it is imperative to study how Spy1 affects various cellular events that are known to be targeted during tumorigenesis. Cellular events such as cellular migration, adhesion, invasion and stemness are processes that are essential for a cell to behave properly during normal development. However, misregulation of such processes has been linked to mammary tumorigenesis and can promote metastasis (Mahoney et al., 2002). These sets of experiments will involve documenting the role of Spy1 in migration, adhesion and invasion, and stemness. This work will further clarify the role of Spy1 in mammary tumorigenesis.

Cell line Rationale

Throughout these studies, four different cell lines were utilized: MCF7, MDA-MB-231, HC11 and HEK 293. <u>*MCF7 & 231:*</u> The MCF7 cell line represent a cancerous breast cell line that are estrogen receptor (ER) positive as well as E-cadherin positive, and are representative of a luminal breast cancer subtype. The 231 cell line represent a basal breast cancer cell line that is ER and E-cadherin negative. Since 231 and MCF7 cell lines contain high levels of Spy1, it was essential to knockdown Spy1 expression to assess how various cellular processes would be affected. <u>*HC11 & 293:*</u> HC11 cells are normal mouse mammary cells derived from a mid-pregnant Balb/c mouse that have mutant p53 alleles. HEK 293 cells are human embryonic kidney cells that express vitronectin. For Spy1 overexpression, it was important to use cell lines that did not express high levels of Spy1. Using these cell lines allow us to adequately investigate the effects of elevated levels of Spy1 on specific aspects of cellular function. In addition, while breast cell types are of

the primary importance to our work, these cell lines are notoriously difficult to manipulate gene expression. Hence, determining whether 293 cell lines follow the same physiological response in response to altered Spy1 levels is essential to facilitate the use of these cells for future pathway/molecular studies.

Spy1 Knockdown Increases Cellular Migration

To assess the role of Spy1 in cell migration, stable knockdown of Spy1 was introduced into MDA-MB-231 (231) and MCF7 cell lines utilizing infection techniques with lentivirus. Stable Spy1 knockdown (pLKO-Spy1) and stable control (pLKO) cell lines were treated with 20 mM lithium chloride (LiCl) and 20 mM sodium chloride (NaCl), which served as a control. 20 mM Lithium chloride is utilized to mimic the Wnt in that Li^+ ions have been shown to inhibit GSK3 activity (Csenlenyi et al., 2008). It was important to study Spy1 expression in the presence and absence of LiCl to see if Spy1 overexpression and knockdown was affected by GSK3 in order to further understand Spy1's role in the Wnt pathway. Cells were subjected to a trypan blue exclusion assay to assess proliferation rates (Figures 1A 231; 2A, MCF7). MCF7 and 231 knockdown pLKO-Spy1 cells have lower cell numbers in comparison to the pLKO –control cells. Spy1 knockdown has been shown in literature to cause a decrease in cell number, so it was to be expected that Spy1 knockdown in both 231 and MCF7 would elicit lower cell numbers (McAndrew et al., 2009).Upon observing differences between 231 knockdown Spy1 cells treated with NaCl and LiCl, it appeared that NaCl treated Spy1 cells had higher cell numbers in comparison to the LiCl treated Spy1 cells. However in MCF7, Spy1 knockdown NaCl cells had lower cells numbers in comparison to LiCl treated cells (Figure 2A). Once cell numbers were obtained, 231 and MCF7 stable cells, with and

without LiCl, were seeded into a migration chambers where 3, 4, 5 and 18 hour timepoints were collected (Figure 1B; 231 & 2B; MCF7). For both 231 and MCF7 stable cell lines knock down of Spy1 expression increased the number of cells migrating through the polycarbonate chambers (Figure 1B; 231 & 2B; MCF7). These data were confirmed by collecting cells from the polycarbonate chambers, staining with crystal violet and measuring optical density at 590 nm (Figure 1C; 231 & 2C; MCF7). For both 231 and MCF7, pLKO-Spy1 cells had higher optical densities than the pLKO control (Figure 1C; 231 & 2C; MCF7). However, an interesting relationship was observed in western blot analysis (Figure 1D). In the knockdown 231 experiments, β-catenin levels appeared to be decreased in the presence of Spy1 knockdown. This may mean that in presence of active GSK3, Spy1 may be affecting β-catenin levels. However in the presence of LiCl., there appeared to be an increase in β-catenin levels, which may show that Spy1 does not affect β-catenin levels in the absence of GSK3 (Figure 1D 231; 2D MCF7). In sum, reduced Spy1 expression results in an increase in cell migration in a manner which may not be dependent on GSK3, meaning that Spy1 may be affecting β-catenin levels in a way that does not fit with the known canonical Wnt pathway.

Spy1 Overexpression Decreases Cellular Migration

To delve deeper into the role of Spy1 in cell migration, overexpression experiments were performed utilizing stable cell lines that were created through infection with lentiviral expression constructs. Empty vector was used as a control (pEIZ) along with pEIZ-Flag Spy1 in non-tumorigenic immortalized mouse mammary cells (HC11). In addition, transient transfection methods were utilized on HEK 293 (293) cells to introduce Spy1 overexpression (myc-Spy1-pCS3) prior to the migration assay. LiCl or

NaCl control treated overexpressing Spy1 cells were subjected to trypan blue exclusion assay to assess proliferation rates. In both cell types, Spy1 overexpression results in significantly higher cell numbers in comparison to pEIZ and pCS3 (Figure 3A; HC11 & 4A; 293). Spy1 overexpressing or control cell lines treated with LiCl or NaCl were then seeded into migration chambers where 3, 4, 5 and 18 hour timepoints were collected. In both cell systems Spy1 overexpressing cells had lower cell numbers migrate through the polycarbonate chamber in comparison to their pEIZ and pCS3 controls (Figures 3B; HC11 & 4B; 293). Then, cells were collected from their chambers, stained with crystal violet and their optical density (OD) was measured at 590 nm (Figure 3C & 4C). For the HC11 cells, there were statistically significant differences between pEIZ-Flag-Spy1 and pEIZ control cells during timepoints 3, 4 and 5 hours at the $p < 0.05$ and $p < 0.001$ levels (Figure 3C). For 293 pCS3-Myc-Spy1 cells, there was a statistically significant relationship between Spy1 overexpressing cells and their pCS3-control during all timepoints at the $p < 0.05$ and $p < 0.001$ levels (Figure 4C). Overexpression of Spy1 increased β-catenin levels in both NaCl and LiCl treated cells in the HC11 cells (Figure 3D). However, 293 overexpressing Spy1 cells only showed higher β-catenin levels when treated with LiCl (Figure 4D). E-cadherin levels appeared to be elevated in LiCl-treated cells in comparison to the NaCl-treated cells (Figure 4E). However, both control and treatment cells elicited constant E-cadherin levels (Figure 4E). To summarize Spy1 overexpression effects on cellular migration, Spy1 caused fewer cells to migrate.

The results of Spy1 on cellular migration were not results that were expected. The original postulate for these experiments was that Spy1 overexpression would increase migration and its knockdown would cause a decrease in migration. The rationale behind

this postulate was that Spy1 has been shown to be upregulated in invasive breast cancers (Zucchi et al., 2004), and so it was thought that if Spy1 promote metastasis; a scenario where cells have acquired the ability to be more motile (Huber et al., 2005). It is possible that that Spy1's potential interactions with Axin and LRP6 could be involved with stabilizing β-catenin levels in metastasis, where β-catenin levels have been shown to be stabilized in EMT (Huber et al., 2005).

Spy1 Knockdown Decreases Adhesion

Cell adhesion is a very important event involved in cell-cell and cell-ECM contact. Adhesion also plays a role in migration, where cell-substrate interactions are vital for permitting a cell to be mobile. It was with great interest to study the role of Spy1 in cell-substrate adhesion after revealing significant effects of Spy1 on cell migration. Stable MCF7 and 231 knockdown Spy1 cell lines, infected with pLKO-control and pLKO-Spy1 lentivirus were subjected to a trypan blue exclusion assay to assess proliferation rates. Both MCF7 and 231 cells expressing pLKO-Spy1 expression demonstrated lower cell numbers than their controls (Figure 5A; 231, 6A; MCF7). 20,000 stable cells were then seeded onto various substrates; plastic, collagen and fibronectin and cells were allowed to attach over 0, 4 and 24 hours. At each particular timepoint media was removed, cells were washed with 1X PBS and then stained with coomassie blue and pictures were taken. In both cell systems pLKO-Spy1 had fewer cells that attached on the collagen, fibronectin and plastic substrates (Figures 5B; 231 & and 6B; MCF7). Once photographs were taken, stained cells were put into lysis buffer and their OD was measured at 590 nm. Optical density of MCF7 and 231 pLKO-Spy1 cells were lower than their pLKO-control (Figures 5C; 231 & 6C; MCF7). The relationship between

Spy1 and β-catenin was again observed in the 231 knockdown Spy1 cells in the presence of LiCl or NaCl control. In 231 cells, lower overall levels of β-catenin were observed in the presence of Spy1 knockdown. (Figure 5D). However for MCF7 cells, β-catenin levels could not be probed and only E-cadherin levels were observed. It appeared that Spy1 caused an increase in E-cadherin levels (Figure 6D), which may mean that the absence of Spy1 could be allowing E-cadherin to recruit β-catenin up to the plasma membrane to promote cell-cell adhesion and disrupt cell-substrate adhesion. To summarize the relationship between Spy1 and adhesion, it was determined that Spy1 knockdown reduces the number of cells that are able to adhere to plastic, collagen and fibronectin substrates in both MCF7 and 231 cells.

Spy1 Overexpression Increases Adhesion

Since the knock down expression of Spy1 causes a decrease in cell adhesion, it was of great interest to see how Spy1 overexpression could affect the ability of cells to adhere to select substrates (plastic, collagen and fibronectin). 293 cells were transiently transfected with Spy1 overexpression vector (pCS3-Myc-Spy1)_ or the empty vector control (pCS3), and HC11 stable pEIZ-control and pEIZ-Flag-Spy1 were utilized in these assays. Proliferative abilities of 293 and HC11 cells overexpressing Spy1 were assessed using trypan blue exclusion assay. 293 pCS3-Myc-Spy1 and HC11 pEIZ-Flag-Spy1 had higher cell numbers than their pCS3 and pEIZ controls respectively (Figures 7A HC11; and 8A 293). 20,000 cells were then seeded onto the different substrates and were permitted to adhere for 0, 4, and 24 hours. At each timepoint, cells were washed with 1X PBS, stained with coomassie blue and photographs were taken. The results from these studies in 293 cells show that Spy1 significantly increases adhesion to the various

substrates over control (Figure 8B). These results were confirmed by measuring absorbance of 293 overexpressing cells over control (Figure 8C). The relationship between 293 overexpressing Spy1 and substrate adhesion yielded statistically significant results at the $p<0.05$ and $p<0.001$ for 293 Spy1 overexpressing cells adhering to collagen and plastic substrates (Figure 8C).

In the HC11 cell line, cells overexpressing Spy1 (pEIZ-Flag-Spy1) had more cells adhere to the plastic and collagen substrates than the HC11-pEIZ controls (Figure 7B). This same trend was seen when optical densities were measured and was highly statistically significant ($p<0.001$) for early timepoints (0 and 4 h plastic and fibronectin) and statistically significant ($p<0.05$) during the last timepoint (24 h plastic, collagen and fibronectin) (Figure 7C). Subsequently, HC11 pEIZ-Flag-Spy1 cells appeared to have adhered less to the fibronectin substrate (Figure 8B), a trend confirmed by absorbance measurement (Figure 7C). In sum, Spy1 overexpression increases adhesion of 293 cells to all substrates tested and HC11 cells for both collagen and plastic matrices. However for the HC11 cells Spy1 overexpression inhibited adhesion to a fibronectin substrate.

Spy1 Effects on Invasion

In cancer, literature has shown that changes in cellular migration, adhesion and ability to invade are hallmarks for metastatic tumors. Given the role of Spy1 on adhesion and migration, it was important to determine whether Spy1 could affect invasion. For these experiments, MCF7 stable Spy1 knockdown or control cells (pLKO-Spy1 or pLKO respectively) were used along with transiently transfected 293 cells to overexpress Spy1 (pCS3-Myc-Spy1) or control (pCS3). Serum-starved cell suspension consisting of 500,000 cells were seeded into cultrex-coated chambers and permitted to invade through

for 24 hours. The results from the study showed that MCF7 Spy1 knockdown cells caused reductions in the ability of cells to invade through the coated membrane (Figure 9A). Along this same trend, 293 cells with elevated levels of Spy1 were able to invade through coated chambers more efficiently than their controls (Figure 9A). This same trend was seen when optical densities were measured (at 590 nm) after the assay. Results showed that MCF7 pLKO-Spy1 cells had statistically significant lower optical densities than the pLKO-control ($p<0.001$) and 293 pCS3-Myc-Spy1 cells had higher optical densities than relevant controls ($p<0.01$) (Figure 9B). In summary, Spy1 knockdown reduced cell invasiveness whereas Spy1 overexpression promotes cell invasiveness.

Spy1 Effects on β-catenin Transcriptional Activity

To gain more insight into the potential role of Spy1 in the canonical Wnt signaling pathway, it was important to study how Spy1 could affect β-catenin signaling. To assess β-catenin signaling in the presence of Spy1 overexpression in 293 cells, a luciferase reporter assay was performed to determine β-catenin's transcriptional activity using a β-catenin reporter plasmid (TOPFLASH) and a mutant β-catenin reporter plasmid (FOPFLASH). β-catenin transcriptional activity was higher in 293 cells overexpressing Spy1 (pCS3-Myc-Spy1) in comparison to the FOPFLASH controls (Figure 10A). To summarize these findings, Spy1 overexpression increases β-catenin transcriptional activity in the presence of activated GSK3, where LiCl treatments prevented Spy1 from affecting β-catenin transcriptional activity (Figure 10A).

Spy1 Effects on Stemness

Previous research has shown that Spy1 is highly expressed during specific times in mammary gland development including puberty, early pregnancy and involution

(Golipour et al., 2008). As previously mentioned, the mammary gland undergoes major changes in structure and function during pregnancy and lactation, where expression of Spy1 has been shown to be elevated during this period of development (Golipour et al., 2008). Specifically, involution is a stage where lactation is no longer needed and the gland undergoes massive restructuring to return back to its virginal state. During involution, signals are being sent to the mammary acini to undergo apoptosis, among other reconstruction events. At the same time, mammary stem cells present within gland are now being recruited to help in the restructuring and reorganization of the mammary gland. Stem cells are highly reliant on their niche, a microenvironment that promotes stem cell self-renewal through controlling events such as proliferation and adhesion (Brisken et al., 2007). The cancer stem cell hypothesis has come to the forefront to begin to explain how tumorigenesis could arise. The hypothesis states that tumors arise from tumor cells that have acquired self-renewal abilities (Tan et al., 2006). Given that Spy1 is involved with causing mammary tumorigenesis, is highly expressed during involution, as well can affect cellular processes like adhesion, migration and proliferation, a few interesting questions stood out: Does Spy1 have any effects on mammary stem cells? What role could Spy1 have in affecting stemness? Or could Spy1 promote the expression "cancer stem cell"? These were a few questions that prompted studying Spy1's role in affecting stem cell self-renewal.

To begin to answer these questions, a novel cell culture technique was utilized where it would be possible to work with stem/progenitor cells through promoting their growth in specialized culturing conditions. This new technique involves the culturing of structures called mammospheres (MS), where cells are seeded in non-differentiating and

anchorage independent conditions which promote the stem cells present in the population to form mammospheres. MCF7 and 231 cell lines were utilized in these experiments because both cell lines contain a small pool of stem cells. The ability for a stem cell to undergo self-renewal has been demonstrated through enriching the stem cell pool by the formation of mammospheres and then passaging these spheres, thereby releasing stem cells to begin to form more spheres with further passaging (Dey et al., 2009). It was of great interest to determine whether Spy1 could affect mammosphere size, numbers, passaging and sphering abilities. Overexpressing stable cell lines were generated using lentiviral pEIZ-control and pEIZ-Flag-Spy1 for the 231 cell system. In addition, MCF7 cells were previously created through retroviral infection using pLXSN-control and pLXSN-Flag-Spy1 (Golipour et al., 2008). Knockdown stable cell lines were created using lentiviral pLKO-control and pLKO- Spy1 for both MCF7 and 231. Stable cells, both overexpression and knocking down Spy1 expression, were seeded into ultra low adhesion plates and grown in serum free media, supplemented with epidermal growth factor (EGF) and fibroblastic growth factor (FGF), for 7 days and then passaged four times.

For the overexpression study, the results show that for both 231 and MCF7 overexpressing Spy1 cells had statistically larger diameters than their controls (Figure 11A I-II; overexpression Spy1 MS & 11A III-IV; Knockdown Spy1 MS). For the MCF7 MS, there was a statistical difference between pLXSN and pLXSN Spy1 in passages 1, 2, and 4 at the $p < 0.05$ and $p < 0.001$ levels (Figure 11B). Whereas, the 231 pEIZ-Flag-Spy1 stables showed statistical differences between pEIZ-control and pEIZ-Flag-Spy1 in all four passages at the $p < 0.001$ level (Figure 11B). Next, the numbers of MSs were counted

between passages comparing numbers between the overexpressing Spy1 cells and their controls. MCF7 pLXSN-Flag-Spy1 generated higher numbers of MSs in the first two passages when compared to the pLXSN control MS's (Figure 11B). High statistical significance was only observed during passage 3 between MCF7 pLXSN-Flag-Spy1 MSs and pLXSN-control MSs (Figure 11B). Conversely, 231 pEIZ-Flag-Spy1 MSs generally had lower numbers of spheres formed than the pEIZ-control (Figure 11B).

For the knockdown studies, the 231 pLKO-Spy1 cells showed a cyclical trend where diameter varied across passages (Figure 11B). For instance, 231 pLKO passage 1, 3 and 4 MSs were larger than 231 pLKO-Spy1 counterparts (Figure 11A IV. and B). However, 231 pLKO-Spy1 passages 2 MSs had larger diameters than pLKO-control (Figure 11A and B). There were differences between 231 pLKO-Spy1 and pLKO-control MSs in every passage (Figure 11B). With respect to number of spheres, there were variations with respect to passage number in number of spheres formed between the control and knockdown Spy1 MSs (Figure 11A). In passages 1 and 4, more spheres formed in the controls than the 231 pLKO-Spy1 spheres. However in passages 2 and 3, more spheres had formed in the 231 pLKO-Spy1 than in the control (Figure 11B).

For the MCF7 knockdown study, it appeared that the MCF7 pLKO-control were forming MSs that were larger than the MCF7 pLKO-Spy1 in passage 1 (Figure 11A III). However in passage 2, no spheres formed in the MCF7 pLKO Spy1 cells (Figure 11A III). There were statistical differences between the MCF7 pLKO control and MCF7 pLKO-Spy1 mammospheres (Figure 11B). Subsequently, the number of MSs was counted and numbers were compared between MCF7 pLKO control and MCF7 pLKO-Spy1 MSs across passages (Figure 11B). For the MCF7's, the pLKO-Spy1 MSs yielded

lower numbers of spheres formed than the pLKO-control and this relationship was shown to be statistical significant in both passages, at the $p<0.05$ and $p<0.001$ levels respectively (Figure 11B). From a biological standpoint, Spy1's effects on mammospheres shows that Spy1 could be stimulating the stem cell self-renewal as well as proliferation. This is biologically relevant since stem cells are necessary in the mammary gland to rebuild the gland after involution. In a tumorigenic context, these mammary stem cells could transform and become cancerous where Spy1 could be stimulating these cancerous stem cells and promoting mammary tumorigenesis.

