# Differences in the Regulation of Thrombospondin-1 Expression Between Epithelial Cells and Fibroblasts

By

Roberto Karlo Rodriguez

B.S. Biochemistry
University of California, Davis, 1999

Submitted to the Department of Biology in Partial Fulfilment of the Requirements for the Degree of Doctor of Philosophy in Biology

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ABSTRACT

Induction of angiogenesis is a critical and rate-limiting step in the progression of cancer. It is widely acknowledged that this induction requires the concomitant stimulation of pro-angiogenic and repression of anti-angiogenic proteins. It has been demonstrated that in human epithelial cells repression of the angiogenesis inhibitor Thrombospondin-1 (Tsp-1) requires stimulation of Myc in combination with hyper-physiologic levels of oncogenic Ras.

This work demonstrates that in human mammary epithelial cells, repression of Tsp-1 requires the activation of Myc by a Ras-induced pathway that activates the MAPK p38. This work also demonstrates that repression of Tsp-1 in human fibroblasts requires the combined inhibition of the tumor suppressors p53 and pRb. These results suggest that the molecular requirements for the induction of angiogenesis differ significantly between carcinomas and sarcomas.

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# Chapter 1. Introduction

## I: The role of angiogenesis in tumorigenesis

Angiogenesis is the process of new capillary blood vessels arising from preexisting blood vessels (Clark and Clark 1939). As such, it is an essential component of development, reproduction and wound healing (Dvorak et al. 1981). Angiogenesis also occurs in tissues where there is a change in mass or metabolism (Dvorak et al. 1981). It is in these ways that physiologic, i.e. non-pathogenic, angiogenesis allows the remodeled tissue to maintain adequate levels of oxygen (Dvorak et al. 1981). The process of angiogenesis was first described in rabbit ear chambers in 1939 and has been extensively studied since (Clark and Clark 1939). One of the major breakthroughs occurred when, using cultured endothelial cells, Folkman et al were able to establish *in vitro* models of capillary network formation (Gimbrone et al. 1973; Folkman and Haudenschild 1980).

Induction of angiogenesis occurs through growth factor-dependent activation of endothelial cells. This activation is most often initiated in response to stimuli such as hypoxia, inflammation and mechanical stress (Ausprunk and Folkman 1977; Hobson and Denekamp 1984). These stimuli induce the production of growth factors, such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), and epidermal growth factor (EGF), from endothelial cells and pericytes, undifferentiated fibroblast-like cells (Arbiser 2004). These growth factors initiate signaling cascades in endothelial cells that lead to the activation of transcription factors, which enhance expression of cell cycle mediators, integrins, and proteases, that promote the progression of angiogenesis (Milkiewicz et al. 2006). The

activated transcription factors further stimulate production of angiogenic growth factors and receptors, which provides a positive feedback loop that results in a more robust response (Milkiewicz et al. 2006). Induced proteins such as matrix metalloproteinases (MMPs) can also serve as components of negative feedback loops downregulating the angiogenic response by activating angiogenesis inhibitors like endostatin and angiostatin (O'Reilly et al. 1994; O'Reilly et al. 1997). This regulatory network helps to coordinate the complex process of angiogenesis. Induction of angiogenesis can be broken down into five basic steps: endothelial cell infiltration, endothelial cell proliferation, breakdown of the basement membrane, endothelial cell migration, and endothelial tube formation and stabilization (Ausprunk and Folkman 1977; Hobson and Denekamp 1984).

Permeability, or endothelial cell infiltration, is the first step in angiogenesis, and is controlled through adherens junctions located on the surface of endothelial cells (Dejana 2004). Adherens junctions are formed through the interactions of adhesive molecules, the most prominent of which is vascular endothelial-cadherin (VE-cadherin) (Corada et al. 1999). Anchoring to the actin cytoskeleton is essential for optimal adhesion of VE-cadherin, and is mediated by binding in complex with $\alpha$- and $\beta$-catenins (Lampugnani et al. 1995; Corada et al. 1999). Dissociation of adherens junctions leads to an increase in endothelial cell permeability (Dejana 2004). Growth factors like EGF and HGF can induce endothelial cell permeability by phosphorylating $\beta$-catenin via their receptor tyrosine kinases (Hoschuetzky et al. 1994).

Subsequent to increasing permeability, activated endothelial cells undergo cell proliferation. VEGF binding to VEGF receptor 2 (VEGFR2) stimulates endothelial cell

proliferation (Quinn et al. 1993; Bernatchez et al. 1999). VEGFR2 activates mitogen activated protein kinases (MAPKs) such as ERK-1, ERK-2 and p38, as well as phosphatidylinositol 3-kinase (PI3K) (Yu and Sato 1999). Activation of these signaling pathways leads to induction of endothelial cell proliferation by modulating cell cycle regulators. For example, VEGF-induced Akt signaling leads to the repression of the CDK inhibitor p27 (Potente et al. 2003). Activation of the Akt pathway is also involved in promoting endothelial cell survival (Gerber et al. 1998). This is accomplished through induction of anti-apoptotic molecules such as Bcl-2 (Flusberg et al. 2001).

Once endothelial cells are activated, the sheath-like covering of the existing blood vessel (also known as the basement membrane) needs to be broken down to create space for the new vessel that will sprout. The basement membrane is degraded by active MMPs (Birkedal-Hansen 1995). The two MMPS that are most critical in this process are MMP-2 and MT1-MMP (Nguyen et al. 2001; Ohno-Matsui et al. 2003; Langlois et al. 2004). One way in which production of active MMPs is stimulated is via the mechanical forces involved in reshaping the vasculature (Boyd et al. 2005). Signaling through VEGFR2 induces degradation of the basement membrane at the leading edge of migrating endothelial cells (Pepper 2001). The VEGFR2-mediated degradation effect occurs via the activation of the trypsin protease plasmin following proteolytic cleavage of plasminogen (Pepper 2001).

After the basement membrane is degraded at the site of the new budding vessel, migrating endothelial cells home to the bud in order to elongate the new blood vessel. Endothelial cell migration occurs through cellular attachment to matrix

proteins like integrins and through modulation of components of the cytoskeleton (Ingber 2002). VEGF and bFGF are the primary mediators of endothelial cell motility (Shono et al. 2001; Lavenburg et al. 2003). VEGF can induce cell migration through activation of the Rho family of GTPases, which regulate the dynamic function of the actin cytoskeleton (Lavenburg et al. 2003). Basic-FGF can act as a chemoattractant to induce endothelial cell motility, which is mediated through MAPK signaling (Shono et al. 2001).

Once the endothelial cells have migrated to the site of vessel budding, they must form the lumen, which will become the new vessel, and stabilize the lumen to complete the process of angiogenesis. Lumen formation occurs through morphological changes in endothelial cells that involve cell elongation along with either intercellular or intracellular lumenization (Egginton et al. 2001). Integrins play an essential role in lumen formation. For example, endothelial cells can form the lumen by generating and fusing intracellular vacuoles, and the mechanism of vacuole formation is integrin $\alpha_2\beta_1$-dependent (Davis and Camarillo 1996).

Endothelial cells not only undergo terminal differentiation during lumenization, but they also undergo replicative quiescence. At a molecular level, it has been shown that MAPKs like ERK aid in both lumen formation and replicative quiescence (Yang et al. 2004). Angiopoietin1, through the activation of its cognate receptor Tie2, can induce quiescence in endothelial cells by promoting cell survival through the MAPK and PI3K signaling pathways (Peters et al. 2004). Undifferentiated fibroblast-like cells called pericytes and smooth muscle cells aid in the stabilization of the lumen by providing structural support to the new vessel (Park et al. 1994; Green et al. 2001;

Ishida et al. 2001; Luttun et al. 2002). Pericytes and smooth muscle cells also stimulate an increase in the diameter of the new blood vessel via placental growth factor (PlGF) mediated signaling through the VEGF receptor-1 on the surface of these cells (Park et al. 1994; Green et al. 2001; Ishida et al. 2001; Luttun et al. 2002).

The complex process of angiogenesis is regulated at many different steps by a variety of cytokines and signaling molecules. As mentioned above, VEGF is one of the most potent angiogenic stimulators. VEGF can induce endothelial cell permeability, proliferation, survival, and migration. VEGF transcription is also induced in response to hypoxia, through the transcription factor HIF-1$\alpha$ (Forsythe et al. 1996). Additionally, VEGF can be induced in response to an increase in inflammatory mediators like interleukins and transforming growth factor $\beta$ (TGF- $\beta$ )(McColl et al. 2004). In fact, many of the commonly studied growth factors mediate their activities in part by stimulating production of VEGF (Dulak et al. 2000). For example, EGF binding to HER2 (human epidermal growth factor receptor 2) increases synthesis of HIF-1$\alpha$, which leads to VEGF induction (Laughner et al. 2001).