In order to determine whether Spy1 affects the ability of a cell to form a MSs, a clonal assay was performed to calculate the efficiency of sphere formation. A small number (10 cells) of control and manipulated Spy1 cells were seeded individually in a 96 well low attachment plate. Specifically, cells utilized in this assay were derived from dissociated passage 1 spheres. Cells had 7 days to form MSs at which time, wells were scored according to whether spheres had formed (see Design and Methodology for how sphering percentage was calculated). For the MCF7 overexpression, the pLXSN-control sphere-forming percentage was 17.7% whereas the MCF7 pLXSN-Flag-Spy1 sphere-forming percentage was 8.1%, which was determined to be statistically significant at the $p<0.001$ level (Figure 11C). For the 231 cells, pEIZ-Flag-Spy1 cells yielded a 7.5% for their ability to form spheres whereas pEIZ cells yielded a 9.1% (Figure 11C). For the knockdown MCF7 cells, the pLKO-control sphering percentage was 4.5% and the pLKO-Spy1 sphering percentage was 5.7% (Figure 11C). Finally for the 231 knockdown Spy1 clonal assay, pLKO-control cells obtained a 3.8% in sphere formation abilities and the pLKO-Spy1 cells yielded a 4.9% (Figure 11C).

Diameter size was measured at the end of the clonal assay (Figure 11C). For the MCF7 spheres formed in the clonal assay, pLXSN-Flag Spy1 MSs were slightly smaller (128.8 um) than the pLXSN-control spheres (129.1 um) (Figure 11C). For 231 Spy1 overexpression, pEIZ-Flag-Spy1 MSs formed larger spheres (104.8 um) than pEIZ-control (93.3 um), which proved to be statistically significant relationship at the $p<0.001$ level (Figure 11C). For Spy1 knockdown in the MCF7's, pLKO-Spy1 MSs were larger (172.1 um) than the controls (117.1 um; $p<0.001$) (Figure 11C). As well, 231 pLKO-Spy1 formed spheres with small diameters (88.4 um) than pLKO-control (95.7 um) ($p<0.001$) (Figure 11C). In essence, the resultant MS diameters determined in the clonal assay were affected by Spy1 manipulation, just like MSs from the passaging experiments (Figure 11B & C).

To understand how Spy1 could affect other proteins and how these relationships could explain the effects of Spy1 on stemness, cells were collected and protein was extracted from each passage from the MCF7 cells containing either pLXSN-control and pLXSN-Flag-Spy1or MCF7 pLKO-control and pLKO-Spy1, as well as the 231 cells with the knockdown constructs pLKO-control and pLKO-Spy1 (Figures 11D, E, F).

For MCF7 MSs containing the knockdown constructs, reduced Spy1 (Spy1-pLKO) expression upregulated E-cadherin expression in both MS passages (Figure 11E). Consequently, β-catenin levels were lower in the presence of pLKO-Spy1 expression, which appears to fit with literature that has shown that lower β-catenin expression prevents stem cell self-renewal (Cajanek et al., 2009) (Figure 11E). As well, N-cadherin levels were upregulated across passages 1 and 2 of MCF7 pLKO-Spy1, which is surprising considering E-cadherin expression is also elevated (Figure 11E). With respect

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to E-cadherin and N-cadherin, it is possible that Spy1 may be involved in maintaining the balance between stem cells (increased N-cadherin expression) and progenitors (increased E-cadherin expression) and this may explain why these two proteins are elevated in during MS culture (Klopp et al., 2010). Additionally, MCF7 Spy1 MS elicited higher β-catenin levels than controls, which seems to fit with literature that shows that β-catenin stabilization can promote stem cell self-renewal (Perez-Ruiz et al., 2008).

In Figure 11D, protein levels were assessed for 231 knockdown Spy1 MSs. It must be mentioned that 231's are E-cadherin negative and no expression of E-cadherin was ever observed via western blot analysis. However, it appears that in the early passages, 231 pLKO-Spy1 spheres exhibited higher N-cadherin levels than pLKO-control (Figure 11D). Interestingly, β-catenin levels were also low in the presence of pLKO-Spy1 (Figure 11D). In the MCF7 Spy1 overexpressing MSs, β-catenin levels were elevated with elevated levels of Spy1 however, E-cadherin levels were only modestly elevated (Figure 11F)

To assess potential alterations in gene expression associated with knockdown of Spy1 expression, 231 MSs containing pEIZ control or pEIZ-Flag-Spy1 were selected through passaging, RNA collected and gene expression analysed by Q-RT-PCR. Classical markers for mammary differentiation or stemness were utilized: i) differentiation marker, cytokeratin 18, ii) stemness markers, CD24, iii) another stemness marker, CD44, and iv) an embryonic stem cell marker, Oct4 (Figure 11G). pEIZ-Flag-Spy1 MSs had slightly higher levels of CK18 than pEIZ-controls in the early MS passages and monolayer (Figure 11G). However in passage 4, 231 pEIZ-Flag-Spy1 MSs had slightly lower levels of CK18 than pEIZ-controls (Figure 11G). As previously

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mentioned, researchers have discovered that mammary stem cells exhibit the CD24-/CD44+ on their cell surface. Measuring these markers demonstrated that there was a variation in expression of stem markers across passages where, for instance, monolayer Spy1 overexpressing cells appeared to be more stem-like, in passages 1 and 3 the Spy1 overexpressing MSs exhibited a less stem-like appearance but this was reversed in passages 2 and 4 where pEIZ-Flag-Spy1 MSs exhibited more of a stem-like appearance than control treated cells (Figure 11G). These same irregular variations were noted when measuring the pluripotency marker Oct4. Monolayer 231 pEIZ-Flag-Spy1 cells expressed higher amounts of Oct4 than their control counterparts, in the first MS passage cells overexpressing Spy1 showed lower levels of Oct4 and subsequently in passages 2-4, Spy1 overexpressing MSs expressed higher levels of Oct4 than controls.

In passaging experiments, Spy1 overexpression elicited larger MS, whereas Spy1 knockdown showed a less clear trend where MCF7 knockdown Spy1 MS were larger and 231 knockdown Spy1 MS were smaller. As well, 231 overexpressing Spy1 MS may have exhibited larger MS however, these MS did not elicit more stemness as the QRT PCR data displayed. The QRT PCR data showed that there may be inconsistencies using immortalized cell lines in MS culture. This may explain the irregular variation in the expression of stemness and differentiation markers across passages. The effects of Spy1 on stem cell self-renewal is still in its infancy, so it would be necessary to perform more experiments to further clarify how Spy1 could affect mammary stem cells and how these effects could contribute to mammary tumorigenesis.

A

B

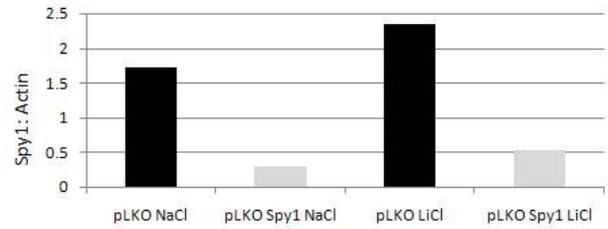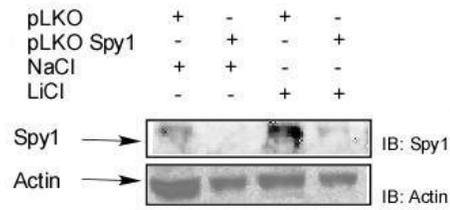|				
pLKO	+	+	+	+
pLKO Spy1	−	−	−	−
LiCl	−	−	+	+
NaCl	+	+	−	−

Collection Time (hours)

3

4

5

18

49
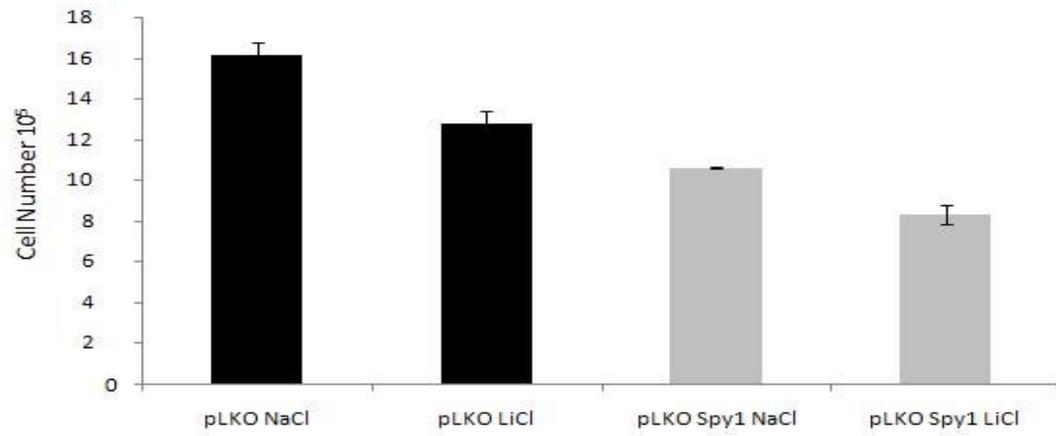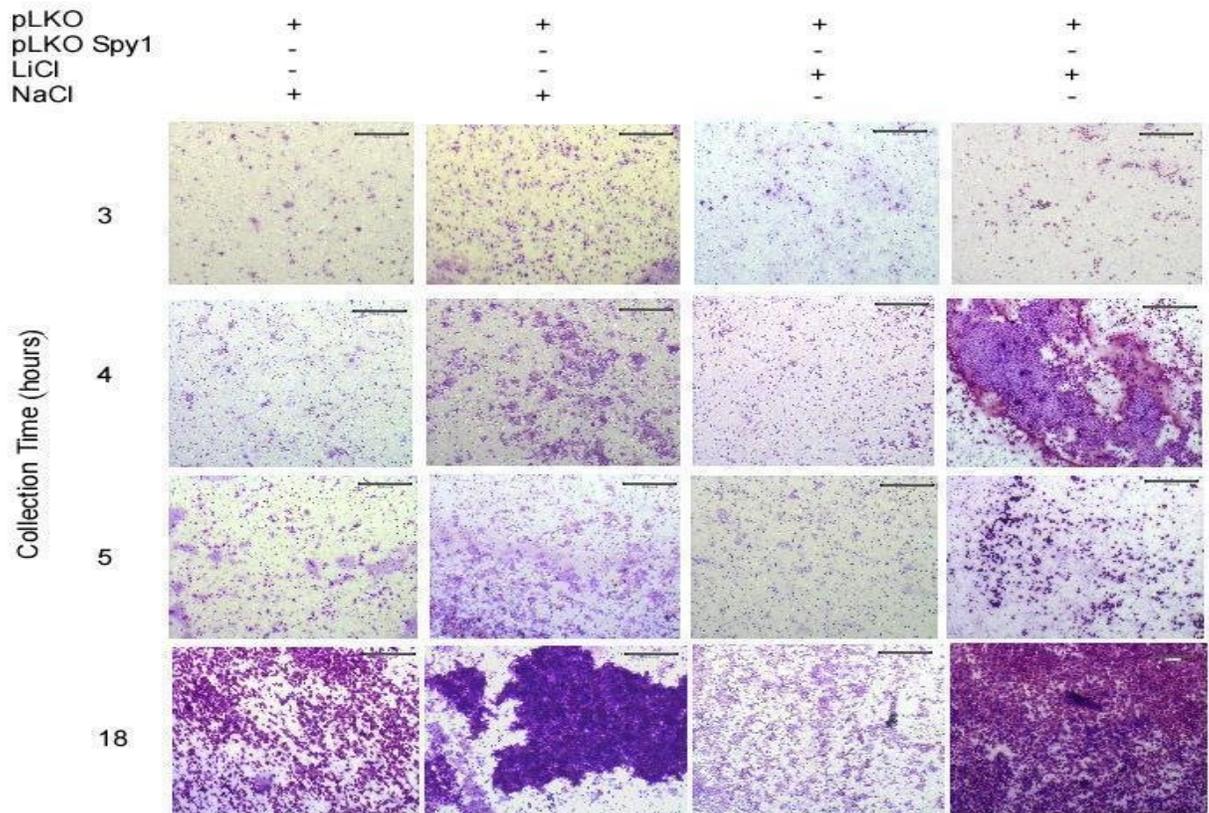
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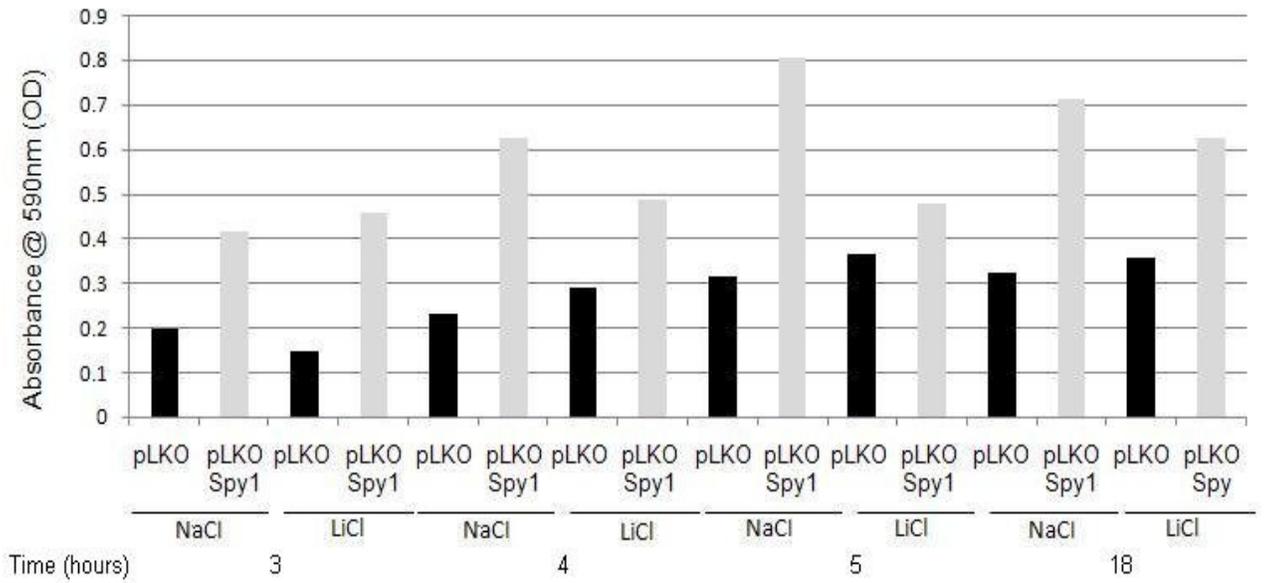
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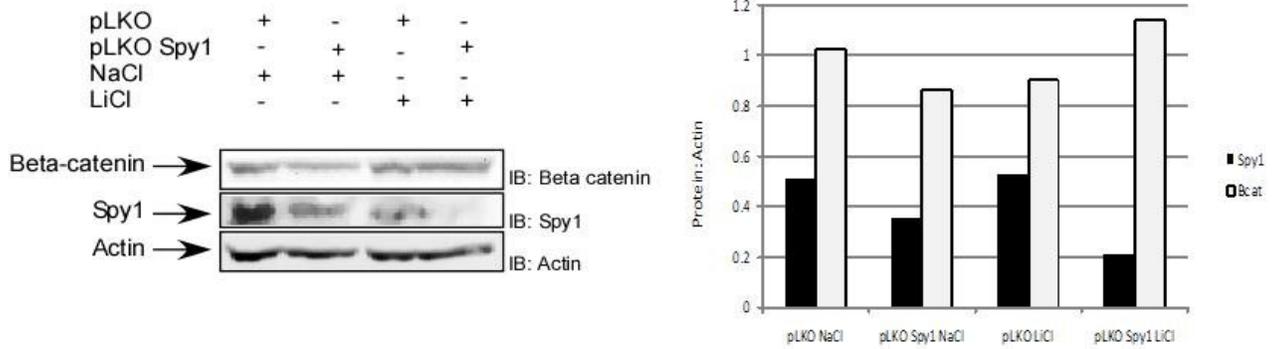
Figure 1. *Spy1 Knockdown Increases Migration in MDA-MB-231 cel1s.* Knockdown Spy1 (pLKO Spy1; grey bars) and Control cells (pLKO; black bars) in the presence and absence of LiCl were utilized for assessing: *A*, Average cell numbers obtained over 2 experiments using trypan blue analysis in triplicate counts. *B*, Migration pictures obtained from one set of experiments at 20x magnification. *C*, Optical density measured at 590 nm over two experiments. *D*, Western blot of migration assay and densitometry graph to represent Spy1 (black bars) and β-catenin (empty bars) expression levels of one experiment.

A

B

C

D

Figure 2. *Spy1 Knockdown Increases Migration in MCF7 cells.* Knockdown Spy1 (pLKO Spy1; grey bars) and Control cells (pLKO; black bars) in the presence and absence of LiCl were utilized for assessing: *A*, Average cell numbers obtained over 2 experiments using trypan blue analysis in triplicate counts. *B*, Migration pictures obtained from one set of experiments at 20x magnification. *C*, Optical density measured at 590 nm over two experiments over 2 experiments. *D*, Western blot of migration assay and densitometry graph to represent Spy1 expression levels of one representative experiment.

A

B

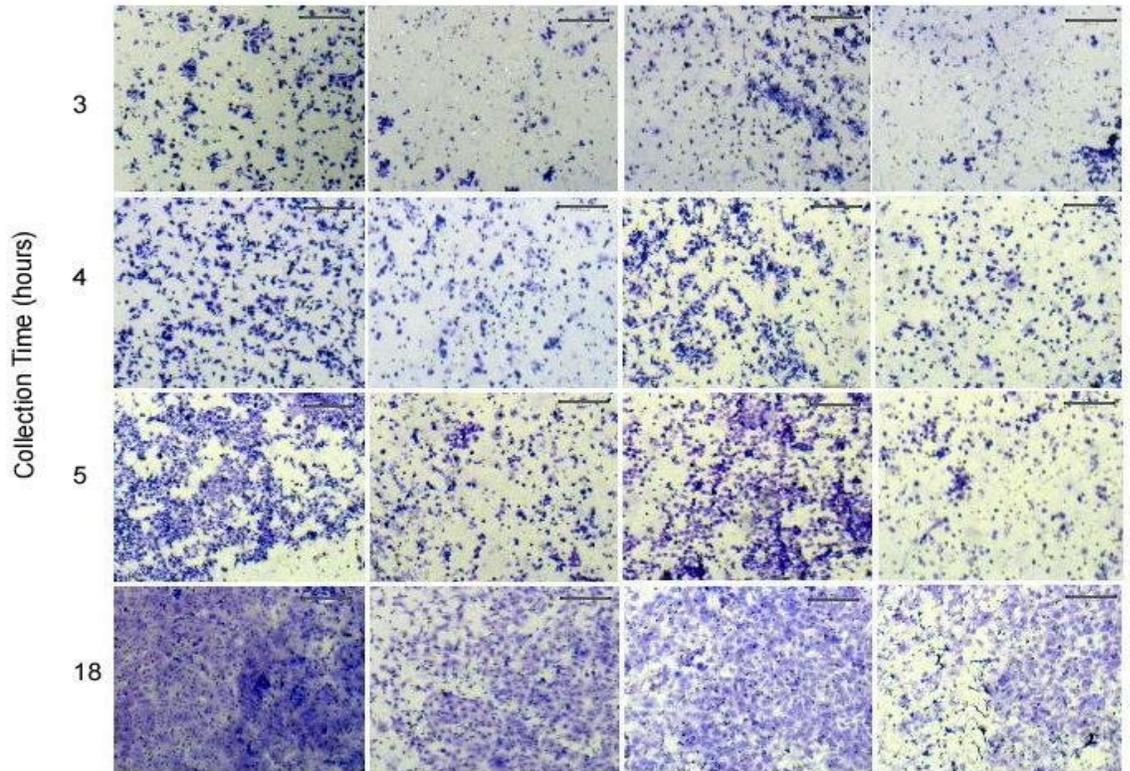| pEIZ | + | + | + | + |
pEIZ Flag Spy1	-	-	-	-
LiCl	-	-	+	+
NaCl	+	+	-	-

Collection Time (hours): 3, 4, 5, 18
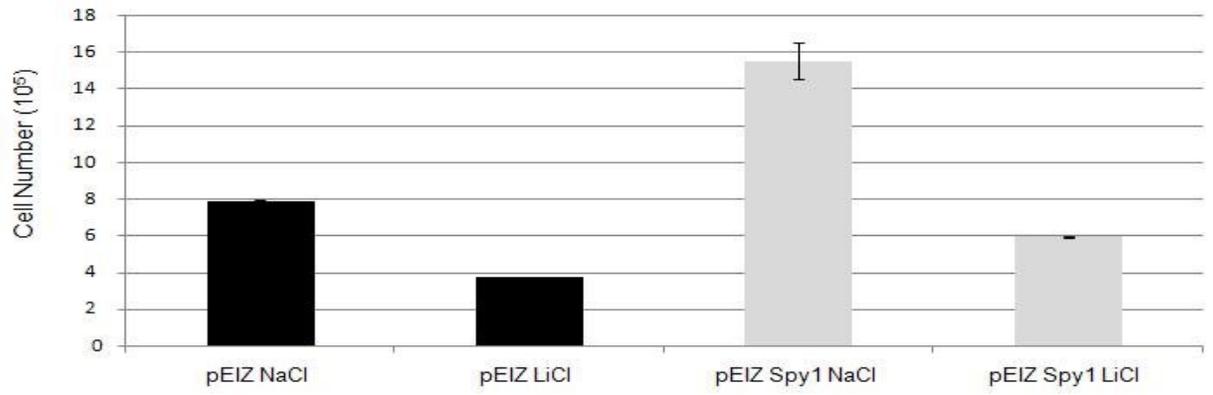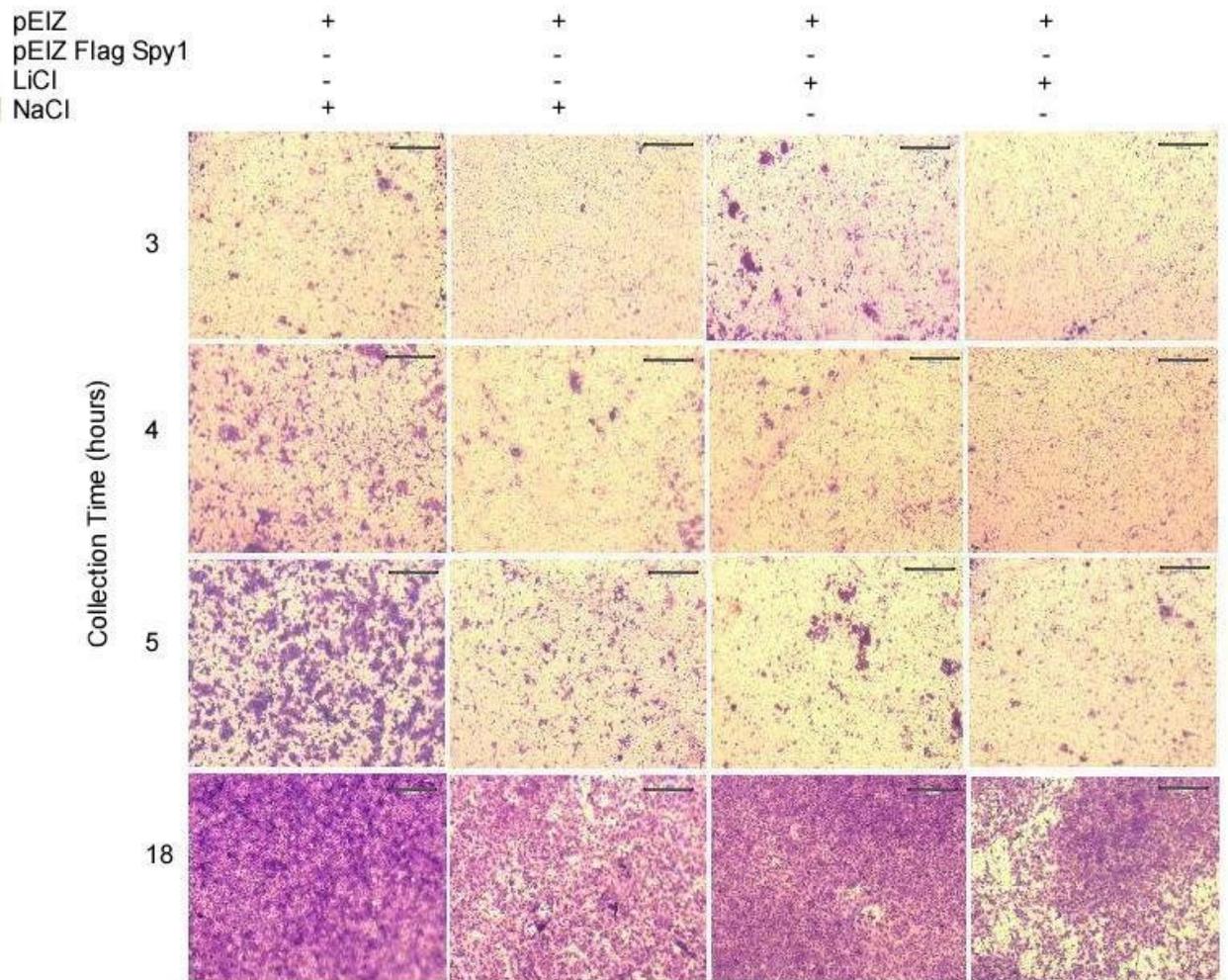
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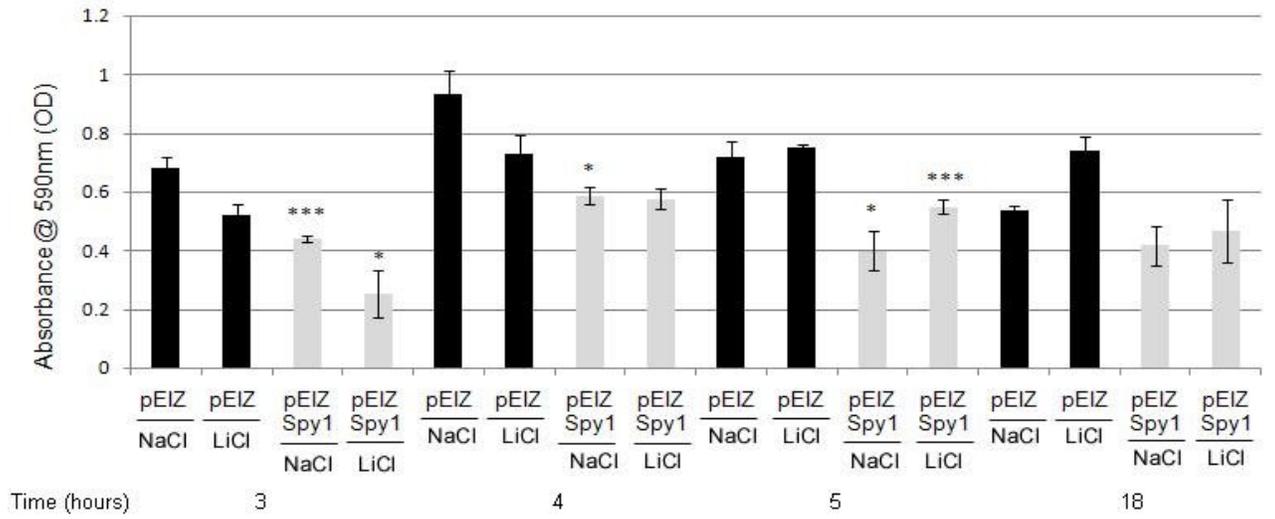
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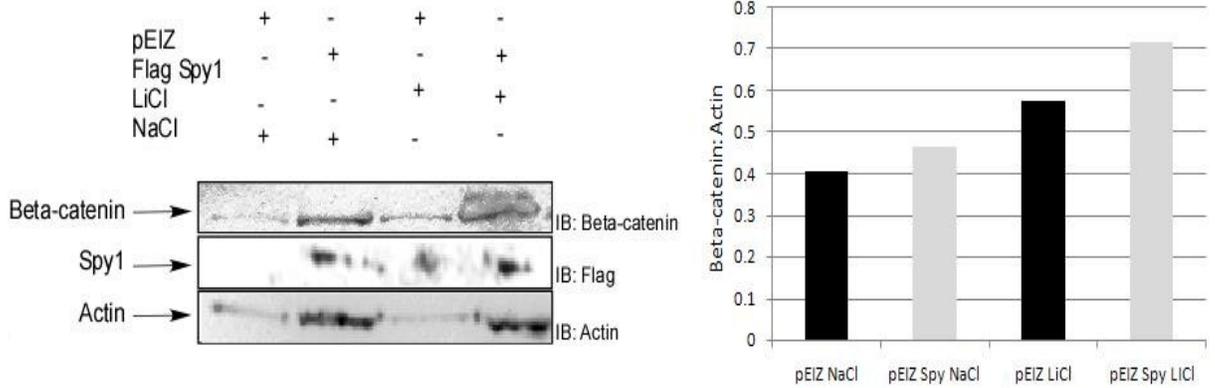
Figure 3. *Spy1 Overexpression Decreases Migration in HC11 cells.* Spy1 Overexpression (pEIZ Flag Spy1; grey bars) and Control cells (pEIZ; black bars) in the absence and presence of LiCl were utilized for assessing: *A*, Average cell numbers obtained over 3 experiments using trypan blue analysis (n=3). *B*, Migration pictures obtained from one set of experiments at 20x magnification. *C*, Optical density measured at 590 nm (n=3, * $p<0.05$, *** $p<0.001$). *D*, Western blot and densitometry graph of β-catenin levels of one representative experiment.
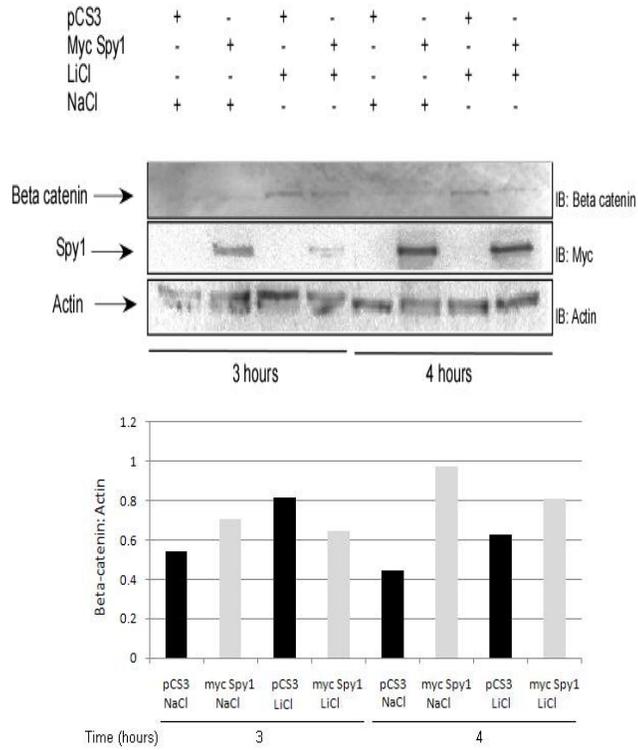
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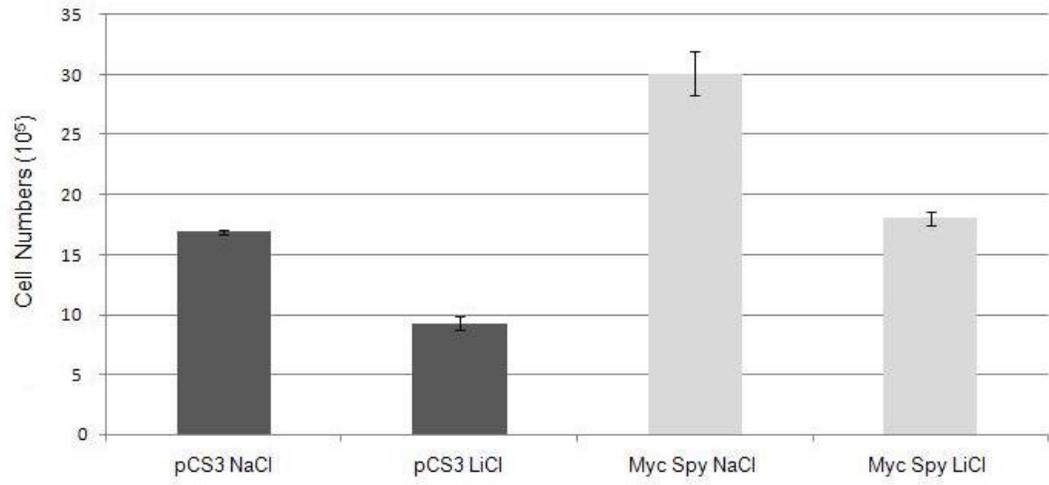
B

pCS3	+	+	+	+
pCS3 Myc Spy1	-	-	-	-
LiCl	-	-	+	+
NaCl	+	+	-	-

Collection Time (hours)

3

4

5

18

C

D

D

|								
pCS3	+	-	+	-	+	-	+	-
Myc Spy1	-	+	-	+	-	+	-	+
LiCl	-	-	-	-	+	+	+	+
NaCl	+	+	+	+	-	-	-	-