Under normal physiological conditions, angiogenesis is a tightly regulated process that is dependent on the balance between endogenous angiogenic promoters and inhibitors. The first demonstrated endogenous molecule to show anti-angiogenic effects was interferon alpha (IFN-$\alpha$), which inhibits endothelial cell migration (Brouty-Boye and Zetter 1980). IFN-$\alpha$ has also been shown to inhibit basement membrane degradation by inhibition of both urokinase-type plasminogen activator (uPA) and MMP-9 expression (Pepper et al. 1994; Ma et al. 2001a; Ma et al. 2001b). Cleavage of plasminogen not only releases the angiogenic stimulator

plasmin, but also releases the inhibitor angiostatin (Nelson and Kastan 1994). Angiostatin inhibits angiogenesis by blocking both endothelial cell proliferation and migration (O'Reilly et al. 1994). Endostatin, another endogenous angiogenesis inhibitor released by proteolysis, is produced when collagen XVIII is cleaved by the cysteine-protease cathepsin L (O'Reilly et al. 1997; Felbor et al. 2000; Maeshima et al. 2001). Endostatin has been shown to specifically inhibit the processes of endothelial cell proliferation, endothelial cell migration, VEGFR2-mediated signaling, and activation of both MT1-MMP and MMP-2 (Dhanabal et al. 1999; Yamaguchi et al. 1999; Kim et al. 2000; Kim et al. 2002b). Additionally, endostatin is known to induce cell apoptosis by repressing Bcl-2 and Bcl-XL (Dhanabal et al. 1999; Yamaguchi et al. 1999; Kim et al. 2000; Kim et al. 2002b).

Thrombospondins represent another class of matrix-derived angiogenesis inhibitors. Thrombospondin-1 (Tsp-1) was the first protein identified to be a naturally occurring inhibitor of angiogenesis (Good et al. 1990). Briefly, Tsp-1 has been shown to promote endothelial cell apoptosis and to inhibit endothelial cell proliferation and migration through activation of TGFβ and CD36 (Baird and Durkin 1986; Frater-Schroder et al. 1986; Dawson et al. 1997; Crawford et al. 1998; Jimenez et al. 2000). Tsp-2 can also function as an angiogenesis inhibitor (Streit et al. 1999). Tsp-2 has been shown to promote endothelial cell apoptosis and inhibit VEGF-dependent endothelial cell migration and lumenization (Noh et al. 2003). These and other endogenous inhibitors help to maintain the balance of positive and negative angiogenic stimuli that regulate physiological angiogenesis.

Tumor growth also requires the induction of angiogenesis (Folkman et al. 1971). Tumor angiogenesis allows solid tumors to grow beyond a microscopic size of approximately 2mm in diameter, the size constraint imposed by the diffusion limit of oxygen and nutrients (Gimbrone et al. 1972). In much the same manner as physiologic angiogenesis, tumor angiogenesis is achieved by altering the balance of inducers and inhibitors towards the side of the inducers. In tumor angiogenesis this shift towards angiogenic induction is accomplished by abrogating many of the negative feedback loops, which results in unrestrained endothelial cell proliferation and blood vessel formation (Dvorak 1986). Interestingly, it has been demonstrated that many tumors also stimulate the production of inhibitors such as angiostatin, a factor that suppresses the angiogenesis and growth of metastatic colonies .

A wide variety of mechanisms can induce tumor angiogenesis. For example, when a tumor grows to a size that limits the diffusion of oxygen, hypoxia induces the production of HIF-1$\alpha$, which in turn induces production of VEGF (Forsythe et al. 1996). VEGF can then induce angiogenesis by stimulating the growth and migration of endothelial cells. Tumors can also induce angiogenesis through production and secretion of the cytokine interleukin 8 (IL-8). IL-8 has been shown to induce the proliferation and migration of endothelial cells, and is thought to be the major angiogenic factor produced in some tumors (Koch et al. 1992; Smith et al. 1994; Lingen et al. 1998). Tumors can also promote angiogenesis and metastasis by inhibiting the expression of angiogenesis inhibitors. For example Tsp-1 expression is repressed in various tumors and fibroblasts, which are part of the tumor microenvironment (Kalas et al. 2005).

Tumors are able to modulate expression of angiogenesis promoters and inhibitors through the activation of oncogenes. For example, the oncogene *ets-1* is a transcription factor that can induce expression of MMPs and urokinase-type plasminogen activator in endothelial cells, thus promoting cell migration and lumen formation (Iwasaka et al. 1996; Lavenburg et al. 2003). Ets-1 has also been found to induce transcription of VEGF in endothelial cells (Hashiya et al. 2004).

Activated Ras is another oncogene that promotes angiogenesis. A recent study has shown that in a model of melanoma, Ras was required for the maintenance of angiogenesis and that inactivating Ras led to endothelial cell apoptosis (Tang et al. 2005). It has also been shown that Ras can lead to the repression of Tsp-1 expression in a genetically engineered tumor model system (Watnick et al. 2003). Expression of the *myc* oncogene can promote tumor angiogenesis by inducing VEGF production and repressing Tsp-1 expression (Janz et al. 2000; Watnick et al. 2003; Knies-Bamforth et al. 2004). A recent study has also found that Myc can activate a micro-RNA cluster that promotes angiogenesis through repression of Tsp-1 (Dews et al. 2006). These studies clearly indicate the essential role of angiogenesis in the progression of cancer, especially in the development of metastasis. Additionally, these studies demonstrate that tumor cells can activate various signaling pathways that lead to the induction of angiogenesis.

## II: The role of the tumor stroma and carcinoma-associated fibroblasts in angiogenesis

During the initial stages of cancer progression, tumor cells are confined as a neoplastic legion within a given tissue (termed carcinoma *in situ*) that is separated from neighboring tissue by the basement membrane (Hanahan and Weinberg 2000a). The basement membrane, immune cells, blood vessels, ECM and fibroblasts surrounding the tumor cells constitute the tumor stroma (Ronnov-Jessen et al. 1996). Tumor progression, including angiogenesis, invasion, and metastasis has been demonstrated to be promoted by fibroblasts located within the tumor stroma (Elenbaas and Weinberg 2001). Fibroblasts within the tumor stroma are known as carcinoma-associated fibroblasts (CAFs) (Olumi et al. 1999). It is believed that CAFs are very similar to, if not the same as, activated fibroblasts found in the stroma of tissues undergoing wound repair (Durning et al. 1984; Tsukada et al. 1987; Schor et al. 1988a; Schor et al. 1988b).

Fibroblasts are elongated cells of the connective tissue of organs that are characterized as not being vascular, inflammatory, or epithelial cells (Tarin and Croft 1969). Though morphologically distinct, the molecular identity of fibroblasts is poorly defined. Fibroblasts are embedded within the matrix of the connective tissue and have many important functions, including synthesis and deposition of ECM, regulation of epithelial cell differentiation, and regulation of inflammatory response (Parsonage et al. 2005; Tomasek et al. 2005). Fibroblasts also play a major role in wound repair (Gabbiani 2003). Specifically, during wound repair, fibroblasts become activated,

invade the wound, produce ECM to anchor other cells involved in the repair process, and facilitate healing wound contractions (Castor et al. 1979; Gabbiani 2003).

Activated fibroblasts proliferate at a greater rate, produce greater amounts of ECM, and express α-smooth-muscle actin (Gabbiani 2003). Injured epithelial cells release growth factors like TGFβ and bFGF that can induce fibroblast activation, or direct cell-cell contacts with leukocytes can also lead to activation (Clayton et al. 1998; Choi and Tseng 2001). In order to accommodate production of greater amounts of ECM, activated fibroblasts contain an oval-sized euchromatic nucleus, rough endoplasmic reticulum, and prominent Golgi apparatus (Castor et al. 1979). Activated fibroblasts also produce proteases that degrade the ECM, such as MMPs, aid in the turnover and reorganization of the ECM, and secrete growth factors like HGH and bFGF (Rodemann and Muller 1991). Once the wound is repaired, the number of activated fibroblasts is greatly decreased, though overall the number of fibroblasts in not significantly changed (Gabbiani 2003). It is not clear whether the decrease in the number of activated fibroblasts is due to apoptosis and repopulation of normal fibroblasts from neighboring tissue, or if the activated fibroblasts revert back to normal fibroblasts. However, it seems more likely that the activation is transient and once wound repair is complete, the fibroblasts revert back to a quiescent phenotype.

Studies have shown that fibroblasts in the tumor stroma exist in a state similar to the activated fibroblasts associated with wound healing (Durning et al. 1984; Dvorak et al. 1984; Tsukada et al. 1987; Ronnov-Jessen et al. 1996). For example, in breast cancer approximately 80% of the fibroblasts within the tumor stroma are

thought to become activated (Sappino et al. 1988). Though it is not clear how fibroblasts become CAFs, *in vitro* studies have shown that TGFβ can induce CAF-like properties in normal fibroblasts (Ronnov-Jessen and Petersen 1993). Unpublished studies presented in a recent review suggest that human carcinoma cells can convert normal fibroblasts into CAFs in a mouse xenograft model (Orimo and Weinberg 2006). Once fibroblasts become CAFs, they can be cultured in the absence of carcinoma cells and retain their CAF phenotype in culture until they undergo senescence (Orimo et al. 2005). It is interesting to note that chickens infected with Rous sarcoma virus develop invasive carcinomas when wounded, showing the oncogenecity of tumor stroma (Dolberg et al. 1985). The tumor stroma has also been shown to be associated with increased blood vessel density (Kalluri and Zeisberg 2006). Taken together, these studies demonstrate the importance of the tumor stroma, and CAFs in particular, to the process of tumor progression.