E-cadherin → IB: E-cadherin

Beta catenin → IB: Beta-catenin

Spy1 → IB: Myc

Actin → IB: Actin

5 hours   18 hours   5 hours   18 hours

60
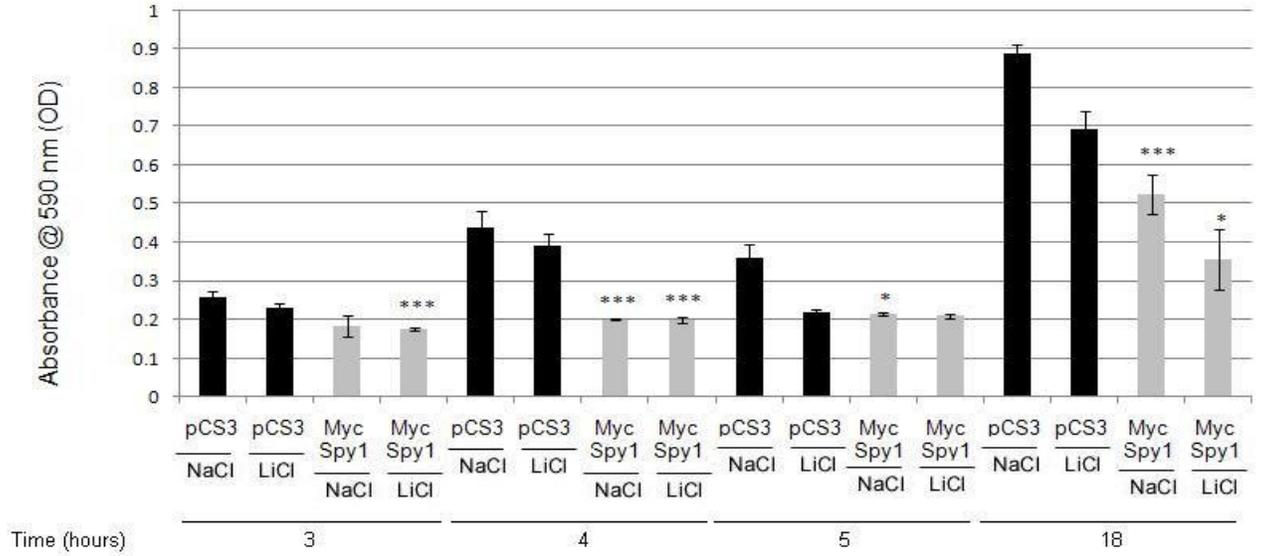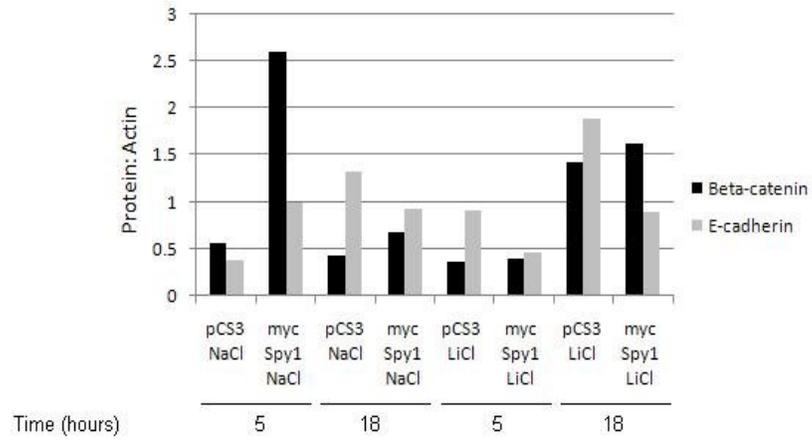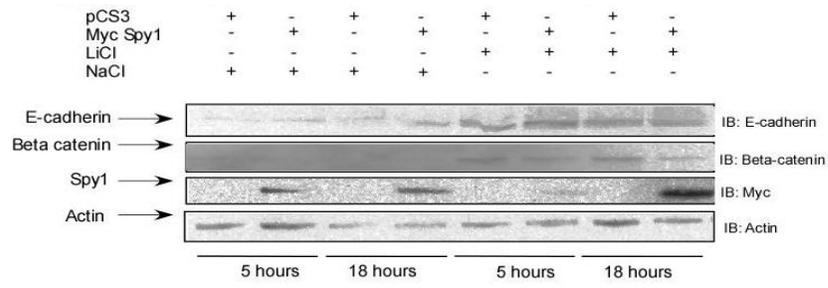
Figure 4. *Spy1 Overexpression Decreases Migration in HEK 293 cells.* Spy1 Overexpression (pCS3 Myc Spy1; light bars) and Control cells (pCS3; dark bars) in the absence and presence of LiCl were utilized for assessing: *A*, Represented graph of average cell numbers obtained from 3 hour timepoint (from 3 experiments) to show trend of Spy1's effects on proliferation through the use of trypan blue analysis. *B*, Migration pictures obtained from one set of experiments at 20x magnification. *C*, Optical density measured at 590 nm (n=3), * $p<0.05$, *** $p<0.001$). *D*, Western blot analysis and densitometry graph of β-catenin (black bars) and E-cadherin (grey bars) levels of one experiment over different timepoints using one representative experiment.
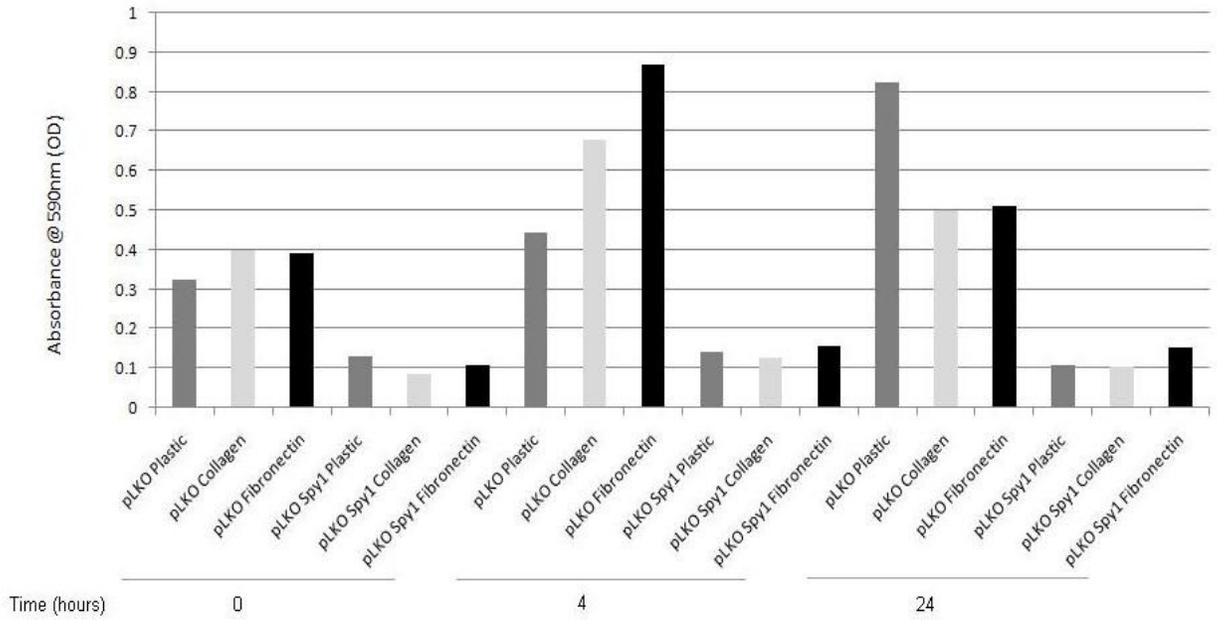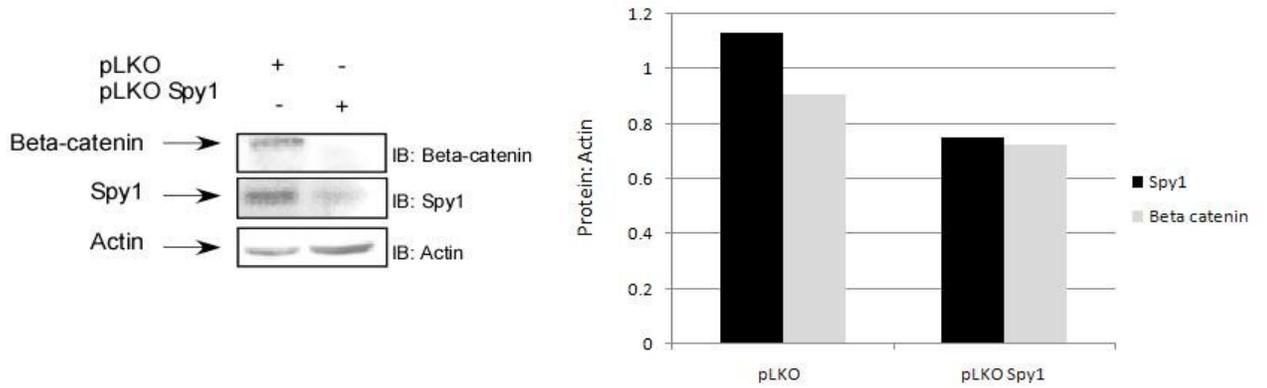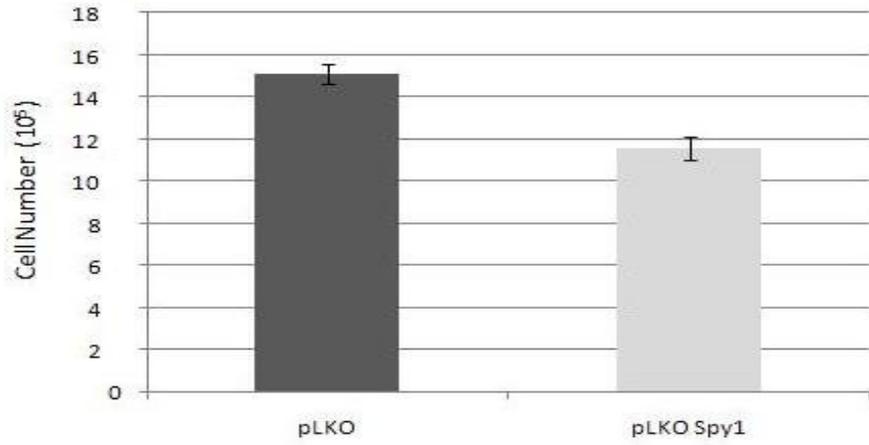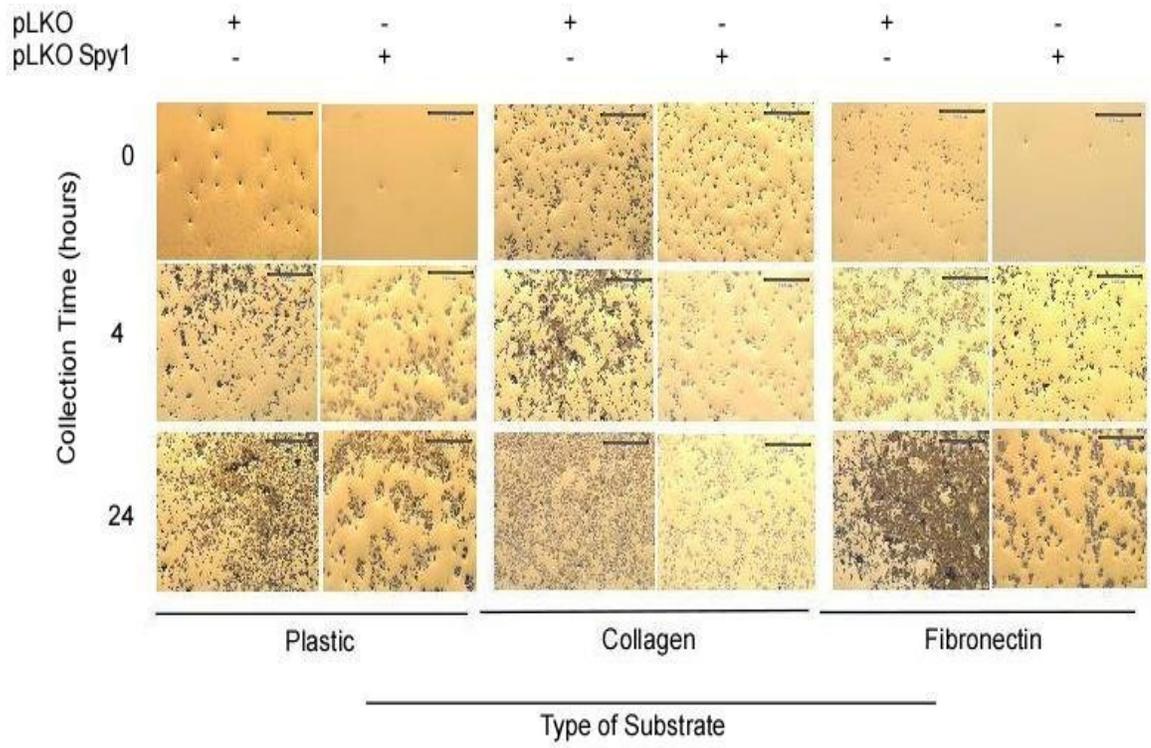
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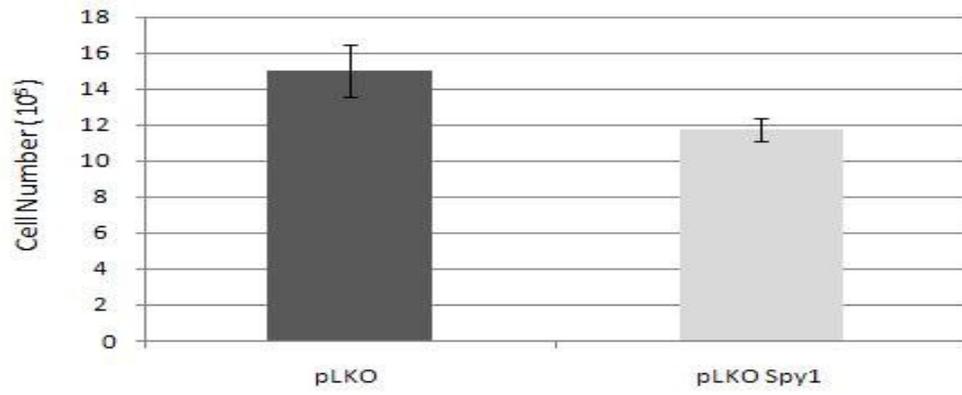
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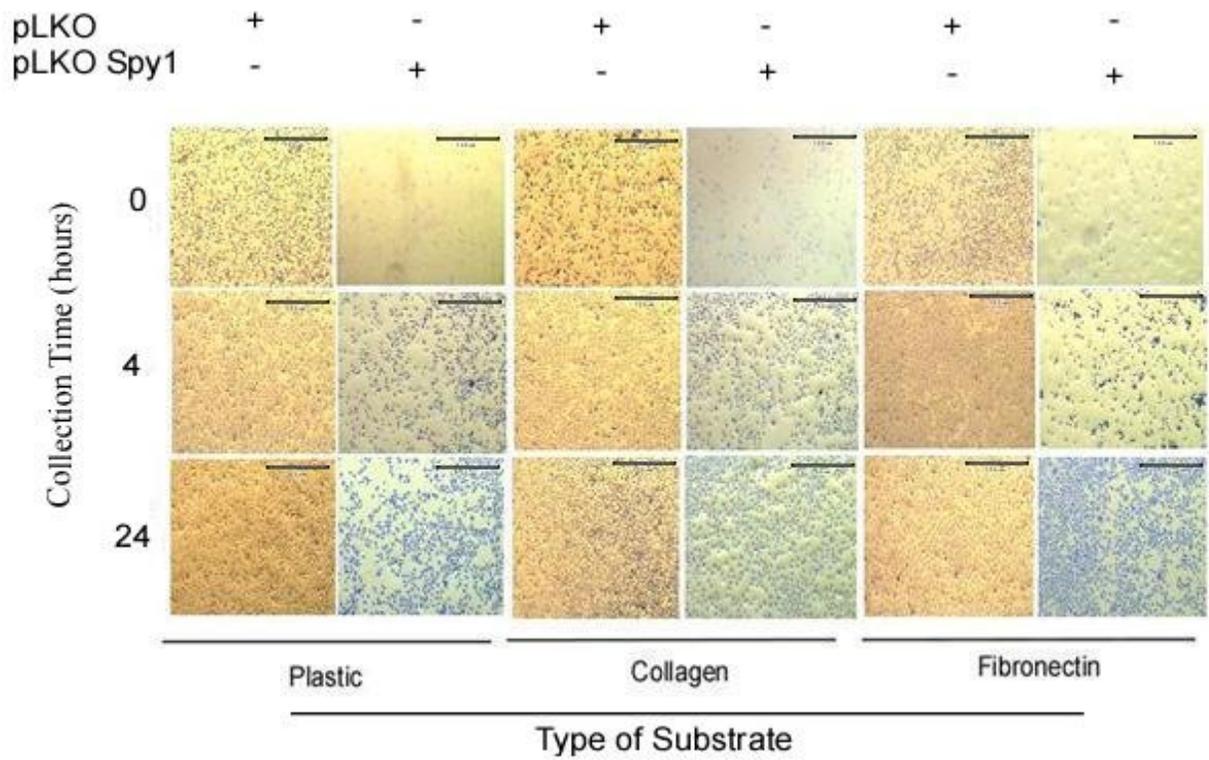
C

D

Figure 5. *Spy1 Knockdown Decreases Adhesion in MDA-MB-231 cells.* Knockdown Spy1 (pLKO Spy1; light bars) and Control cells (pLKO; dark bars) were utilized for assessing *A*, Average cell number obtained over 2 experiments using trypan blue analysis. *B*, Representative pictures from one adhesion timecourse experiment performed on plastic, collagen and fibronectin substrates at 20x magnification. *C*, Optical density measured at 590 nm over two experiments. (n=2). *D*, Western blot of adhesion assay and densitometry graph to represent Spy1 (black bars) and β-catenin (grey bars) expression levels of one representative experiment.
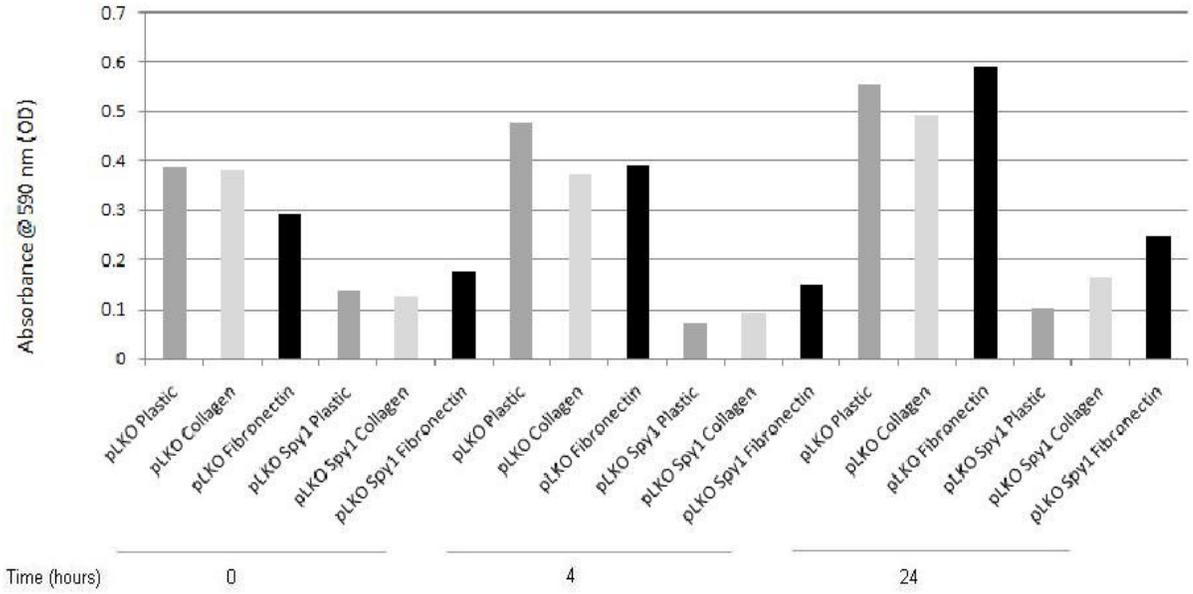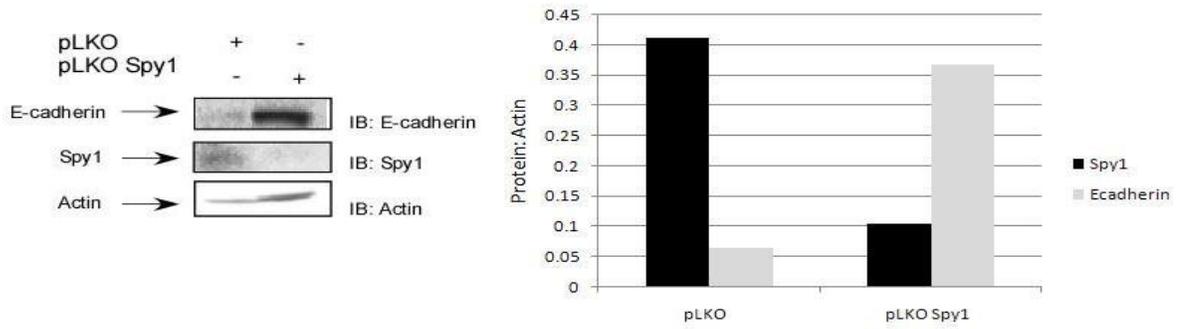
A

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Figure 6. *Spy1 Knockdown Decreases Adhesion in MCF7 cells.* Knockdown Spy1 (pLKO Spy1; light bars) and Control cells (pLKO; dark bars) were utilized for assessing *A*, Average cell numbers obtained over 2 experiments using trypan blue analysis. *B*, Representative pictures from one adhesion timecourse experiment performed on plastic, collagen and fibronectin substrates at 20x magnification. *C*, Optical density measured at 590 nm over two experiments. (n=2). *D*, Western blot of adhesion assay and densitometry graph to represent Spy1 (black bars) and E-cadherin (grey bars) expression levels for one representative experiment.
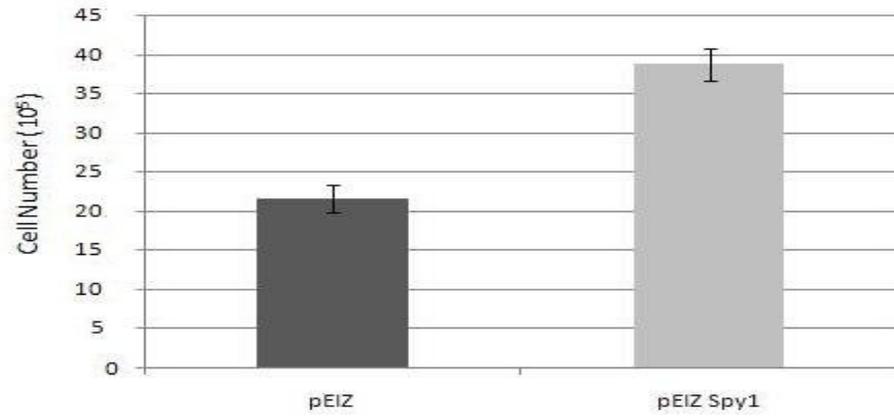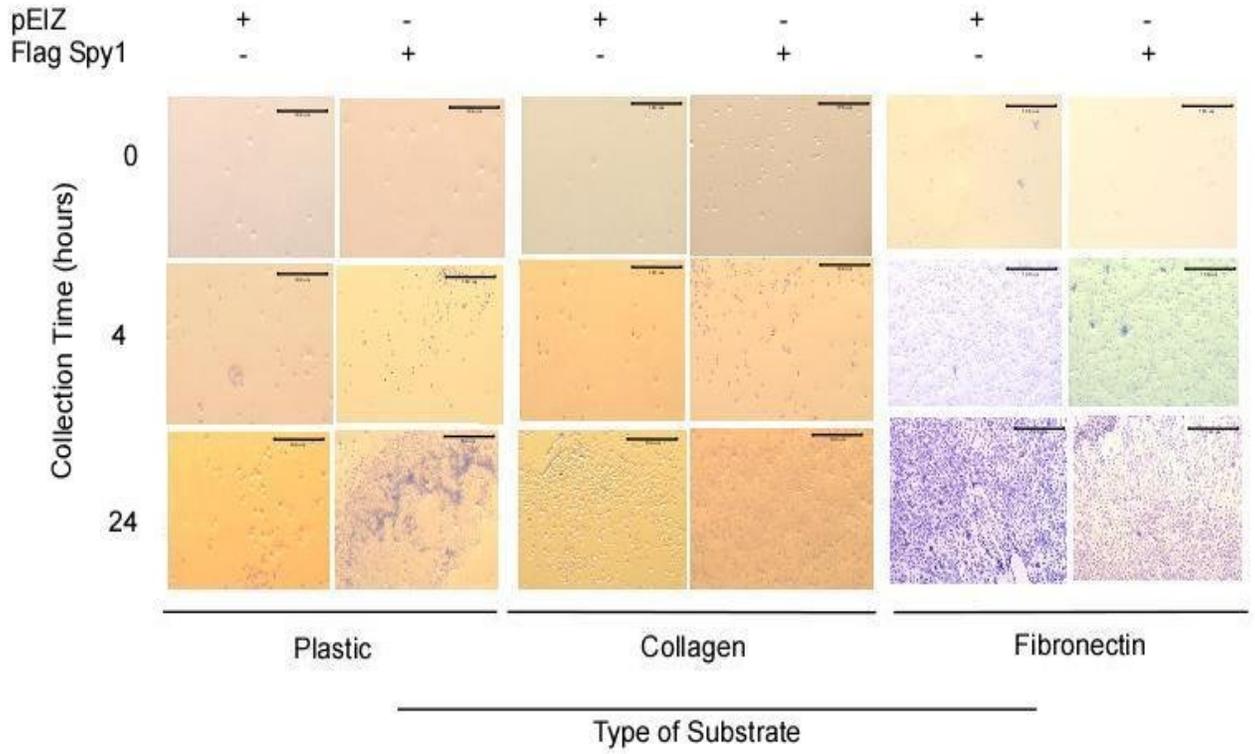
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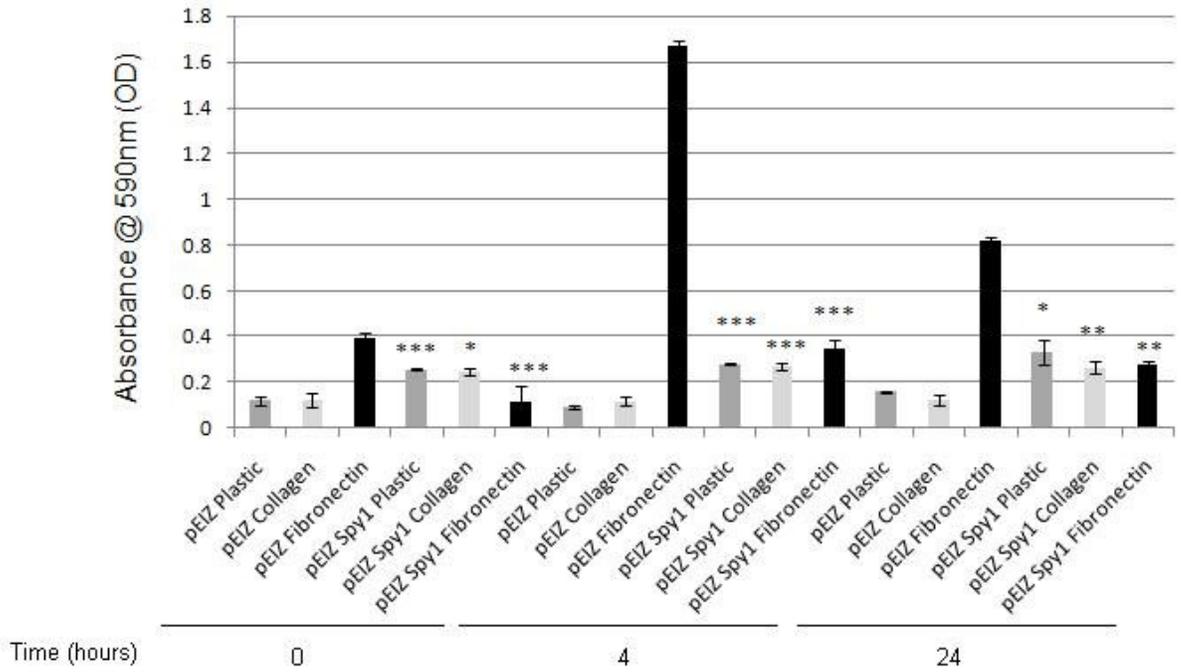
D

Figure 7. *Spy1 Overexpression Increases Adhesion in HC11 cells.* Spy1 Overexpression (pEIZ Flag Spy1; light bars) and Control cells (pEIZ; dark bars) were utilized for assessing: *A*, Average cell numbers from 3 replicates using trypan blue analysis. *B*, Representative pictures from one adhesion timecourse experiment performed on plastic, collagen and fibronectin substrates at 20x magnification. *C*, Optical density measured at 590 nm (n=3), * $p<0.05$, ** $p<0.01$, *** $p<0.001$). *D*, Western blot of adhesion assay of one representative experiment.

A

B
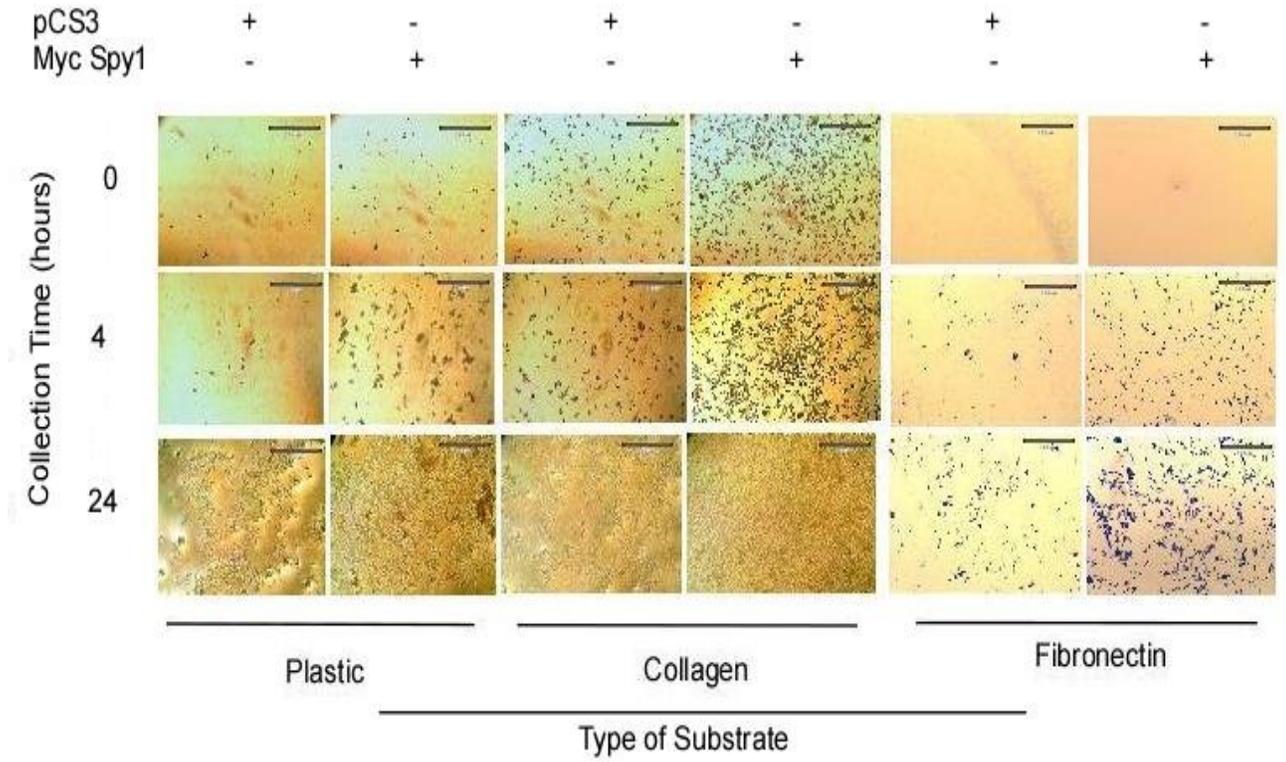
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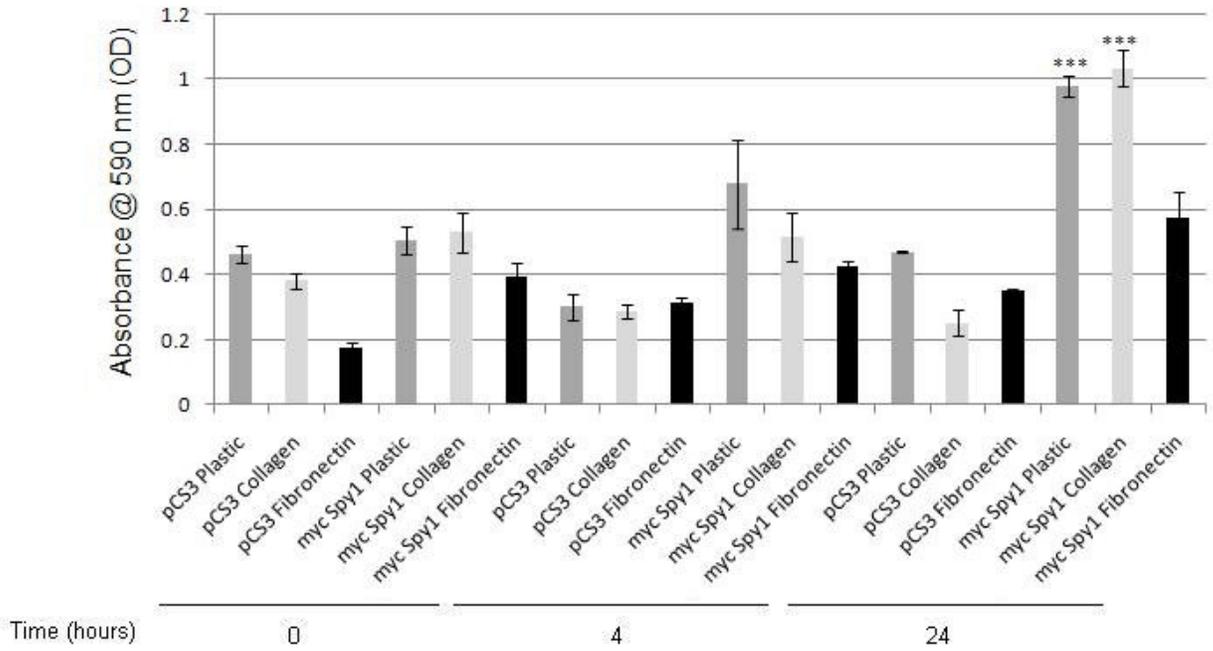
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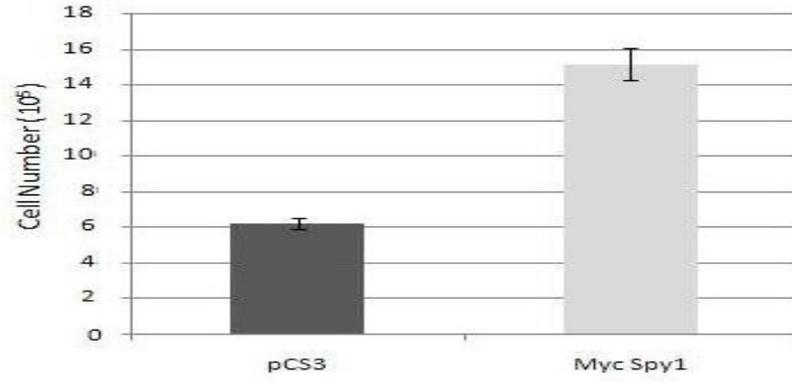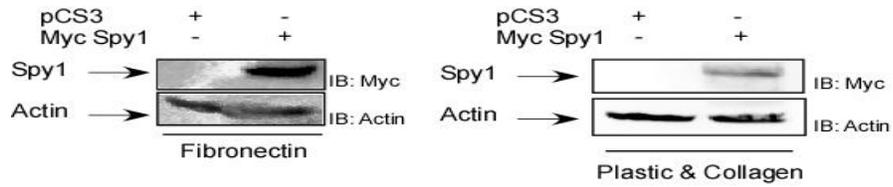**Figure 8.** *Spy1 Overexpression Increases Adhesion in HEK 293 cells.* Spy1 Overexpression (pCS3 Myc Spy1; light bars) and Control cells (pCS3; dark bars) were utilized for assessing: *A*, Average cell numbers from 3 replicates using trypan blue analysis. *B*, Representative pictures from one adhesion timecourse experiment performed on plastic, collagen and fibronectin substrates at 20x magnification. *C*, Optical density measured at 590 nm (n=3), *** p<0.001). *D*, Western blot analysis of adhesion assay of one representative experiment.

A

pLKO	+	-
pLKO Spy1	-	+

invaded cells

Time (hours)   24

pLKO	+	-
pLKO Spy1	-	+

Spy1 →   IB: Spy1

Actin →   IB: Actin

B

pCS3	+	-
Myc Spy1	-	+

invaded cells

Time (hours)   24

pCS3	+	-
Myc Spy1	-	+

Spy1 →   IB: Myc

Actin →   IB: Actin
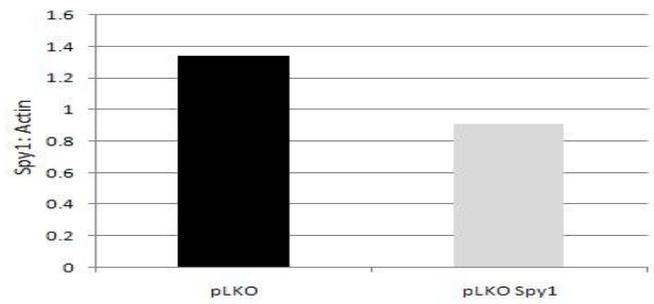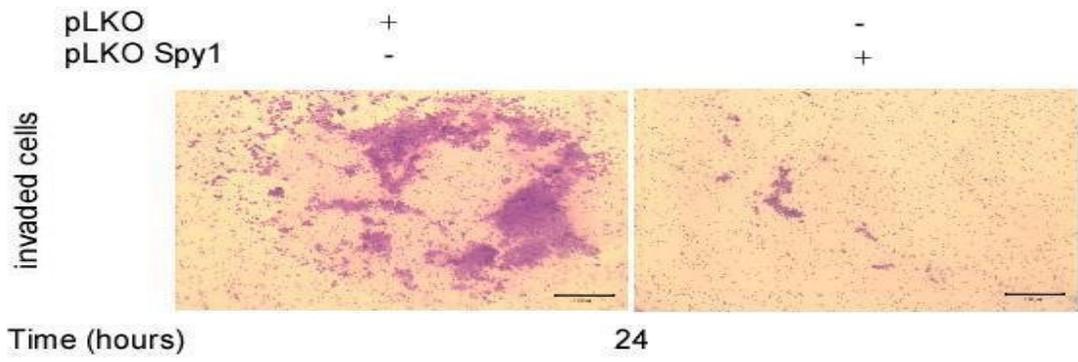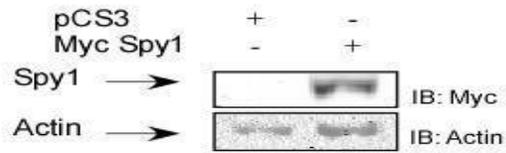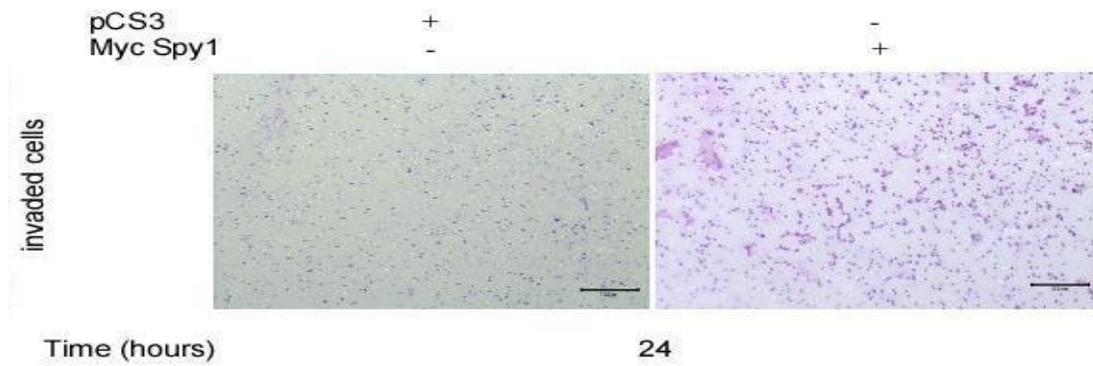
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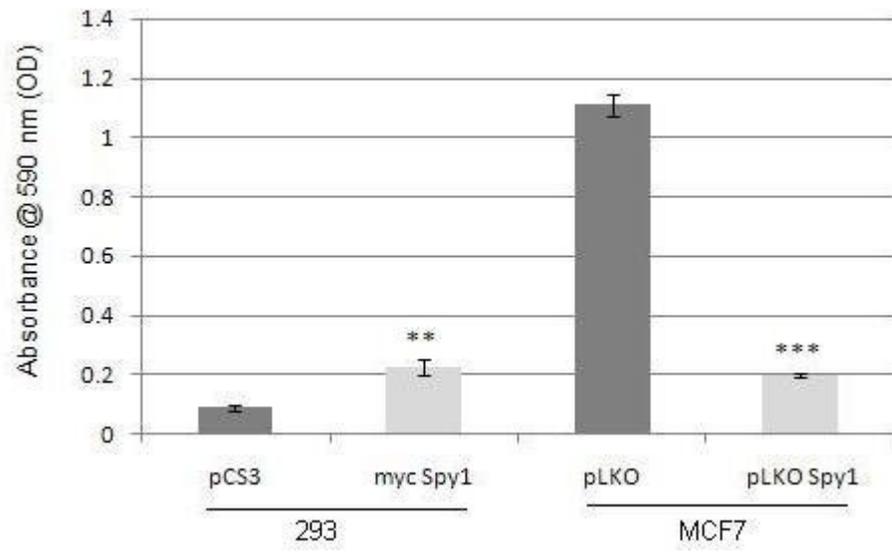
Figure 9. *Spy1 Effects on Invasion.* Spy1 Knockdown (pLKO Spy1; light bars) with Control cells (pLKO; dark bars) as well as Spy1 overexpression (pCS3 Myc Spy1; light bars) with Control cells (pCS3; dark bars) were utilized for assessing: *A*, Pictures from invasion assay of one representative replicate for knockdown and western analysis with quantification (lower panels). *B*, Pictures from invasion assay of one representative replicate for overexpression 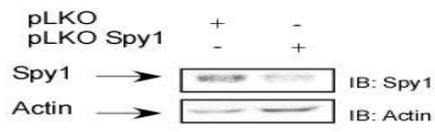experiments (at 20x magnification) and western analysis with quantification (lower panels). *C*, Optical density measured at 590 nm for both overexpression (n=3, ** $p < 0.01$) and knockdown (n=3, *** $p < 0.001$) studies.