The transition of *in situ* carcinoma to invasive carcinoma has been well characterized in breast cancer (Ronnov-Jessen et al. 1996). In normal breast tissue mammary ducts are lined with ductal epithelium which is, in turn, surrounded by the myoepithelial layer and sheathed by the basement membrane (Ronnov-Jessen et al. 1996). Connecting the mammary ducts is the stroma consisting of ECM, blood capillaries, and fibroblasts (Ronnov-Jessen et al. 1996). When oncogenic transformation and proliferation of epithelial cells give rise to ductal carcinoma *in situ*, the epithelial and myoepithelial linings become indistinct, the number of stromal fibroblasts increases, the amount of ECM increases, and capillary density increases (Hanahan and Weinberg 2000a). However, the basement membrane of the ducts

remains intact. When the carcinoma progresses to the invasive state the basement membrane is degraded and stromal cells, including CAFs, inflammatory response cells, and newly formed capillaries, come into contact with the tumor cells (Hanahan and Weinberg 2000a). CAFs in the stroma of invasive carcinoma continue depositing large amounts of ECM, including tenascin C in some cases (Chiquet-Ehrismann et al. 1986; Inaguma et al. 1988). It has been shown that in breast and bladder carcinomas expression of tenascin C correlates with increased tumor invasiveness (Mackie et al. 1987; Brunner et al. 2004). The accumulation of ECM in tumors contributes to increased interstitial fluid pressure that hinders oxygen and nutrient diffusion (Netti et al. 2000; Brown et al. 2004). This CAF-mediated hypoxia could lead to the expression of HIF-1$\alpha$ and the induction of VEGF, thus providing a mechanism by which CAFs can promote angiogenesis in tumors.

As stated above CAFs are associated with tumor cells at most stages of cancer progression. Many studies have shown the ability of fibroblasts to promote cancer. For example patients genetically predisposed to breast cancer contained skin fibroblasts that proliferated more easily *in vitro* (Kopelovich 1982). It has also been demonstrated that CAFs can promote tumor growth in a mouse xenograft model whereas normal fibroblasts cannot (Olumi et al. 1999). In this study, SV40 large T antigen-immortalized prostate epithelial cells were mixed with either CAFs or normal fibroblasts and then implanted into immuno-compromised mice. Olumi et al. demonstrated that large tumors grew in the grafts containing CAFs, whereas no tumors grew in grafts containing normal fibroblasts. This demonstrates how CAFs aid

in the formation of tumors, probably through induction of tumorigenic changes in epithelial cells.

Tumor progression is also mediated by CAFs. It has recently been shown that mixing human breast carcinoma cells with CAFs in a mouse xenograft gave rise to tumors that were larger and more angiogenic than when mixed with normal fibroblasts (Orimo et al. 2005). Furthermore, it was shown that the increase in tumor cell proliferation was mediated by stromal cell-derived factor 1 (SDF1) secreted by the CAFs binding to the CXCR4 receptor on tumor cells. Additionally secreted SDF1 was also shown to mediate angiogenesis by recruitment of endothelial progenitor cells (EPCs), which are recruited into the carcinoma during tumor angiogenesis and differentiate into vascular endothelial cells (Lyden et al. 2001). Another study demonstrated the ability of CAFs to induce invasiveness *in vivo* with rat colon carcinoma cells that were not invasive *in vitro* (Dimanche-Boitrel et al. 1994). CAFs also secrete MMPs that help degrade the basement membrane and promote tumor invasion. For example, MMP3 secreted by CAFs can promote tumor cell invasiveness (Lochter et al. 1997). This is accomplished by MMP3-mediated cleavage of the extra-cellular domain of the adhesive protein E-cadherin on the surface of mammary epithelial cells. Cleavage of E-cadherin causes mammary epithelial cells to disperse and undergo epithelial-to-mesenchymal transition, which promotes tumor cell invasiveness.

CAFs have also been implicated in tumor metastasis, by promoting the proliferation of tumor cells at the metastatic site. For example, a liver metastatic cell line was shown to secrete factors that activate fibroblasts *in vitro* (Olaso et al. 1997).

These activated fibroblasts were shown to be within the tumor stroma of the metastasis, and quiescent fibroblasts taken from the liver of mice were activated when cultured with conditioned media (CM) from the melanoma metastasis. The tumor CM induced fibroblast migration, proliferation and production of MMP2. This suggests that CAFs help to create a niche for tumor cells at metastatic sites (Olaso and Vidal-Vanaclocha 2003). A recent study has shown that mice deficient for the Mts1 protein, which stimulates tumor metastasis, failed to grow metastases when highly metastatic mammary carcinoma cells were grafted onto these mice (Grum-Schwensen et al. 2005). Furthermore, there was a significant delay in tumor uptake as well a decrease in tumor incidence as compared to wild-type mice injected with the carcinoma cells. When the tumor cells were mixed with Mts1-competent fibroblasts and injected into the *mts1* knockout mice, the ability of these tumors to metastasize was partially restored. Additionally, recent work from the Watnick group (manuscript in review) has shown that CM from metastatic human breast and prostate carcinoma cell lines are able to repress the expression of Tsp-1 in fibroblasts from tissues where the carcinoma is known to metastasize. This demonstrates the ability of tumors to prime metastatic sites for angiogenesis by decreasing the levels of an endogenous angiogenesis inhibitor.

It is clear that angiogenesis is an essential step in the progression and metastasis of tumors. Fibroblasts play an important role in promoting not only tumor progression but angiogenesis as well. Carcinoma-associated fibroblasts produce growth factors like VEGF that aid in the recruitment and activation of endothelial cells within the tumor stroma. During tumor invasion, CAFs produce not only angiogenic

growth factors, but also produce proteases that break down the basement membrane of the tumor associated tissue as well as the basement membrane of stromal blood vessels, an essential step in angiogenesis. Finally, during tumor metastasis CAFs are able to create permissive environments for tumor growth and angiogenesis at metastatic sites. Studies from Kalas et al and from the Watnick laboratory have shown that tumors secrete factors that are able to repress the expression of Tsp-1 in fibroblasts (Kalas et al. 2005). These studies underscore the importance of Tsp-1 in the induction of angiogenesis. In order for tumor cells to induce angiogenesis, the balance of angiogenic stimulators and inhibitors must be shifted towards induction of angiogenic stimulators in the tumor stroma. It is becoming clear that repression of the angiogenesis inhibitor Tsp-1 in the tumor stroma is an important step in shifting the balance towards induction of angiogenesis.

## III: The role of Thrombospondin-1 in angiogenesis

First described in 1978, Tsp-1 is a 180 kDa glycoprotein that functions as a homotrimer (Lawler et al. 1978). Apart from its anti-angiogenic activity, Tsp-1 is also involved in cell adhesion, migration, and apoptosis (Lawler 2002). Tsp-1 is a matricellular protein that is secreted into the extracellular matrix (ECM) of cells (Lawler 2002). Tsp-1 interacts with various components of the ECM through its functional domains. At the N-terminus, Tsp-1 has a heparin binding domain, followed by a procollagen homology region (PHR), three distinct repeat sequence regions (termed type 1, type 2 and type 3), and a C-terminal globular region that contains a calcium binding domain as well as binding domains for various integrins (Lawler 2002). The major anti-angiogenic effects of Tsp-1 are mediated through its type 1 repeats (termed TSR). Within the TSR are binding domains for TGF-β1 and CD36, which can inhibit tumor angiogenesis (Crawford et al. 1998).

TGF-β1 is a cytokine that functions as a tumor suppressor and a regulator of angiogenesis (Markowitz and Roberts 1996). TGF-β1 has been shown to inhibit tumor growth both *in vitro* and *in vivo* (Tessier and Hoang 1988; Gomella et al. 1989; Braun et al. 1992; Cui et al. 1996). TGF-β1 is secreted in an auto-inhibitory state and becomes functional when its latency-associated peptide (LAP) is cleaved (Nunes et al. 1997; Crawford et al. 1998). Tsp-1 is one of only a few proteins that can activate TGF-β1, by binding to the TGF-β1 LAP, which causes a conformational change within TGF-β1 (Crawford et al. 1998). TGF-β1 inhibits angiogenesis by inhibiting endothelial cell proliferation and migration (Baird and Durkin 1986; Frater-Schroder et al. 1986; Muller et al. 1987). Though TGF-β1 acts as a tumor suppressor early in tumor

development, it has been shown previously that TGF-β1 can also increase tumor invasiveness and metastasis by interacting with the stroma (Bhowmick et al. 2004; Tian et al. 2004; Kang et al. 2005).

CD36 is a transmembrane glycoprotein that serves as an adhesion receptor for Tsp-1 (Asch et al. 1987; Asch et al. 1992). CD36 is involved in cell adhesion and is expressed on the surface of platelets and endothelial cells (Barnwell et al. 1989). Through its TSR, Tsp-1 can bind to CD36 on endothelial cells (Dawson et al. 1997). Activation of CD36 by Tsp-1 in endothelial cells leads to apoptosis and inhibition of cell migration (Dawson et al. 1997; Jimenez et al. 2000). Tsp-1 binding to CD36 activates a signaling cascade, involving Fyn, Caspase-3, p38, and MAP kinase, that leads to increased expression of Fas ligand and apoptosis in endothelial cells (Jimenez et al. 2000; Volpert 2000).

Integrin activation through the C-terminal domain of Tsp-1 has also been shown to have anti-angiogenic effects. For example, Tsp-1 activation of the integrin associated protein CD47 has been shown to block angiogenesis by inhibiting endothelial cell tube formation (Kanda et al. 1999; Freyberg et al. 2000). Tsp-1 can also inhibit angiogenesis by inhibiting the activation of matrix metalloproteinase-9 (MMP-9) (Bein and Simons 2000; Rodriguez-Manzaneque et al. 2001). MMP-9 is a matricellular proteinase that degrades components of the ECM (Shapiro 1998). One of the TSRs in Tsp-1 has been demonstrated to mediate the inhibition of MMP-9 activation by inhibiting the MMP-3-mediated processing of proMMP-9 to the active form (Bein and Simons 2000; Rodriguez-Manzaneque et al. 2001). One of the functions of MMP-9 in tumor progression is to release VEGF from the ECM (Bergers

et al. 2000). Thus, inhibiting MMP-9 activation inhibits VEGF activity in the tumor stroma.

Studies utilizing Tsp-1 knockout mice have helped to further elucidate the role of Tsp-1 in development and adult angiogenesis (Lawler et al. 1998; Wang et al. 2003; Wang et al. 2005). Lawler et al. demonstrated that Tsp-1 knockout mice develop normally and are fertile, yet these mice display a mild lordotic curvature of the spine and develop extensive and acute pneumonia (Lawler et al. 1998). There is also an increase in epithelial cell proliferation in the lung airways in these mice, leading to the conclusion that Tsp-1 is involved in normal lung homeostasis (Lawler et al. 1998). In addition, they found that the levels of Tsp-2, Tsp-3, and Tsp-4 mRNA were not increased in the Tsp-1 knockout mice compared to wild-type controls (Lawler et al. 1998). This result suggests that at least no single thrombospondin protein is compensating for the loss of Tsp-1 in these mice.

Further studies of the Tsp-1 knockout mice revealed a role for Tsp-1 in vascular homeostasis (Wang et al. 2003). Studies of retinal vascular development demonstrated that Tsp-1 knockout mice have an increase in retinal vascular density due to an increased number of retinal endothelial cells (Wang et al. 2003). This study of retinal vascular development also concluded that while Tsp-1 is not required for primary development of retinal vasculature, Tsp-1 is essential for the remodeling and maturation of retinal vasculature (Wang et al. 2003). A later study by the same group demonstrated that Tsp-1 knockout mice retain an isoform of platelet-endothelial cell adhesion molecule-1 (PECAM-1) during the maturation stage of retinal development (Wang et al. 2005). PECAM-1 is a cell adhesion molecule expressed on the surface

of endothelial cells, and is thought to be a regulator of endothelial cell adhesion and migration (Almendro et al. 1996). This study suggests that the retention of this isoform in retinal endothelial cells prevents the cells from attaining a quiescent, differentiated phenotype (Wang et al. 2005). Wang et al also showed that Tsp-1 knockout retinal endothelial cells (RECs) proliferate and migrate at a higher rate than wild-type control RECs, and they suggest that this is mediated by the presence of this isoform of PECAM-1 (Wang et al. 2005; Wang and Sheibani 2006). Further studies elucidated how proliferation is increased in Tsp-1 knockout RECs (Wang et al. 2006). It was demonstrated that cell cycle regulators (cyclins and cyclin dependent kinases) are expressed at higher levels in Tsp-1 knockout RECs than in wild-type RECs (Wang et al. 2006). Also, Tsp-1 knockout RECs expressed lower levels of active Fyn and JNK2 (effectors of endothelial cell apoptosis), which increased REC survival (Wang et al. 2006). Furthermore, the increased migratory phenotype of Tsp-1 knockout RECs was attributed to increased signaling through the Src/PI3-kinase signaling pathways (Wang et al. 2006). Taken together these Tsp-1 knockout studies support the role of Tsp-1 as an angiogenesis inhibitor and as a regulator of vascular maturation and homeostasis.

Despite the overwhelming evidence suggesting that Tsp-1 is an inhibitor of angiogenesis, there are some cases where Tsp-1 seems to induce angiogenesis (Varani et al. 1986; Qian et al. 1997). For example, Tsp-1 was found to induce activation of MMP-9 in gastric, pancreatic, and breast cancers (Qian et al. 2001; Albo et al. 2002; Wang et al. 2002; Zhang et al. 2003). One possible explanation could be that increased amount of Tsp-1 induces some tumor cells to produce more pro-

angiogenic factors, like VEGF, that counteract the inhibitory effects of Tsp-1 and creates a Tsp-1 resistant tumor (Filleur et al. 2001). Another explanation could be the outgrowth of tumor cells that are insensitive to Tsp-1. The Tsp-1 insensitive cells would have a growth advantage during tumor progression to the angiogenic phenotype. Furthermore, it could be that Tsp-1 is proteolytically cleaved in the matrix of these tumors rendering Tsp-1 nonfunctional.

In addition to their role in tumor cell-autonomous growth regulation, tumor suppressors and oncogenes also play a role in regulating tumor angiogenesis (Slack and Bornstein 1994; Grant et al. 1998; Kawahara et al. 1998; Fontanini et al. 1999; Janz et al. 2000; Ravi et al. 2000; Watnick et al. 2003; Kalas et al. 2005). In fact, acquisition of the angiogenic phenotype requires not only the repression of Tsp-1, but also the inactivation of tumor suppressors (Grant et al. 1998; Kawahara et al. 1998; Fontanini et al. 1999; Ravi et al. 2000). For example, the tumor suppressor p53 has been shown to be a positive regulator of Tsp-1 expression (Dameron et al. 1994). Studies have also demonstrated that aerosol delivery of p53 into a mouse with lung tumors leads to inhibition of tumor metastasis and up-regulation of Tsp-1 (Gautam et al. 2002). Loss of p53 has been demonstrated to induce the repression of Tsp-1 expression (Dameron et al. 1994; Grant et al. 1998). For example, in fibroblast cell lines derived from patients containing a mutant allele of p53 (Li-Fraumeni syndrome), it was found that loss of functional p53 leads to a reduction of Tsp-1 protein (Dameron et al. 1994). Also, it has been shown that in malignant melanomas expression of mutant p53 correlates with reduced Tsp-1 expression and the metastatic phenotype (Grant et al. 1998). It should be noted, however, that in both of these cases

repression of Tsp-1 expression could have resulted from a collaborating event that occurred when p53 activity was lost or mutated. In support of this hypothesis there have been various reported cases where no correlation was found between p53 status and Tsp-1 expression in tumor progression (Kawahara et al. 1998; Fontanini et al. 1999; Reiher et al. 2001; Grossfeld et al. 2002).

Oncogenes have been shown to negatively regulate Tsp-1 expression (Slack and Bornstein 1994; Janz et al. 2000; Watnick et al. 2003; Kalas et al. 2005). For example, expression of v-Src in rodent fibroblasts results in the repression of Tsp-1 gene expression (Slack and Bornstein 1994). Also, overexpression of c-Jun in rat fibroblasts leads to the transcriptional repression of Tsp-1 through binding of the WT1 tumor suppressor to the Tsp-1 promoter (Mettouchi et al. 1994; Dejong et al. 1999). Studies involving Id1 knockout mice also revealed that Tsp-1 transcription is inhibited by the Id1 transcription factor during angiogenesis (Volpert et al. 2002). Other studies demonstrate that expression of the oncogene Myc leads to the repression of Tsp-1 expression through an increase in the rate of mRNA turnover, thus destabilizing Tsp-1 transcripts (Janz et al. 2000). Furthermore, it has been shown that in epithelial cells repression of Tsp-1 expression occurs through phosphorylation of Myc induced by the Ras-Rho-ROCK signaling pathway (Watnick et al. 2003). Others have also shown that expression of Ras leads to the repression of Tsp-1 expression in fibroblasts (Kalas et al. 2005). While these findings clearly show the role of oncogenes and tumor suppressors in the regulation of Tsp-1, it remains unclear if the same regulatory pathways are utilized in all cell types or if there are cell type specific effects. It is also unclear if repression of Tsp-1 expression is the primary mechanism by which tumors

induce an angiogenically permissive environment in the tumor stroma, and if the same signal transduction pathways affect these changes.