A
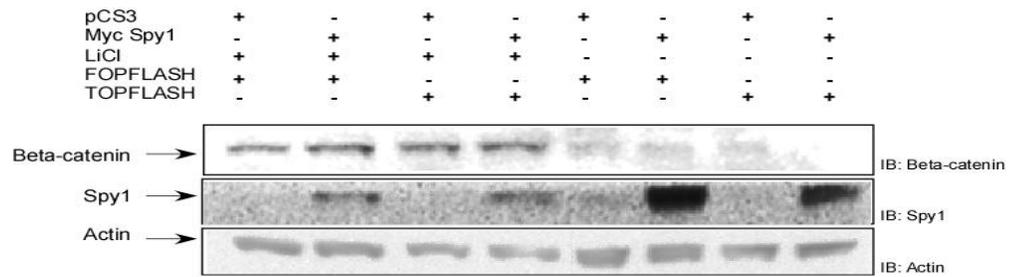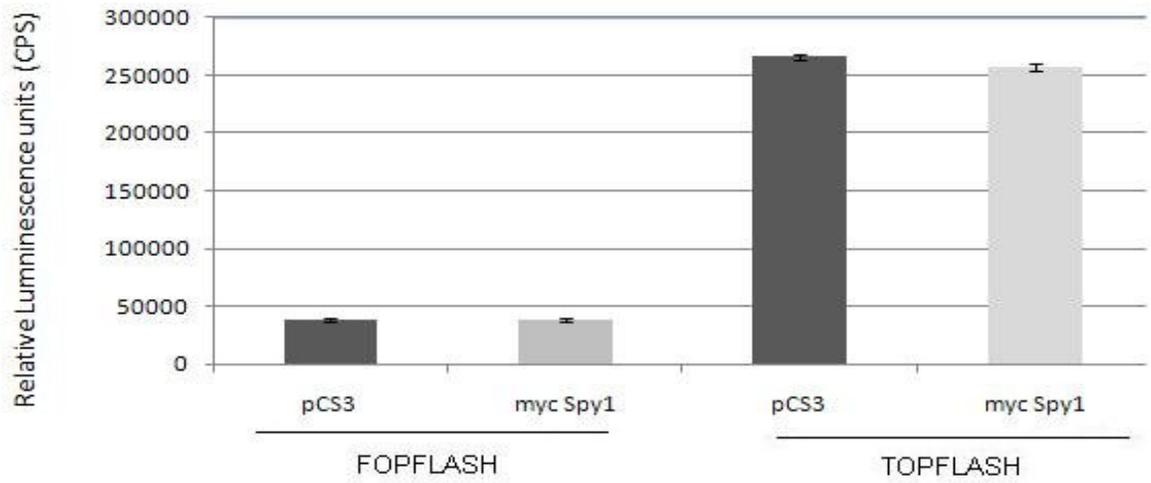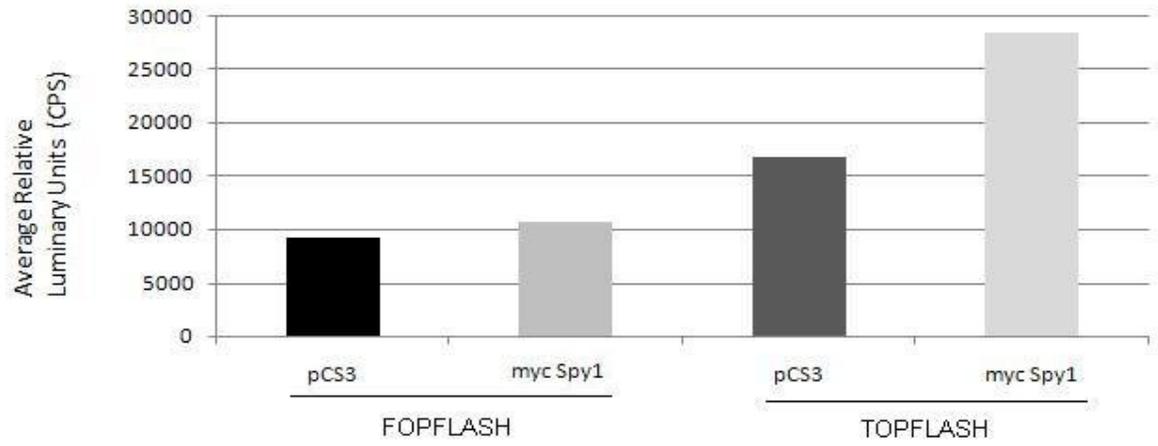
Figure 10. *Spy1 Effects on β-catenin's Activity.* HEK 293 cells were transfected with β-catenin luciferase reporter plasmid (TOPFLASH) and mutant β-catenin luciferase reporter plasmid (FOPFLASH) in the absence LiCl (top graph) and with LiCl (bottom graph) to assess the effect of Spy1 on β-catenin transcriptional activity. *A*, Graphs of luciferase assay of pCS3 (control) and pCS3 Myc Spy1, where the amount of bioluminescence was measured in counts per second (cps); Western blot analysis of β-catenin reporter assay with 293 Spy1 Overexpression without LiCl (n=1) and with LiCl (n=3).

A
I.

pLXSN   +   -
pLXSN Spy1   -   +

Passage 1

Passage 2

II.

pEIZ   +   -
pEIZ Flag Spy1   -   +

Passage 1

Passage 2

III.

	pLKO	+	−
	pLKO Spy1	−	+
Passage 1			
Passage 2			

IV.

	pLKO	+	−
	pLKO Spy1	−	+
Passage 1			
Passage 2			

B

C

D

E

F

G

Fold Change (RQ)									
1	100	0.7	100	1	20	3	16	0.8	13

Fold Change (RQ)									
1	1.2	1.2	1.58	1	1.6	4	1	2	2.4

Fold Change (RQ)									
1	1.4	6.3	5	1	1.1	0.7	1.6	1.1	1.3
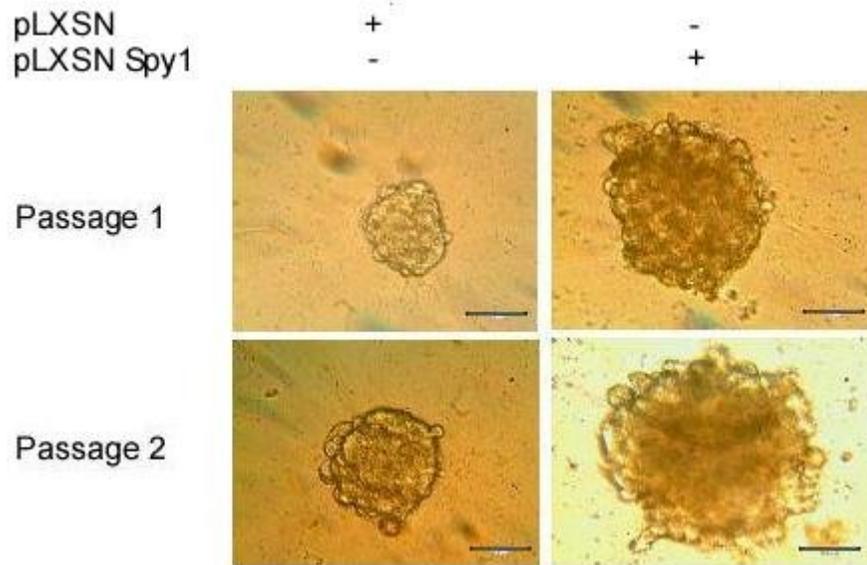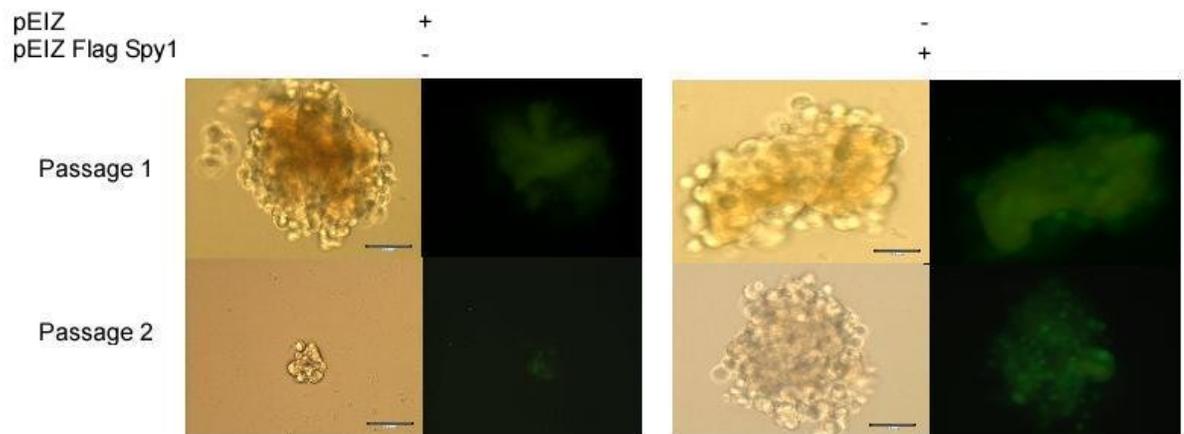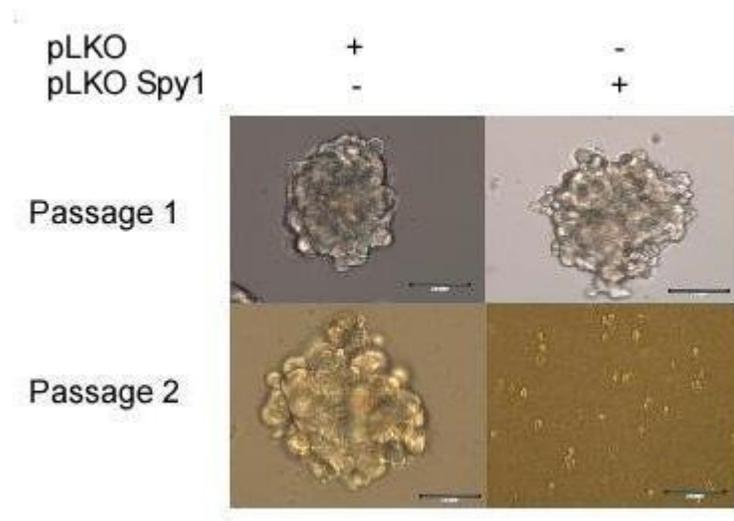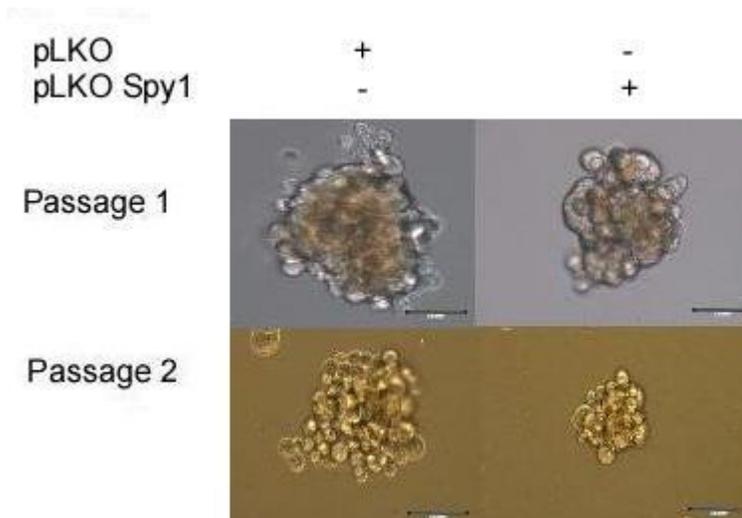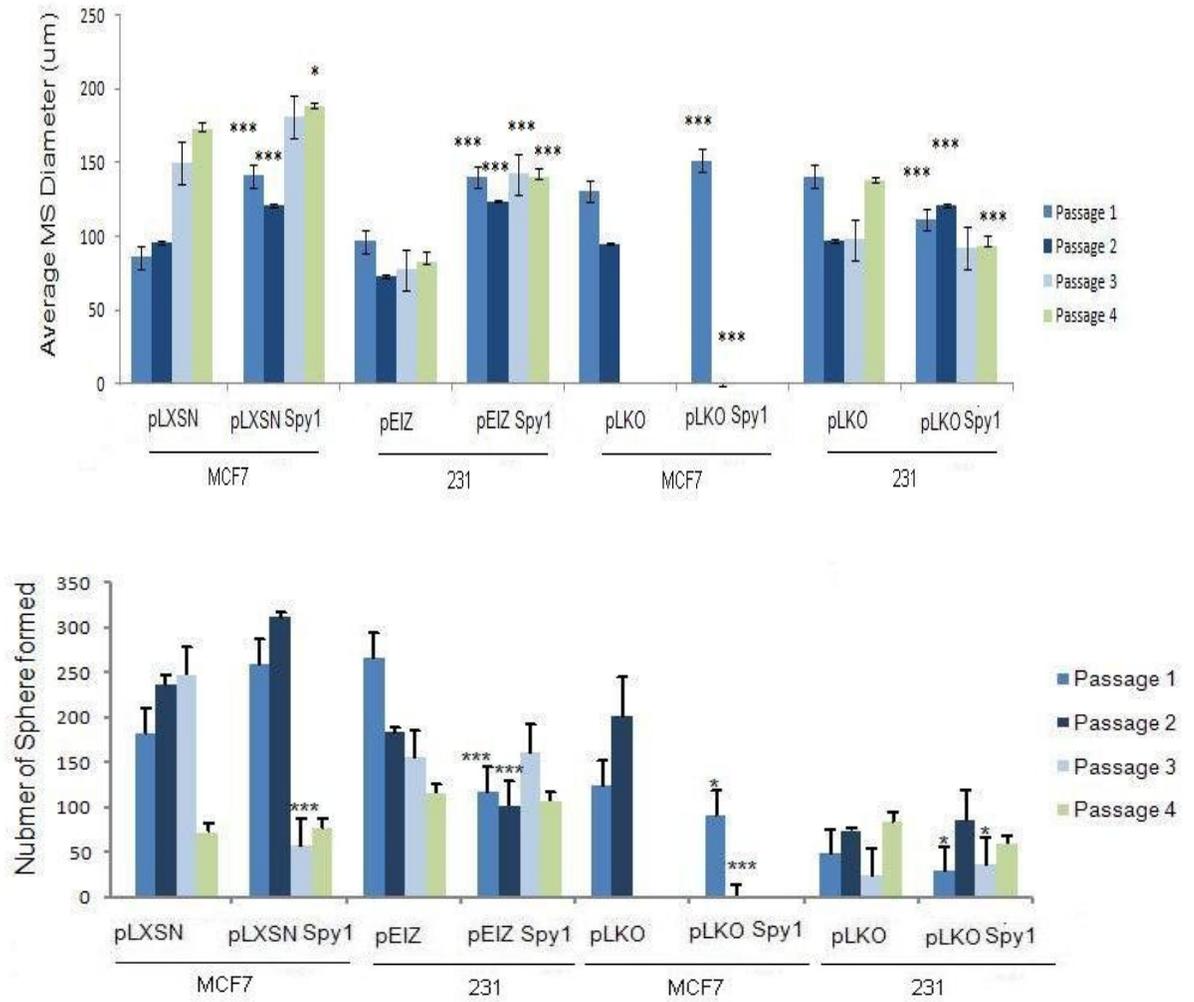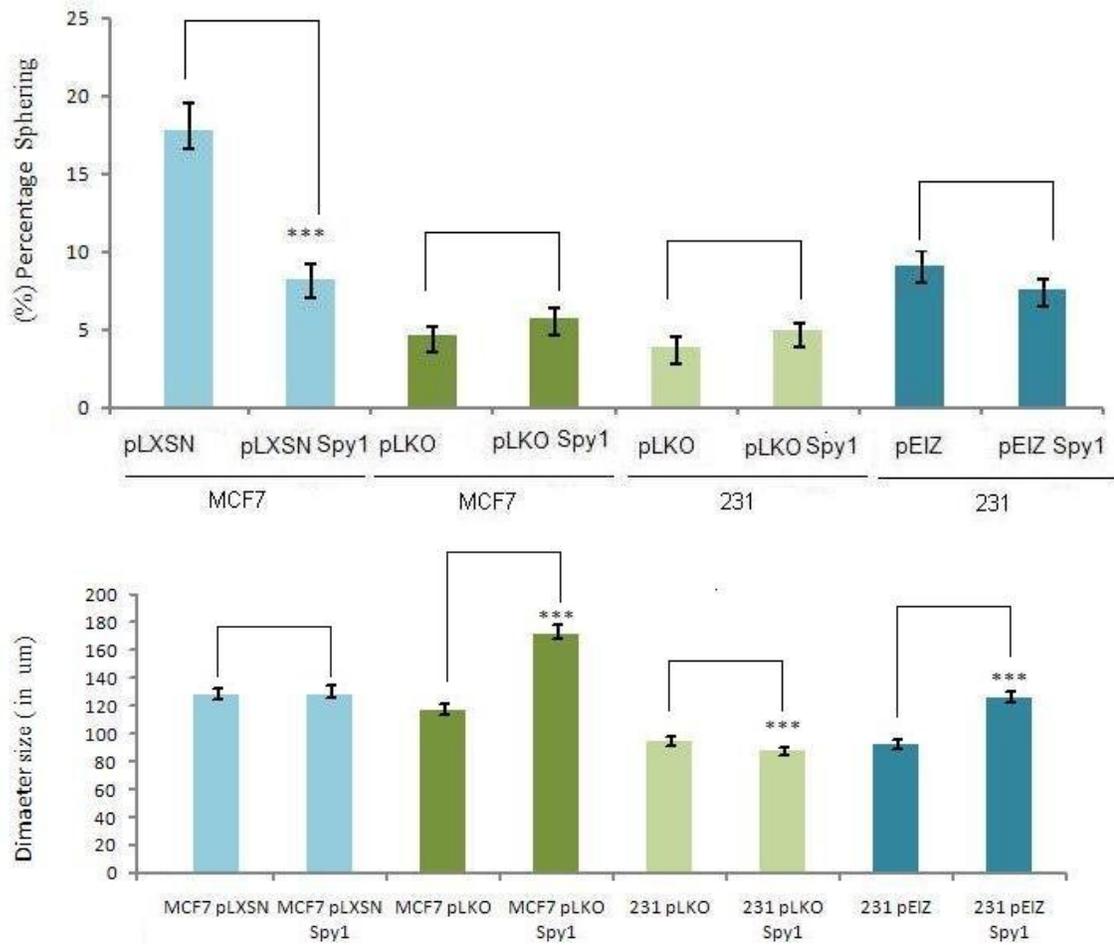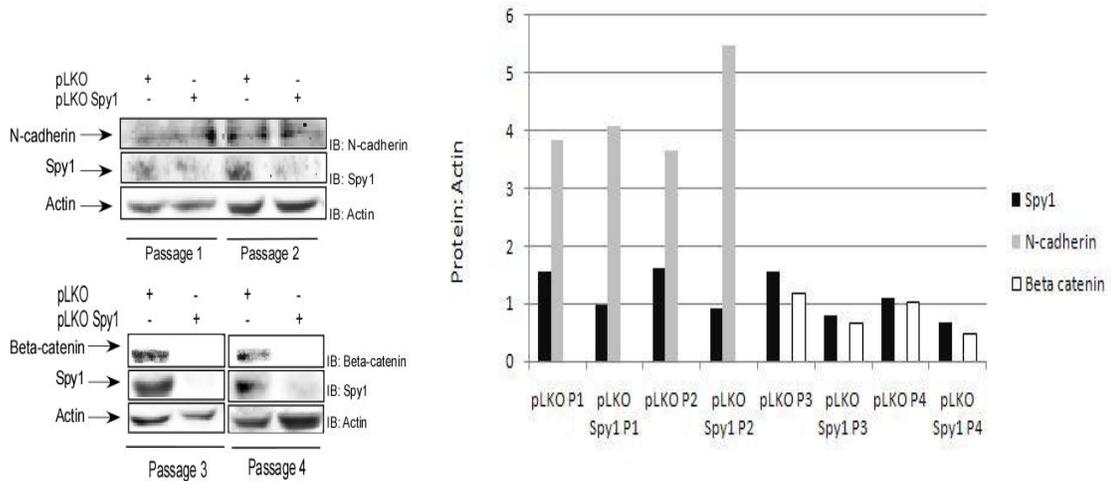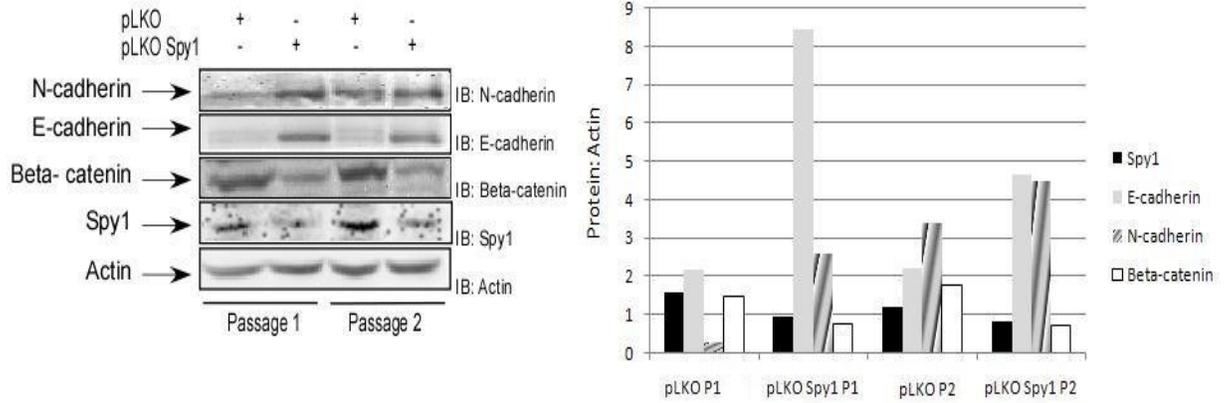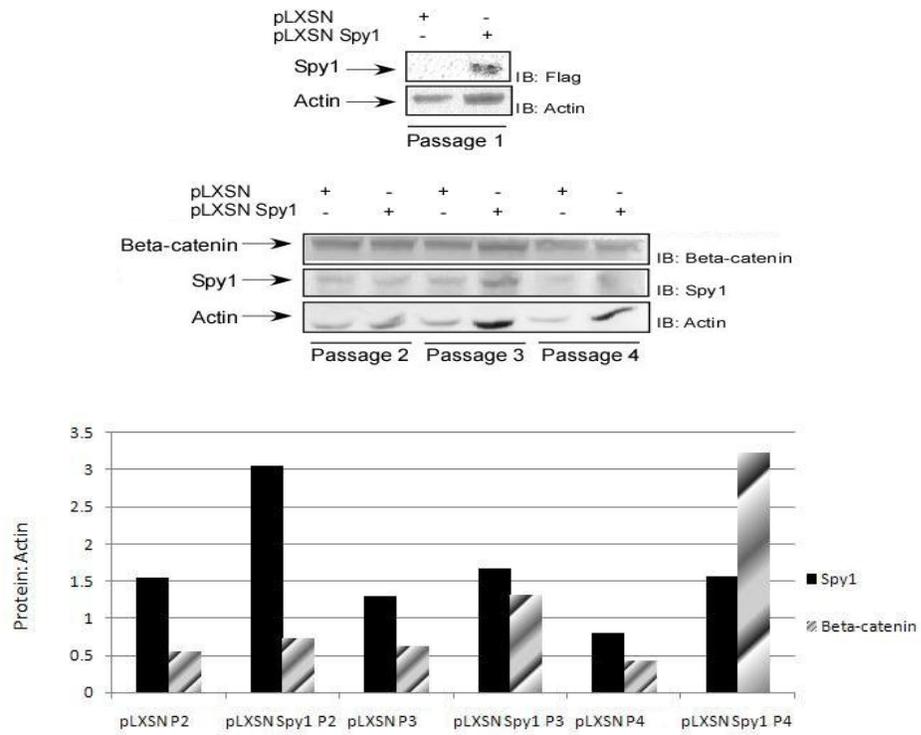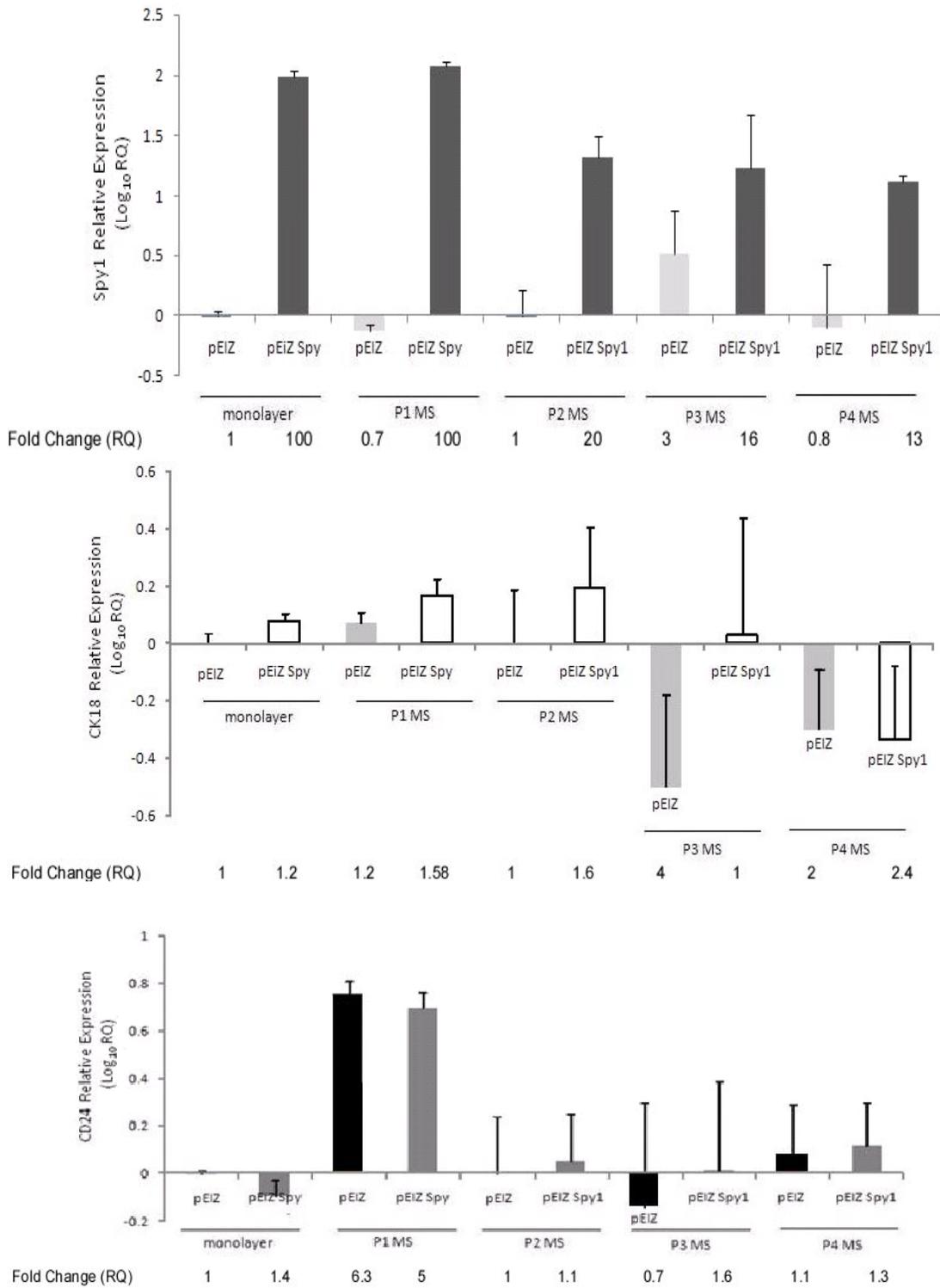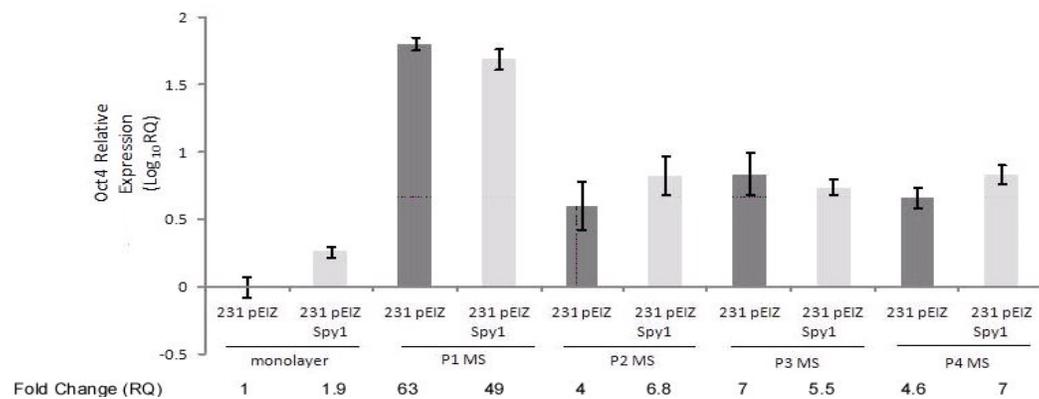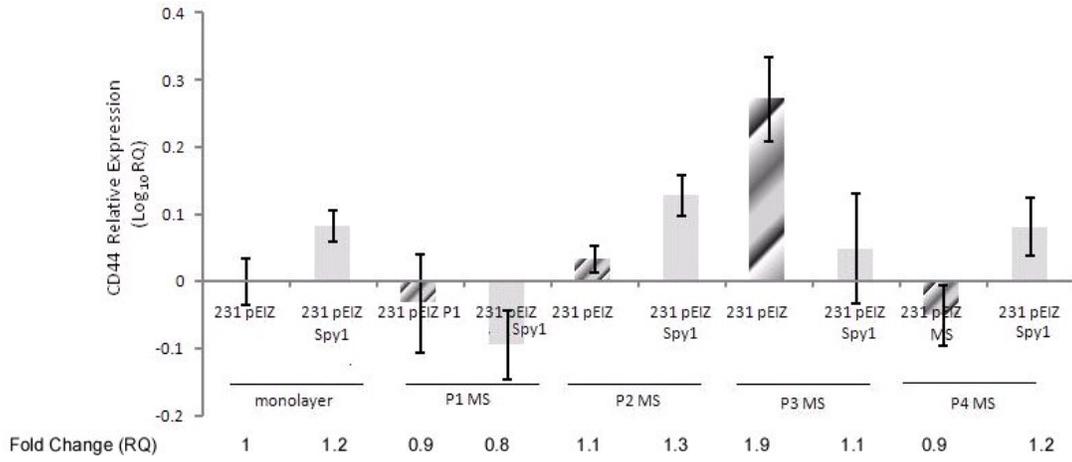
Figure 11. *Spy1 Effects on Stemness. A*, Pictures of representative passages at 20x magnification. I, Two representative passages of MCF7 Control (pLXSN) mammospheres and MCF7 pLXSN Spy1 Overexpressing mammospheres and knockdown Spy1 mammospheres. II, Two representative passages of 231 Control (pEIZ) spheres and 231 Overexpressing mammospheres (pEIZ Flag Spy1). III, Two representative passages of MCF7 Control (pLKO) and MCF7 Knockdown Spy1 (pLKO Spy1) mammospheres. IV, Two representative passages of 231 Control (pLKO) mammospheres and 231 Spy1 Knockdown (pLKO Spy1) mammospheres. *B*, Graph for average diameter size across four passages of mammospheres for knockdown Spy1 and overexpression (n=3). Graph for average number of spheres formed during four passages of mammospheres for knockdown and overexpression of Spy1 (n=3). *C*, Graphs for sphering abilities (in percent) for stable celllines of Spy1 knockdown and overexpression and average diameter size of spheres formed in clonal assay (* $p<0.05$, *** $p<0.001$). *D* Western blot analysis and densitometry of pooled 231 pLKO and pLKO Spy1 (shSpy1) mammospheres for Spy1, N-cadherin, β-catenin protein expression across mammosphere passages (passages 1-4). *E,* Western blot analysis and densitometry of pooled protein lysats for MCF7 pLKO and pLKO Spy1 (shSpy1) mammospheres for Spy1, N-cadherin, β-catenin protein expression across mammosphere passages (passages 1-4). *F,* Western blot analysis and densitometry of pooled MCF7 pLXSN and pLXSN Spy1 (overexpressing Spy1) mammospheres for Spy1, N-cadherin, E-cadherin, and β-catenin protein expression across mammosphere passages (passages 1-4). *G,* QRT PCR analysis of 231 overexpressing Spy1 mammospheres mRNA, where Spy1, Cytokeratin 18, CD24, CD44 and Oct4 were assessed across four mammosphere passages and monolayer and is representative of one experiment done in triplicate.

IV. DISCUSSION

Although the mechanism of how Spy1 promotes mammary tumorigenesis is not completely understood, it is becoming well established that Spy1 contributes to mammary carcinogenesis through affecting essential cellular processes. Migration, adhesion, invasion, self-renewal and morphology are all processes that are affected in cancer, and understanding the effects of Spy1 on these processes is crucial in elucidating the multifaceted mechanisms of cancer.

Potential Mechanisms involving Spy1 in Mammary Tumorigenesis

Research has shown that Spy1 is involved in regulating the cell cycle through its interactions with Cdk2 (Porter et al., 2002) and that Spy1 overexpression can promote murine mammary tumorigenesis *in vivo* (Golipour et al., 2008). It has also been shown that the expression of Spy1 is elevated in more aggressive tumors as well as in invasive, metastatic cell lines like MDA-MB-231 and MCF10CA1a (Al Sorkhy et al., 2010). Hence, it was of great interest to determine the importance of Spy1 expression in mammary tumorigenesis. Through studying the effects of Spy1 on cell migration and adhesion, it was determined that reducing levels of Spy1 could increase migration and decrease adhesion, two events that are essential for epithelial-mesenchymal transition (EMT) or metastasis to occur (Figure 1B, 2B, 5B, & 6B). As previously mentioned, EMT is a process that plays a crucial role in development, where cells are said to transition from a less motile, adherent, epithelial type cell into a motile, fibroblastic cell, which is necessary for the formation of the neural tube during mammalian development. In addition, loss of E-cadherin has been classified as biomarker for EMT and most data supports the hypothesis that a loss of cell-cell adhesion leads to EMT which in turn

promotes metastasis (Huber et al., 2005). Blanco and colleagues discovered that in a rat model of lung carcinogenesis that there was a consistent loss of adherens junctions and E-cadherin, during tumor metastases (Blanco et al., 2004). EMT is also known to play a role in stimulating metastasis (Huber et al., 2005), where research has shown that EMT can cause the upregulation of mesenchymal markers, such as N-cadherin and vimentin, and downregulation of epithelial marker, mainly E-cadherin. Given this data, how could Spy1 fit into this metastasis/EMT model of tumorigenesis? The answer may lie with two proteins which Spy1 is intimately associated: c-Myc and p21. Spy1 has been shown to mimic c-Myc patterns during mammary gland development and to be upregulated by c-Myc expression (Golipour et al., 2008); as well, Spy1 is able to inhibit p21 expression (Nebreda et al., 2000). Liu et al showed that when p21 was deleted that this caused an increase in c-Myc induced EMT and the presence of cancer stem cells as well, they showed that endogenous p21 could repress EMT in transgenic mice (Liu et al., 2009).
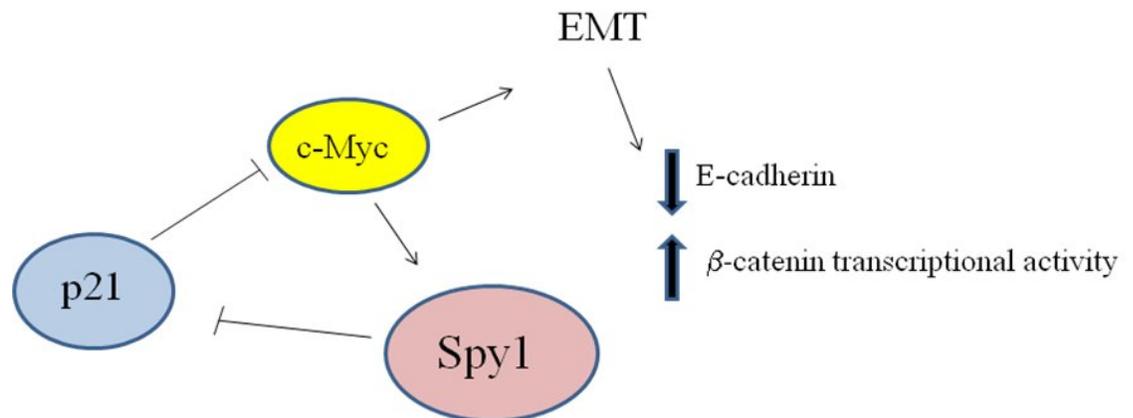
Figure 12. Spy1 effects in contributing to EMT.

Putting all this information together, endogenous levels of Spy1 may be essential to help c-Myc promote EMT (Figure 12). Research shows that c-Myc overexpression can induce EMT through upregulating mesenchymal markers (Liu et al., 2009). However

results from our lab have shown that c-Myc overexpression can affect Spy1 expression (Golipour et al., 2008). In this scenario, it is possible that Spy1 endogenously initiates EMT through overriding p21, and as a result enhances c-Myc and transcriptional upregulation of the necessary markers for EMT to occur. It is important not to disregard that not all EMT models behave in a manner that is "typical" of EMT. In the literature, another protein has shown effects which appears to be comparable to Spy1 expression in tumorigenesis and metastasis. Caveolins are integral membrane proteins that are found in the majority of adherent, mammalian cells but have also been linked to tumors with a metastatic phenotype (Li et al., 2001). Lin and colleagues have demonstrated that an upregulation of caveolin-1 in pancreatic cancer cells led to a decrease in cellular migration, and downregulation of caveolin-1 caused an increase in cellular migration through activation of the p38 MAPK pathway; both scenarios promoted EMT by inhibiting RhoC (Lin et al., 2005).

However, keeping in mind the classic EMT model, it is possible that Spy1 expression in tumorigenesis may not be so clear cut. It has been observed that Spy1 knockdown can affect migration and adhesion to fit the EMT model (Figure 1B, 2B, 5B, 6B). In addition, Spy1 overexpression has shown to increase invasion (Figure 9A) as well as increase β-catenin transcriptional activity (Figure 10A), which are events that fit the EMT model. However, Spy1 overexpression effects on migration and adhesion exhibit opposing trends, where Spy1 overexpression causes a decrease in migration and an increase in cell-substrate adhesion. It is possible that Spy1 knockdown data is preliminarily showing that endogenous Spy1 levels may be involved in maintaining the balance between a regular tumor or metastasizing tumor (Figure 1B, 2B, 5B, 6B).

Although the role of Spy1 in mammary tumorigenesis is still unknown, potential mechanisms involving Spy1 could function in the following manner: Spy1 levels become ablated due to transformation, which may involve stimulation of c-Myc to induce EMT, and the downregulation of Spy1 leads to non-adherent and more motile cells. On the other hand, it is possible that Spy1 overexpression could be involved in helping tumor cells invade (Figure 9A) as well as colonize into a new microenvironment and promote tumor cell proliferation to form new metastases (Figure 7B & 8B). The ability of Spy1 overexpressing cells to promote invasion fits the classical model of metastasis, which corroborates much of the literature that is known about metastasis and EMT (Jia et al., 1999) (Leber et al., 2009). However, further experimentation is crucial for dissecting the exact mechanism that Spy1 uses to promote mammary tumorigenesis. Even though Spy1 is a novel protein whose role in tumorigenesis has yet to be defined, it is quite possible that Spy1 may be involved in promoting events to stimulate EMT or metastasis.