## IV: The role of c-Myc in oncogenesis and angiogenesis

The nuclear protein Myc was one of the first identified oncogenes (Sheiness et al. 1978). Like Src, Myc was first described as a viral oncogene from the avian carcinoma virus MC29 that caused various carcinomas in chickens and that could transform cells *in vitro* (Duesberg et al. 1977; Sheiness et al. 1978). Numerous studies have shown that Myc expression plays an oncogenic role in tumorigenesis, and that elevated levels of Myc are one of the most common characteristics in human cancers (Little et al. 1983; Nau et al. 1984; Trent et al. 1986; Seshadri et al. 1989; Spencer and Groudine 1991; Nesbit et al. 1999). The precise mechanism by which Myc exerts its oncogenic effects on cells remains unclear; however it is thought that Myc-mediated cell cycle induction plays a role (Evan et al. 1994). Myc has also been implicated in promoting tumor angiogenesis in carcinomas (Pelengaris et al. 1999; Brandvold et al. 2000; Watnick et al. 2003).

Myc is a short-lived nuclear protein that functions as a transcription factor when bound to the nuclear protein Max in a heterodimer (Kato et al. 1992a). Myc contains two nuclear localization domains near the C-terminus (Persson and Leder 1984). Myc also contains an N-terminal transactivation domain and a basic helix-loop-helix leucine zipper domain at its C-terminus (bHLHZ) (Kato et al. 1990). The Myc bHLHZ domain resembles those of certain families of transcription factors, where the bHLHZ domain mediates dimerization and sequence specific DNA binding (Kato et al. 1990). Myc cannot function as a transcription factor on its own because it does

not form homodimers and is unable to bind DNA (Kato et al. 1992b). However, it was demonstrated that Max, through its own bHLHZ domain can bind to Myc and recognize a sequence-specific DNA-binding domain termed the E-box domain (Blackwell et al. 1990; Fisher et al. 1991a; Fisher et al. 1991b; Kerkhoff and Bister 1991; Kerkhoff et al. 1991). Furthermore it was shown that Myc/Max heterodimers are able to function as transcriptional activators (Amati et al. 1992). Studies have shown that the Myc/Max dimer mediates transcriptional activation, at least in part, by acetylating H4 histones in the area where it binds the promoter (Eisenman 2001). This acetylation activity is mediated through binding of TRRAP to the Myc/Max complex, since TRRAP can bind the histone acetyltransferase GCN5 (Bouchard et al. 2001; Frank et al. 2001).

Unlike Myc, Max is a stable protein that is constitutively expressed in both growing and resting cells (Berberich et al. 1992; Blackwood et al. 1992). One of the functions of Max dimers is the regulation of differentiation, which is mediated by the differential expression of Max binding partners (Ayer and Eisenman 1993; Larsson et al. 1994). The unstable proteins Mad and Mxi-1 compete with Myc for Max binding, and their level of expression increases during differentiation while the level of Myc decreases. If Myc binds Max then differentiation is inhibited; however, if Mad or Mxi-1 bind Max then cell differentiation is induced.

The oncogenic role of Myc is mediated primarily through its ability to promote cell proliferation, even in response to cell cycle arrest signals such as p53 activation (Hermeking et al. 1995). Myc induces proliferation through modulation of cell cycle regulators by promoting G1-S transition in the cell cycle (Santoni-Rugiu et al. 2000).

When cells pass the restriction point (R-point) in G1 they are committed to undergoing DNA synthesis (S-phase) (Aguda and Tang 1999). Inactivation of the protein pRb through phosphorylation is one of the best understood events in passage through the R-point (Resnitzky and Reed 1995). One way that Myc stimulates cell proliferation is by promoting activation of the kinase complex cyclin E/Cdk2 (Amati et al. 1998). The cyclin E/Cdk2 complex is one of the kinases responsible for pRb phosphorylation during passage through the R-point (Resnitzky and Reed 1995). Myc can also induce the activation of cyclin E/Cdk2 by suppressing the inhibitory action of the CDK inhibitor p27 (Amati et al. 1998). During G1 p27 is bound to the cyclin E/Cdk2 complex, which inhibits the complex from phosphorylating pRb (LaBaer et al. 1997). Myc can induce expression of cyclin D2, which leads to sequestration of p27 and targets it for ubiquitin mediated degradation (Pagano et al. 1995; Bouchard et al. 1999). Additionally, Myc can induce the expression of a component of the ubiquitin complex that targets p27 (O'Hagan et al. 2000). Furthermore, the Myc/Max complex is able to bind to the transcriptional activator Miz-1 and inhibit transcriptional expression of the CDKIs p15INK4b and p21 (Staller et al. 2001; Seoane et al. 2002; Wu et al. 2003).

Myc induction is highly dependent on mitogenic signals, and is suppressed by growth inhibiting signals (Grandori et al. 2000). For example, Myc is rapidly induced in quiescent cells when growth factors, or serum, are introduced (Grandori et al. 2000). Over-expression of Myc leads to a decrease in growth factor requirements (Keath et al. 1984; Eilers et al. 1991). Furthermore, ectopic expression of Myc can immortalize cultured fibroblasts (Keath et al. 1984). The immortalization of fibroblasts

by Myc is believed to be due in part to the ability of Myc to induce expression of hTERT, the catalytic domain of telomerase (Greenberg et al. 1999). Though Myc is unable to transform cells on its own, combined expression of activated Ras and Myc in rat embryo fibroblasts does lead to full oncogenic transformation in rodent fibroblasts (Land et al. 1986). Co-expression of Ras and Myc *in vivo* also leads to tumorigenesis (Sinn et al. 1987; Langdon et al. 1989; Haupt et al. 1992).

Myc has also been shown to be involved in tumor maintenance (Jain et al. 2002). In an inducible Myc osteosarcoma mouse model, where tumors are induced by overexpressing Myc, it has been shown that a brief inactivation of Myc expression leads to sustained tumor regression by differentiation of the tumor cells into normal bone (Jain et al. 2002). Furthermore, this study demonstrated that reactivation of Myc after tumor regression did not lead to re-formation of the tumor but instead to apoptosis of the Myc-expressing tumor cells. This and other studies have led to the idea that tumors become physiologically dependent on oncogenic signaling for the retention of their malignant phenotype, and inactivation of a critical oncogene leads to the differentiation or apoptosis of the malignant cells (Weinstein 2002).

Along with stimulating proliferation and promoting cell immortalization and transformation, Myc also induces apoptosis (Askew et al. 1991; Evan et al. 1992). In these studies, it was demonstrated that cells constitutively expressing Myc induced apoptosis when growth factor concentrations were low and when cells were arrested at various stages of the cell cycle (Evan et al. 1992). It has been shown that Myc can induce apoptosis through a multitude of signaling pathways. For example, Myc can induce p53 dependent apoptosis by inhibiting p21 (Seoane et al. 2002). In this case

Myc can determine the choice between cell cycle arrest and apoptosis in the p53 DNA damage response. Alternatively Myc can induce p53-independent apoptosis by indirectly stimulating the release of cytochrome C from the mitochondria (Juin et al. 1999). Cytochrome C release appears to be mediated through Myc-dependent activation of Bax (Narita et al. 1998; Mitchell et al. 2000). The apoptotic function of Myc is mediated through the same structural domains that mediate cell proliferation, growth, and transformation (Evan et al. 1992). Myc-induced apoptosis has been postulated to be an intrinsic safety mechanism that prevents unnatural levels of cell proliferation from occurring in cells overexpressing Myc (Askew et al. 1991; Evan et al. 1992; Harrington et al. 1994). Thus tumor cells constitutively expressing Myc must be able to inhibit the apoptotic effects of Myc. Indeed, one study suggests that activation of Myc in some cancers like Burkitt's lymphomas leads to the transactivation of mutant p53, which has been shown to inactivate p53-mediated apoptosis (Roy et al. 1994).

Recent studies have also implicated Myc in non-cell autonomous mediated apoptosis, which may explain the outgrowth of cells constitutively expressing Myc in tumors (de la Cova et al. 2004; Moreno and Basler 2004). Two studies in *Drosophila* found that cells expressing high levels of dMyc in the wing imaginal disk out-compete adjacent cells expressing lower level of dMyc (de la Cova et al. 2004; Moreno and Basler 2004). This process is thought to help regulate the size of adult appendages (de la Cova et al. 2004). Moreover both studies found that dMyc-mediated competition resulted in the induction of apoptosis in the cells expressing lower levels of dMyc. These experiments demonstrate that Myc-induced apoptosis is not

necessarily cell autonomous, and suggest a method by which Myc-expressing cells might grow into a tumor. Cells expressing Myc and resistant to Myc-induced apoptosis might elicit this competition response and induce apoptosis in adjacent cells. This would lead to an outgrowth of preneoplastic cells expressing high levels of Myc and resistant to apoptosis (de la Cova et al. 2004; Moreno and Basler 2004).

Myc not only promotes tumorigenesis through its effects on proliferation and cell growth, but also on its effects on angiogenesis. Various reports have demonstrated that Myc-induced malignancies display increased angiogenesis (Pelengaris et al. 1999; Brandvold et al. 2000). However, these studies did not explain how Myc induced angiogenesis in the tumors. One of the first reported effects of Myc on angiogenesis regulation was the observation that in rat fibroblasts over-expressing Myc, Tsp-1 mRNA levels were repressed (Tikhonenko et al. 1996). It was also shown in a mouse model that Myc-induced tumorigenesis led to the repression of Tsp-1 expression (Ngo et al. 2000). Additionally, a recent study has shown that Myc can activate a micro-RNA cluster that is responsible for the repression of Tsp-1 (Dews et al. 2006). In this study p53- null mouse colonocytes expressing both K-Ras and Myc were shown to upregulate expression of the microRNA cluster miR 17-92 and expression of miR 17-92 was shown to repress expression of Tsp-1. It should be noted that K-Ras was not implicated in the upregulation of miR 17-92 because colonocytes only expressing K-Ras did not upregulate miR 17-92 and Tsp-1 expression was not repressed. However, it is interesting to note that in these mouse colonocytes, repression of Tsp-1 required both

the repression of p53 as well as the Myc-mediated expression of the miR 17-92 cluster.

Myc can also induce production of VEGF in cells. It has been shown in *cmyc* knockout mouse embryonic stem cells that defects in growth and differentiation potential are due in part to the requirement for VEGF, and expression of VEGF in these cells can partially abrogate these effects (Baudino et al. 2002). Furthermore, a recent study has shown that Myc can induce the production and release of VEGF in hypoxic regions of Myc-induced neoplastic legions in a mouse model of melanoma (Knies-Bamforth et al. 2004). In this study Myc expression *in vivo* led to VEGF transcription in keratinocytes at sites of tissue hypoxia. In cultured keratinocytes, expression of Myc promoted VEGF secretion and expression of Myc under hypoxic conditions led to an increase in VEGF protein levels.

Watnick et al. have also shown that in epithelial cells Ras activates a ROCK-dependent signaling pathway that phosphorylates Myc. Myc phosphorylation results in an activated form of the protein and induces Tsp-1 repression (Watnick et al. 2003). Interestingly, a recent study has shown that hypoxia induces VEGF expression in colon cancer cells through the same Ras-ROCK-Myc signaling pathway that represses Tsp-1 in epithelial cells (Mizukami et al. 2006). This study demonstrates a HIF-1$\alpha$-independent pathway that induces hypoxia-mediated VEGF induction.

## V: The role of p53 in tumor development and angiogenesis

One of the most commonly mutated genes in human cancers is *p53* (Levine 1993; Greenblatt et al. 1994). The protein p53 was first discovered as a cellular protein that associated very tightly with Simian virus 40 (SV40) large T antigen (Lane and Crawford 1979; Linzer and Levine 1979; Linzer et al. 1979). Early experiments with cloned p53 described it as having oncogenic potential, though later studies revealed that these p53 clones were obtained from mutant versions of the p53 gene (Parada et al. 1984; Eliyahu et al. 1988; Finlay et al. 1988; Eliyahu et al. 1994). These studies, however, demonstrated that mutation of p53 often leads to a tumorigenic phenotype. Subsequent studies with wild-type p53 conclusively showed that p53 functions as a tumor suppressor (Eliyahu et al. 1989; Finlay et al. 1989; Baker et al. 1990; Chen et al. 1991).

The main function of p53 is in the response to cellular stress, particularly DNA damage (Kastan et al. 1991). In response to DNA damage, p53 can either arrest the cell cycle or induce apoptosis (Kastan et al. 1991; Yonish-Rouach et al. 1991; Kuerbitz et al. 1992; Clarke et al. 1993; Lowe et al. 1993). The activity of p53 is primarily a function of its ability to modulate transcriptional expression of target genes (Luria and Horowitz 1986; Fields and Jang 1990). The N-terminal domain of p53 contains the transactivation domain, while the rest of the protein is composed of a sequence-specific DNA-binding domain, a nuclear localization signal, a C-terminal oligomerization domain, and a lysine-rich C-terminal ubiquitination domain (Kern et al. 1991; el-Deiry et al. 1992; Sturzbecher et al. 1992; Rodriguez et al. 2000). Through

the oligomerization domain, p53 forms a functional active tetramer (Sturzbecher et al. 1992).

Under normal conditions, p53 is maintained at low levels by ubiquitin-mediated proteolysis (Maki et al. 1996). This process is regulated by the ubiquitin ligase MDM-2 (Momand et al. 1992). MDM-2 binds p53 at the N-terminal transactivation domain and inhibits p53 function by ubiquitinating the p53 C-terminal lysines, thereby targeting it to the proteasome for degradation (Momand et al. 1992; Oliner et al. 1993; Honda et al. 1997; Fuchs et al. 1998). Activated p53 induces transcription of MDM-2, thus establishing an autoregulatory loop that ensures attenuation of p53 signaling (Momand et al. 1992). Mouse studies have shown the critical role that MDM-2 plays in regulating p53 expression to allow for proper cell cycle progression during development. Whereas the *mdm2* knockout mouse is embryonic lethal, the *p53 mdm2* double knockout is viable (Jones et al. 1995; Montes de Oca Luna et al. 1995).

Under conditions of cellular stress, such as DNA damage, p53 becomes stabilized through post-translational modifications, is relieved from MDM-2 binding, and becomes active (Canman et al. 1994). For example, gamma-irradiation activates the kinase ATM, which activates the CHK-2 kinase, and both kinases phosphorylate serines in the N-terminal transactivation domain of p53 (Canman et al. 1998; Chehab et al. 2000; Shieh et al. 2000). It is also worth noting that p53 can be activated in response to inappropriate cell proliferation due to oncogenic signaling, which is one mechanism by which p53 suppresses tumors. In response to oncogenic signaling, p19ARF is induced, which inactivates MDM-2 and releases p53 (Kurokawa et al. 1999).

The two main cellular responses arising from p53 activation are cell cycle arrest and apoptosis. The p53 protein can arrest the cell cycle at both the G1 and G2 phases. Arrest at G1 can be mediated through activation of p21, which can inhibit cyclinD1/Cdk4 and cyclinE/Cdk2 (Harper et al. 1993; Xiong et al. 1993). These two CDK complexes promote progression through G1. Conversely, activation of 14-3-3 sigma by p53 causes cdc-25 and cyclinB1/Cdk1 to be sequestered in the cytoplasm, which helps maintain a G2 block (Hermeking et al. 1997). It has also been shown in fibroblasts that constitutive expression of Myc can induce a p53-mediated G2 arrest of the cell cycle (Felsher et al. 2000).

Apoptosis is induced by p53 through the transactivation of pro-apoptotic genes like the mitochondrial pore protein Bax and the BH3 domain proteins PUMA and NOXA, which mediate the release of cytochrome C from the mitochondria (Miyashita et al. 1994; Miyashita and Reed 1995; Oda et al. 2000; Nakano and Vousden 2001; Schuler and Green 2001). It has also been shown that p53 can transcriptionally repress the cell survival gene *bcl-2* (Miyashita et al. 1994).

Myc is one of the targets of p53-mediated transcriptional repression (Moberg et al. 1992; Levy et al. 1993; Ragimov et al. 1993). Moberg et al. and Ragimov et al. demonstrated the ability of p53 to block promoter activation of Myc by blocking formation of the transcriptional pre-initiation complex on the promoter. Levy et al. demonstrated that expression of p53 promotes a G0-like state in leukemic cells and represses transcription of Myc. Furthermore, a recent study has indicated that p53 represses Myc expression by direct association with the Myc promoter and histone H4 deacetylation (Ho et al. 2005). This study also demonstrates that Myc repression

leads to cell cycle arrest at G1 and highlights the interrelation between p53 and Myc. The interaction of Myc with p53 activation seems to play a critical role in determining the cellular response to stress, since overexpression of Myc can lead to apoptosis and repression of Myc can lead to G1 cell cycle arrest (Seoane et al. 2002; Ho et al. 2005).

Studies of murine cancer models have revealed that p53 does not play an essential role during development (Donehower et al. 1992; Jacks et al. 1994; Purdie et al. 1994). However, consistent with its role as a tumor suppressor, p53 knockout mice develop spontaneous tumors (Donehower et al. 1992; Jacks et al. 1994; Purdie et al. 1994). Mice heterozygous for p53 also develop spontaneous tumors, though the tumors develop later in life (Donehower et al. 1992; Jacks et al. 1994; Purdie et al. 1994). Interestingly heterozygous mice develop different types of tumors than the p53 null mice. Knockout mice develop mostly lymphomas and soft tissue sarcomas, while heterozygous mice have an equal distribution of lymphomas, soft tissue sarcomas and osteosarcomas (Jacks et al. 1994). Heterozygous mice also develop carcinomas at a much higher frequency than p53 knockout mice (Jacks et al. 1994).