Alternative Mechanisms involving Spy1 in Mammary Tumorigenesis

Another way of interpreting Spy1's role in tumorigenesis could be through studying the effects of Spy1 on cell polarity. Cell polarity is an important event regulated by signals from the cellular environment to organize the cell into axes with known directionality. In the mammary gland, cell polarity plays a major role in the formation of the mammary acini, where epithelial cells receive signals from neighboring cells and the ECM as to their role and position in the acini (Debnath et al., 2008). Spy1 overexpression causes disruptions in acini morphology *in vitro* (Golipour et al., 2008). As well, 3-dimensional (3D) culturing of HC11 overexpressing Spy1 acini showed that these acini displayed disrupted morphology, specifically in shape and size, which could also point to

problems in cell polarization (data not shown). Disruptions in cell polarity have often

been linked to disruptions in morphology (Hirata et al., 1998). In this study, the trend of a

decrease in migration in the presence of Spy1 overexpression seems to fit well with cells

that may have disrupted polarity (Figure 3B & 4B). Bershadsky and colleagues

determined that when Golgi apparatus function was disrupted in mouse 3T3 cells that

these cells lost their typical polarized morphology and lead to a decrease in cellular

migration (Bershadsky et al., 1994). Disrupted cell polarity has also been identified as a

characteristic of EMT (Huber et al., 2005).
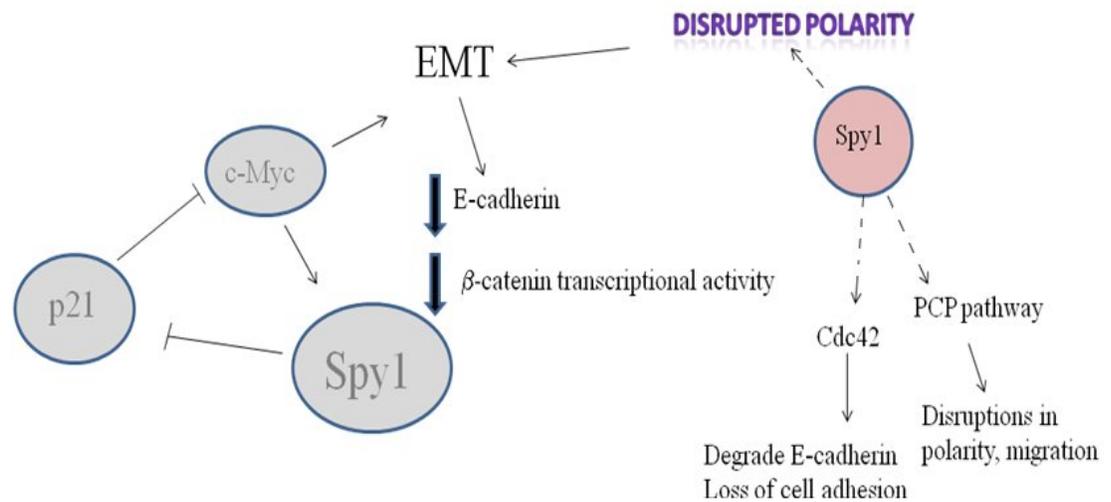
Figure 13. The Effect of Spy1 on Cell Polarity and its Contributions to Tumorigenesis

Since Spy1 overexpression can disrupt morphology and migration, this may imply

that Spy1 could be affecting cellular polarity by exhibiting effects on proteins from the

non-canonical Wnt pathway, planar cell polarity (PCP), which has been a pathway

involved in regulating developmental events such as cell polarization. Proteins from this

pathway (Rho, Rac and cdc42) are all known regulators of the actin cytoskeleton. These

proteins play important roles in regulating cellular events such as cell polarity and

migration (Allen et al., 1997). Migrating cells undergo reorganization of the actin cytoskeleton as well as intracellular components (Allen et al., 1997). However one of these proteins, cdc42 has shown a governing role of cell polarity, where researchers observed that cdc42 overexpression could increase degradation of E-cadherin; an event that promoted EMT (Shen et al., 2008). The absence of E-cadherin has been correlated to disruptions in cell polarity (Shen et al., 2008). Although it is premature to speculate the role of Spy1in polarity, one can hypothesize an involvement in polarity based on the data demonstrating the effects of abrogated levels of Spy1 in mammary morphology (data not shown). Although one could speculate about how Spy1 could affect mammary tumorigenesis, it is possible that Spy1 may be involved regulating cell polarity through affecting the PCP pathway, where *in vitro* work showed that Spy1 could disrupt acini morphology (Figure 13) (Golipour et al., 2008). Since Spy1 could be affecting cellular polarity, it may also be possible that Spy1 could have effects on cdc42, a PCP protein that has been shown to regulate cell polarity (Shen et al., 2008). Effects of Spy1 in cellular polarity could also cause defects in cell adhesion, which is a phenotype that Spy1 knockdown cells exhibited (Figure 5B & 6B). Conversely, effects may be more global in that Spy1 could affect various PCP mediators (Rac and Rho) and disrupt their expression and how they regulate the actin cytoskeleton and hence migration and adhesion, which could explain how Spy1 could be disrupting cellular migration, in both knockdown and overexpressing experiments.

Potential Mechanisms involving Spy1 in Regulating Self-Renewal

Stem cell research has become more prevalent in the recent years where researchers are beginning to establish links between stem cell regulation and

tumorigenesis. In the literature, many mechanisms that govern stem cells and their niche have been observed to be greatly affected during tumorigenesis. The cancer stem cell hypothesis states that a small pool of cells, contained within the tumor, acquire self-renewing abilities which can promote tumorigenesis (Tan et al., 2006). The expression of Spy1 has shown to be elevated during puberty, early pregnancy and involution (Golipour et al., 2008). As previously mentioned, involution is a period where mammary stem cells are recruited to begin to rebuild the mammary gland, so it was especially interesting to see if Spy1's expression during involution could be correlated to stem cell self-renewal. Mammospheres, which are derived from stem cells, were utilized to assess Spy1's effects on stem cell self-renewal with respect to effects on mammosphere passaging, numbers and mammosphere formation in the presence of Spy1 manipulation.

The present evidence supports that Spy1 may affect stem cells self-renewal. Results showed that Spy1 overexpression elicited larger spheres (Figure 11A I) and Spy1 knockdown caused smaller spheres to form in basal breast cancer cells (Figure 11A IV). So questions to ask here are: How does Spy1 affect stem cell self-renewal? How could Spy1's effects on stem cells contribute to tumorigenesis?

How does Spy1 affect Stem Cell Self-Renewal?

Stem cell literature remains divided in terms of identifying a true stem cell phenotype, however research has shown that stem cells can produce larger and more mammospheres (Dey et al., 2009) (Grimshaw et al., 2008). The best way to establish a mammary stem cell phenotype is through utilizing Fluorescence Activated Cell Sorting (FACS), where putative stem cell surface markers CD44 and CD24 were identified (Dontu et al., 2003). In the QRT PCR data, these data seem to fit with certain passages of

231 pEIZ Spy1 mammospheres, where in certain passages Spy1 overexpressing mammospheres exhibited a more stem like appearance than their controls. However looking at stemness markers (CD24, CD44 and Oct4), there were no clear trends that were visualized that exhibited an increasing or decreasing trend of stemness (Figure 11G). It appeared that both stemness and differentiation markers varied in an irregular pattern, which made it very hard to discern a clear relationship between Spy1 and stemness and to fully prove effects of Spy1 in stem cell self-renewal. Interestingly in monolayer, Spy1 overexpressing cells exhibited a more stem like appearance (low CD24, high CD44 and high Oct4 expression) in comparison to all the mammosphere passages (Figure 11G).

Self-renewing abilities were also assessed utilizing the clonal assay which gave a measure of how efficiently certain immortalized cell lines could form mammospheres. Spy1's results from the clonal assay showed some perplexing results where overexpressing Spy1 cells had reduced abilities to form spheres whereas knockdown Spy1 cells had enhanced abilities to form spheres (Figure 11C). In order to corroborate the claim that Spy1 is a positive regulator for stemness, it is necessary to refer back to Spy1's ability to affect adhesion. As previously studies shown, Spy1 overexpression could enhance adhesion, which may help to explain Spy1 overexpressing cells' lower sphering percentages. In this scenario, Spy1 could be increasing adhesion between stem cells, thereby inhibiting stem cells from forming more spheres. In the knockdown Spy1 assay, stem cells do not have to overcome Spy1's adhesion effects and so are able to form more spheres. In addition, the mammosphere passaging experiments gave stem cells ample room in a six well plate to form spheres whereas in a 96 well plate, stem cells were

faced with an obstacle of adhesion effects by Spy1. In essence, these results marginally relay Spy1's ability to affect stem cell self-renewal. Coupled with the gene expression data, it is difficult to characterize Spy1 as a direct stem cell effector. However, further experimentation would be necessary to establish whether Spy1 can actually affect stem cell self-renewal.

Spy1 is a known cell cycle regulator that has been shown to bind and activate cdk2 as well has been shown to cause p27 degradation (Porter et al., 2002) (Porter et al., 2003). Interestingly, p27 and cdk2 both have shown association with affecting stem cells. Jablonska and colleagues demonstrated that cdk2 was essential for maintaining neuronal stem cell self-renewal (Jablonska et al., 2007). As well, Cheng and colleagues demonstrated that p27 does not affect stem cell number or self-renewal but p27 overexpression can enhance the progenitor cell pool (Cheng et al., 2000). In essence, Spy1 could promote self-renewal through its interactions with cdk2 and in this complex, lower levels of p27, which could act as a mechanism to maintain homeostasis with stem and progenitor cell pools within their niche. Another Spy1 related protein that functions in this manner is c-Myc where it was shown that c-Myc was involved in maintaining stem cell differentiation through creating a balance between stem and progenitor cells (Baena et al., 2007). Looking at all the stemness data, it is possible that Spy1 could be affecting stemness in a manner similar to c-Myc. Through its interaction with Cdk2 and p27, Spy1 could be maintaining homeostasis between stem and differentiated cells. So if the stemness/differentiation balance is shifted in either direction, Spy1 overexpression could compensate for both pools of cell types and establish homeostasis once again.

Additionally, β–catenin is a major mediator in the Wnt signaling pathway and its elevated expression has been correlated with many types of tumors and has been linked to influencing stem cell self-renewal (Chen et al., 2007). So throughout all the experiments performed in this thesis, it appeared that a relationship between Spy1 and β–catenin was developing, where β–catenin were low with Spy1 knockdown cells and were elevated in Spy1's overexpressing cells (Figure 1D, 2D, 3D, 4D). As well, Spy1 overexpression was able to enhance β–catenin's transcriptional activity (Figure 10A). It is not surprising that this relationship became more evident throughout these studies because a yeast 2 hybrid assay performed in our lab showed that Spy1 could interact with Wnt mediators, Axin and LRP6. Zeng and colleagues postulated another manner of interpreting the Wnt pathway where Axin, active GSK3 and LRP6 form a complex to promote β–catenin stabilization (Zeng et al., 2008). So it is possible that Spy1 is interacting with Axin/GSK3 and LRP6 to keep β–catenin levels high to help stem cell undergo self-renewal in their niche (Figure 14). Or Spy1 could be functioning with β–catenin to maintain homeostasis between stem and progenitor cells within the stem cells niche. In this context, β–catenin would be involved in maintaining the stem cell numbers and Spy1 could be maintaining the progenitor cells. Or Spy1 with β–catenin could be working synergistically to enhance the stem and progenitor cell pools. Additionally, Spy1's effects in migration and adhesion may also play a role in maintaining the stem cell niche through promoting adherence and preventing migration from their niche. Like with the previous studies, supplementary work would need to be done in order to prove how Spy1 and β–catenin contribute to affecting stem cells and how this relationship contributes to mammary tumorigenesis.
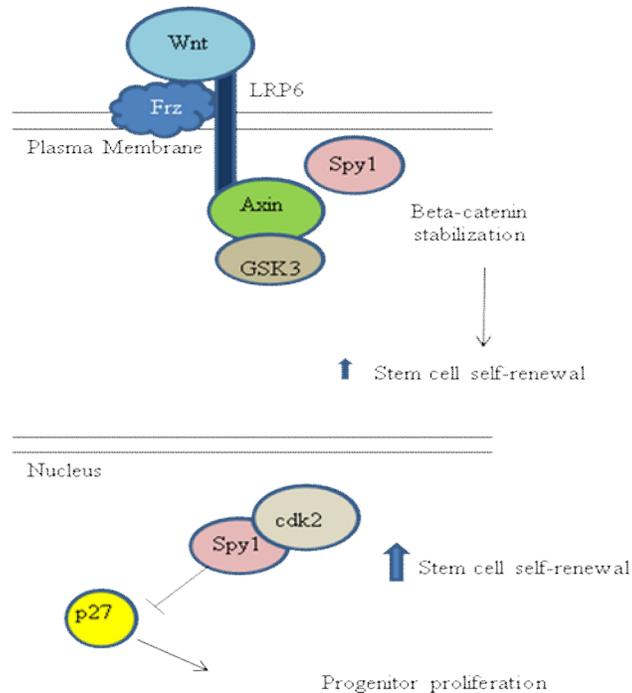
Figure 14. Effects of Spy1 in Stem Cell Self-Renewal

How do effects of Spy1 on Stem Cells Contribute to Tumorigenesis?

Spy1's overexpression has shown its effects in stemness through its ability to enhance mammosphere diameter size and number. However from a tumorigenic standpoint, one can ask the question: How do Spy1 effects on stemness contribute to tumorigenesis? In order to answer this question, it is imperative to revisit the stem cell hypothesis, which essentially states that tumors contain a small population of tumorigenic cells, with self-renewable abilities, that perpetuate tumor formation. Could Spy1 affect these cancer stem cells?

It is quite possible that Spy1 could affect how cancer stem cells adhere to their niche, promote their self-renewal and proliferation. Results showed that Spy1 overexpression could affect mammosphere size and number, which could suggest that Spy1 is essential to self-renewal for both normal and cancerous stem cells. As Figure 4 shows, Spy1 could be enhancing stem cell self-renewal in two ways: i) through its

interactions with cdk2 and effects on p27 and ii) through its interactions with Axin, LRP6 to help stabilize β-catenin (Figure 14). It is possible that Spy1 could help cancer stem cells through providing a suitable microenvironment where cancer stem cells can: adhere better to their niche, enhance their proliferation, which in turn could help propel their self-renewal abilities. If Spy1 could have potentially this much control over cancer stem cells, then it is possible for Spy1 to become an essential biomarker for breast cancer, where Figure 5 shows how targeting Spy1 could be targeting a tumor at it source. Additionally, Spy1's effects with stemness as well as with its links to EMT could provide a potential mechanism of forming more aggressive types of tumors that have a high likelihood to metastasize, which would make Spy1 a very favorable marker for breast cancer.
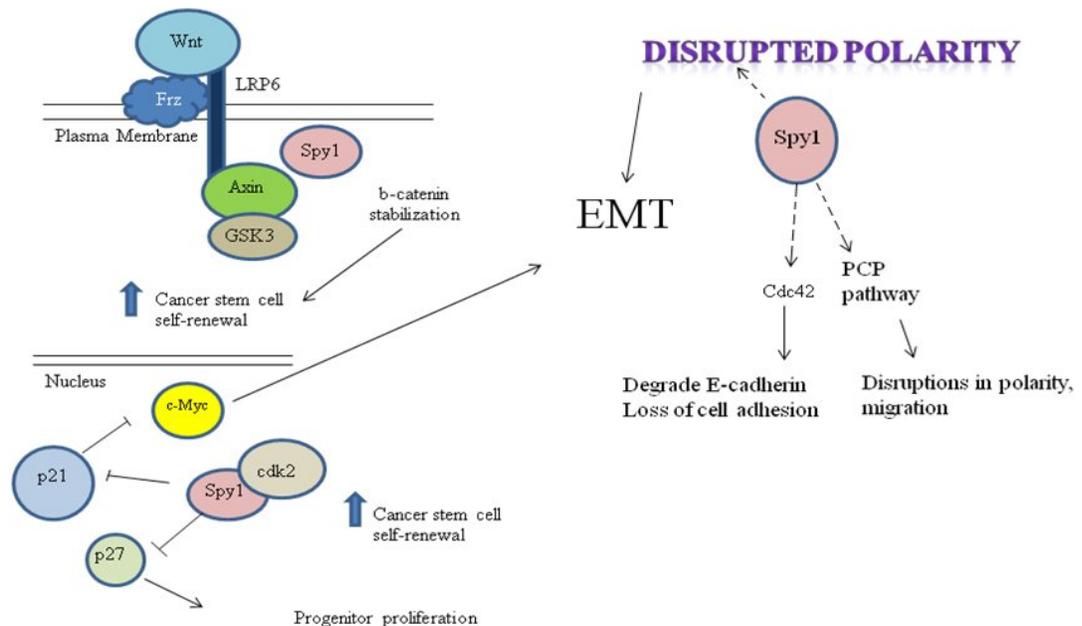
Figure 15. Mechanism involving potential role of Spy1 in Mammary Tumorigenesis.

Breast cancer is an extremely intricate process, however how Spy1 fits into the cancer mechanism remains to be seen. It will take time to fully clarify Spy1's role in

mammary tumorigenesis, however results so far have made small strides in understanding this difficult disease.

V. FUTURE DIRECTIONS

Understanding the role of Spy1 in mammary tumorigenesis is far from being complete, there is still much more work that needs to be done to prove the potential of this novel cell cycle regulator as biological target for breast cancer and metastasis. Although Spy1 overexpression had lead to decreases in migration, it would be vital to clarify how Spy1 is affecting migration through determining links between Spy1 and pathways known to regulate cellular migration. As previously mentioned, it would be interesting to establish links between Spy1 and the Rho pathway, specifically looking at cdc42. Cdc42 has been shown to regulate polarity in migrating cells, so it would be of great interest to knockdown cdc42 and re-perform the migration assays or perform wound healing assays and see how migration and polarity are affected in Spy1's presence and absence.

To better clarify the role of Spy1 in adhesion, it would be essential to determine the effects of Spy1 on adhesion to various substrates in the presence of knocked down expression of E-cadherin, N-cadherin and β-catenin. Additionally, it would be important to perform experiments to determine the interactions between Spy1 and p21 and how this could potentially impact c-Myc induced EMT. For instance, one could utilize overexpressing myc primary tumor cells and knockdown Spy1 and determine protein and mRNA levels of epithelial and mesenchymal markers to determine the relevance of Spy1 and p21 in c-Myc induced EMT. It would also be essential to perform a microarray analysis, derived from a Spy1 overexpressing primary tumor, and study the expression changes of various mesenchymal and epithelial markers. It would of great interest to take Spy1 overexpressing primary cells and use flow cytometry to determine the expression of

mesenchymal and epithelial with respect to Spy1. It is essential to implicate Spy1 with EMT markers that are well established in literature to begin to fit Spy1 signaling into these known pathways thereby strengthening the claim of Spy1's role in tumorigenesis and metastasis.

For Spy1 affecting stem cell self-renewal, it is imperative to perform a fluorescence activated cell sorting (FACS) of Spy1 overexpression and knockdown in primary mammary cells on various stem and differentiation markers and then compare these results against a microarray to establish a expression signature for Spy1 (Andrechek et al., 2008). After performing FACS, Spy1 manipulated cells would be sorted, and then mammospheres could be grown in culture to be injected into mice to determine Spy1 effects on tumor formation.

Preliminary work with the immortalized but non-tumorigenic cell lines MCF10a and HC11 3D acini has further corroborated the ability of Spy1 to disrupt normal mammary morphology. In the MCF10a overexpressing Spy1 acini, it was observed that these acini formed disrupted, multi-acinar structures, where HC11 overexpressing Spy1 acini were larger and also displayed a disordered morphology. Since these experiments were just beginning to come together at the conclusion of this thesis it is essential to grow 3D acini with Spy1 overexpression and knockdown to study the effects. In addition to this, it would be essential to create stable Spy1 overexpression and knockdown cell lines using the cancer series cell lines to further clarify Spy1's role in tumorigenesis in a relevant human breast model. Specifically, it would be important to grow these cell lines in 3D to determine consequences of Spy1 on acini development as well as count and measure mammary acini to get a full developmental summary of the effects of Spy1 on

mammary acini under normal and abnormal conditions. In addition, it would be essential to perform localization studies to verify Spy1's expression in acini development specifically looking at effects on proliferation, apoptosis and polarity. Furthermore, assessing Spy1 protein levels in this model system along with markers to verify localization and morphology is a vital next step for this work.

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