Angiogenesis has also been shown to be regulated by p53. Many studies have demonstrated that expression of wild-type p53, in various types of carcinoma and sarcoma cell lines, leads to the repression of VEGF both *in vitro*, and in mouse xenograft models (Mukhopadhyay et al. 1995; Bouvet et al. 1998; Zhang et al. 2000). Supporting studies have shown that in angiosarcomas expression of mutant p53 or overexpression of MDM-2 increases levels of VEGF expression (Zietz et al. 1998). p53-mediated VEGF repression has been demonstrated to be at the transcriptional

level, as evidenced by the decrease in the levels of VEGF transcript and promoter activity (Mukhopadhyay et al. 1995). Subsequent studies have revealed that p53 inhibits VEGF transcription by associating with and inactivating the transcription factor SP1 (Pal et al. 2001). It has also been demonstrated in carcinoma cell lines that loss of p53 activity leads to increased levels of HIF-1α and transcriptional activation of VEGF (Ravi et al. 2000). However, it seems that in bladder carcinomas repression of p53 does not lead to an increase in VEGF expression (Reiher et al. 2001). Furthermore, this study also showed that loss of p53 did not affect expression of the angiogenesis inhibitor Tsp-1.

Angiogenesis inhibitors can also be induced by p53 activation. A recent study has shown that expression of p53 in human cancer cell lines can stimulate the expression of a collagen prolyl hydroxylase (Teodoro et al. 2006). This study demonstrates that p53-mediated transcriptional activation of α (II) collagen prolyl-4-hydroxylase induces the production of the angiogenesis inhibitors endostatin and tumstatin. An early study has also demonstrated that human fibroblast cell lines derived from Li-Fraumeni patients display a decrease in Tsp-1 expression upon loss of p53 (Dameron et al. 1994). Li-Fraumeni syndrome is a condition in which patients contain one wild-type p53 allele and one nonfunctional mutant p53 allele (Malkin et al. 1990; Srivastava et al. 1990). The second p53 allele is often lost in patients with Li-Fraumeni syndrome leading to a 50% chance of developing cancer by the age of 30. It has also been shown that ectopic expression of p53 in Li-Fraumeni-derived fibroblasts lacking p53 protein expression stimulates Tsp-1 transcription (Dameron et al. 1994). Interestingly, there have been no published reports demonstrating p53-

mediated regulation of Tsp-1 in wild-type cells. In the report by Dameron et al., the use of human cells derived from Li-Fraumeni patients could complicate experimental analysis because it is not clear what other changes may have occurred in the cells when loss of heterozygocity occurred. Therefore the role of p53 in Tsp-1 regulation may not be as clear cut as one would assume.

## VI: The role of pRb in tumor development and angiogenesis

Rb was the first tumor suppressor to be identified (Friend et al. 1986). Early studies determined that complete loss of pRb function leads to the development of the heritable form of retinoblastoma (Cavenee et al. 1983; Godbout et al. 1983). Retinoblastoma is a childhood tumor of the eye that has two distinct forms (Knudson 1971). The first form is sporadic and is characterized by the development of a single tumor (Knudson 1971). The second form of retinoblastoma is hereditary and can be transmitted as an autosomal dominant trait (Knudson 1971). Patients with the heritable form of retinoblastoma have early eye tumor development and the number of tumors increases over time (Knudson 1971). Based on this data it was proposed and later verified that patients with the heritable form of retinoblastoma have a germline mutation in the *Rb* gene and through loss of heterozygosity become completely deficient in Rb functions, thus developing tumors (Knudson 1971; Cavenee et al. 1983; Godbout et al. 1983; Friend et al. 1986; Fung et al. 1987; Lee et al. 1987). It has now been shown that the majority of human cancers have lost pRb activity (Sherr 2004). It has also been shown that viral oncoproteins like SV40 large T antigen and adenovirus E1A can sequester pRb, thus inhibiting pRb from functioning

as a tumor suppressor (Whyte et al. 1988; Dyson et al. 1990). Mouse studies have also shown that loss of pRb function leads to tumor development(Jacks et al. 1992; Hu et al. 1994; Williams et al. 1994; Vooijs et al. 1998). Taken together, these findings clearly establish the role of pRb as a tumor suppressor.

The tumor suppressor pRb is expressed in most tissues and regulation of its activity is cell-cycle-dependent (Goodrich and Lee 1993). pRb is a nuclear phospho-protein with many phosphorylation sites located throughout the protein (Goodrich and Lee 1993). pRb has no catalytic activity and has weak nonspecific DNA-binding ability (Goodrich and Lee 1993). pRb is a member of a family of proteins called the pocket proteins that include p107 and p130 (Classon and Dyson 2001). All three proteins contain a conserved pocket domain that is divided into two by a spacer region (Classon and Dyson 2001). The main function of the pocket proteins is in transcriptional regulation through binding of transcription factors at the pocket domain and subsequent modulation of transcription factor activity (Cobrinik 2005). One of the major interaction partners of the pocket proteins is the E2F family of transcription factors, whose activity is repressed by pRb (Chellappan et al. 1991; Kaelin et al. 1992; Nevins 1992).

The E2F family of transcription factors contains a conserved DNA binding domain and a dimerization domain (Slansky and Farnham 1996). The E2Fs function as transcription factors by forming heterodimers with the DP protein (Helin et al. 1993). E2Fs 1-5 all have a C-terminal activation domain and an overlapping pRb binding domain, which is how pRb inhibits E2F activity (Dyson 1998). E2Fs 1-3, which are known as the activating E2Fs, contain an N-terminal Cdk-binding domain

and nuclear localization sequence (Lees et al. 1993; Dynlacht et al. 1994). These activating E2Fs generally induce genes that are important for cell cycle progression (Dyson 1998). The repressive E2Fs (E2F 4 and 5) lack the Cdk-binding domain and have a nuclear export sequence instead of an import sequence near their N-terminus (Muller et al. 1997; Gaubatz et al. 2001; Apostolova et al. 2002). These repressive E2Fs are though to be required for exit from the cell cycle and differentiation (Du and Pogoriler 2006).

One of the main roles of pRb is regulation of cell cycle progression. During quiescence and early G1, pRb is fully active (i.e. unphosphorylated) and through recruitment of co-repressors binds and represses E2F transcriptional activators (Chellappan et al. 1991). As the cell initiates cell cycle progress in response to mitogenic signals, pRB becomes increasingly phosphorylated, which progressively destabilizes its ability to bind and repress E2F (Mittnacht 1998). For example, during G1 cyclin D/Cdk4 phosphorylates pRb and during the G1/S transition cyclin E/Cdk2 and cyclin A/Cdk2 phosphorylate pRb (Hinds et al. 1992; Ewen et al. 1993; Kato et al. 1993; Resnitzky et al. 1995; Du et al. 1996). Through phosphorylation at discrete sites, CDKs active at different times during G1 can exert temporal control over pRb activity (Mittnacht 1998). By the end of G1 pRB becomes completely inactivated and E2F becomes active, thus allowing expression of E2F target genes essential for S phase entry (Mittnacht 1998). pRb remains inactive for the duration of the cell cycle, suggesting that pRb suppression is essential for cell cycle progression (Mittnacht 1998).

Another mechanism by which pRb functions as a tumor suppressor is through the negative regulation of oncogenic proteins like Myc. Studies have shown that pRb activity is correlated with repression of Myc transcription both *in vitro* and *in vivo* (Murphy et al. 1991; Salcedo et al. 1995; Zhao and Day 2001). Another study demonstrated that active pRb could repress a *c-myc* promoter construct while E2F-1 induced activity of the *c-myc* promoter (Oswald et al. 1994). Furthermore, it has long been established that the Myc promoter is a target of E2F activation in cell culture and in cancer cells (Hiebert et al. 1989; Thalmeier et al. 1989; Elliott et al. 2001). Taken together, these results indicate that pRb inhibits Myc through suppression of E2F. It is worth noting that Myc also promotes E2F activity by inducing expression of the CDKs involved in pRb phosphorylation (Leone et al. 1997). However, a recent study has demonstrated that Myc can induce expression of a microRNA cluster that produces two microRNAs that repress expression of E2F1 protein (O'Donnell et al. 2005). This study illustrates a complex regulatory network that can mediate both the expression and repression of a protein by the same effector.

The pRb tumor suppressor can also modulate p53 activity. Through its suppression of E2F, pRb promotes p53 degradation by inhibiting expression of ATM and Chk2 kinases (Rogoff et al. 2004). Both ATM and Chk2 promote the dissociation of p53 from MDM-2 by phosphorylating p53 (Rogoff et al. 2004). Ectopic expression of pRb also inhibits p53-induced apoptosis in HeLa cells (Haupt et al. 1995). It has been shown that pRb can bind to the Bcl-2 promoter and, with the transcription factor AP-2, induce expression of Bcl-2 in epithelial cells (Decary et al. 2002). These observations demonstrate a mechanism by which pRb is able to overcome p53-

induced apoptosis. However, it also seems that pRb can promote p53-dependent apoptosis. It has been proposed that pRb can form a trimeric complex with p53 and MDM-2, and that this trimer stabilizes p53 and inhibits the transactivation function of p53 (Hsieh et al. 1999). This interaction of pRb with MDM-2 and p53 was found to enhance p53-mediated apoptosis. One potential explanation for this observation is the fact that in this complex p53 is still able to trans-repress the expression of certain genes, such as Bcl-2 (Hsieh et al. 1999). Regulation of cell cycle progression involves many complex interactions that seem to have contradictory effects, but each individual effect may be cell-type and context-specific.

The pRb tumor suppressor family also plays a role in regulating angiogenesis. For example, it has been shown that the pRb family member p130 can inhibit angiogenesis both *in vitro* and *in vivo* by inhibiting VEGF expression (Claudio et al. 2001). It has also been shown that pRb can inhibit the expression of transcriptional activators that induce VEGF (Beischlag et al. 2004; Lasorella et al. 2005). One study has demonstrated that in pituitary tumors the transcription factor Id2 can induce VEGF expression and that it is negatively regulated by pRb (Lasorella et al. 2005). A second study has demonstrated that pRb inhibits expression of TRIP230, which is an essential co-activator of HIF-1-mediated VEGF expression under hypoxic conditions (Chang et al. 1997; Beischlag et al. 2004). Interestingly, expression of active pRb can also trans-activate Hif-1$\alpha$ under normoxic conditions (Budde et al. 2005). The complex nature of pRb function on HIF-1 seems to be dependent on the context of cellular response, and activation of HIF-1 by pRb, which promotes dephosphorylation

of pRb, may be a way to release cell cycle arrest upon return to normoxia form a hypoxic state (Budde et al. 2005).

Expression of VEGF, which promotes endothelial cell proliferation, has also been found to be inhibited by E2F1 (Qin et al. 2006). In this study it was demonstrated that E2F1 knockout mice display increased angiogenesis, and under hypoxic conditions E2F1-deficient cells display increased levels of VEGF. Furthermore, promoter assays demonstrated that ectopic expression of E2F1 can repress VEGF promoter activity in E2F1 deficient cells. This study also demonstrated that the inhibitory effect of E2F1 is mediated by p53. VEGF is transcriptionally repressed by p53 and E2F1 activates p53 expression (Qin et al. 2006). This observation is striking because it demonstrates that a transcriptional activator like E2F1 can act as a transcriptional repressor in certain contexts.

The role of pRb in the regulation of endogenous angiogenesis inhibitors is not well defined. There is one report demonstrating that expression of pRB can activate a Tsp-1 promoter construct in both wild-type and $Ni^{2+}$-transformed Chinese hamster embryonic cells (Salnikow et al. 1994). This is the only published report that links pRb and Tsp-1 expression. However, given the number of signaling pathways that are modulated in response to pRb, it would not be surprising if pRb promotes changes in Tsp-1 expression.

## VII: The role of SV40 large T antigen in cellular transformation

Simian virus 40 was first discovered as a contaminant in poliovirus vaccines (Sweet and Hilleman 1960). Its discovery led to the observation that SV40 is able to transform human cell lines *in vitro* (Shein and Enders 1962). Subsequent studies revealed that two proteins, large T antigen and small T antigen, mediate the oncogenic effect of SV40 (Sleigh et al. 1978). Small T antigen alone cannot transform cells, but its disruption of the serine/threonine phosphatase PP2A helps large T antigen transform cells (Sleigh et al. 1978; Mungre et al. 1994).

Large T antigen (LT) has three domains that function as the primary mediators of its transforming activity (Pipas et al. 1983). At the N-terminal domain, LT contains a J binding domain that is able to bind the molecular chaperone HSC70 and aids in inactivating the pRb family of proteins (Srinivasan et al. 1997; Stubdal et al. 1997). The second domain of LT is the LxCxE binding motif, which is able to bind and sequester the pocket proteins, including pRb, p130 and p107 (Ludlow et al. 1989). The pRb binding motif is also capable of mediating p130 degradation (Zalvide et al. 1998). The third domain is the C-terminal p53 binding domain (Peden et al. 1989; Srinivasan et al. 1989). LT physically associates with the DNA binding domain of p53 and both stabilizes and inactivates p53 (Bargonetti et al. 1992; Mietz et al. 1992; Jiang et al. 1993; Segawa et al. 1993).

The ability of LT to inactivate both p53 and pRb makes it a powerful tool in elucidating the molecular mechanisms of cellular transformation. Expression of LT, in conjunction with the catalytic subunit of telomerase and oncogenic proteins like Ras, can induce cellular transformation of primary human epithelial and mesenchymal cells

(Hahn et al. 1999; Elenbaas et al. 2001). Studies have also demonstrated that LT can contribute to an angiogenic phenotype. For example, expression of LT and Ras in human epithelial cells leads to the repression of Tsp-1 expression (Watnick et al. 2003). Thus the use of LT can also aid in determining the role of tumor suppressors in the modulation of the expression of endogenous angiogenesis inhibitors.

## VIII: Summary

Angiogenesis is a tightly regulated process that is dependent on the balance between angiogenic stimulators and repressors. Based on the available data, it seems likely that the angiogenesis inducer VEGF and the angiogenesis inhibitor Tsp-1 are the central components of this balance. Studies highlighted above clearly show how VEGF expression can be induced in tumor cells and in carcinoma-associated fibroblasts (CAFs). The studies described above also reveal how Tsp-1 can be repressed in tumor cells. However, it remains unclear how tumor cells are able to repress Tsp-1 in the tumor stroma or in the stroma associated with metastatic sites.

Studies of oncogene and tumor suppressor signaling have given us clues as to how tumors are able to repress Tsp-1. For example it has been shown that Ras and Myc can cooperate to inhibit Tsp-1 in epithelial cells and fibroblasts (Watnick et al. 2003; Dews et al. 2006). Furthermore, it has been demonstrated that p53 can induce Tsp-1 expression and that under certain circumstances loss of p53 can result in the loss of Tsp-1 expression in fibroblasts (Dameron et al. 1994). It is also worth noting that pRb can inhibit p53 expression though E2F1 (Qin et al. 2006).

These studies raise the question of whether carcinoma and non-transformed epithelial cells regulate Tsp-1 by different mechanisms than CAFs and inactivated fibroblasts. Watnick et al. demonstrated that Tsp-1 repression in epithelial cells requires both Ras activation and the repression of p53 and/or pRb (through SV40 LT) (Watnick et al. 2003). Alternatively, Dameron et al. demonstrated that in fibroblasts derived from Li-Fraumeni patients, loss of p53 is sufficient to repress Tsp-1 expression (Dameron et al. 1994). These two studies highlight the differences in the angiogenic potential of epithelial cells and fibroblasts. It would appear that in epithelial cells additional genetic changes are required to tip the balance toward an angiogenic phenotype. It remains to be seen which of the two tumor suppressors inactivated by SV40 LT plays a role in repressing Tsp-1. It also remains unclear how inactivation of either of these tumor suppressors mediates repression of Tsp-1 in conjunction with oncogenic signals like Ras.

In this work I analyze the effects of p53 inactivation in epithelial cells and fibroblasts by studying the resultant differences in Myc and Tsp-1 expression. To this end, I make use of both the SV40 large T antigen (LT) and a short hairpin RNA directed against p53. I postulate that expression of Myc and Tsp-1 in epithelial cells is regulated differently than in fibroblasts. Therefore, disrupting p53 and pRb activity in epithelial cells and fibroblasts should yield novel insight into the molecular events that govern the conditions under which carcinomas develop an angiogenic phenotype, as well as conditions under which an angiogenic phenotype is induced in carcinoma-associated fibroblasts or sarcomas.

# Chapter 2. Regulation of Thrombospondin-1 Expression in Epithelial Cells

An extensive amount of research has been done to catalog the molecular and genetic events involved in the transformation, progression, invasion and metastasis of epithelial derived tumors (Hanahan and Weinberg 2000b). These studies have significantly increased our understanding of the role of many tumor suppressors and oncogenes involved in the development of carcinomas. For example, nearly all carcinomas are characterized by mutations in the p53 and pRb pathways and many also contain amplifications in the *c-myc* oncogene (Spencer and Groudine 1991). However the precise mechanisms by which epithelial cells regulate the angiogenic phenotype in their progression to a carcinoma have yet to be elucidated.

In the following study I investigate the role of p53 in regulating Myc and Tsp-1 in epithelial cells. To this end, I have made use of both the SV40 large T antigen (LT) and short hairpin RNA's directed against p53. Given the role of p53 in tumorigenesis, I postulate that loss of p53 has an effect on Myc and Tsp-1 expression. Thus, disrupting p53 in epithelial cells should yield novel insight into the molecular events that govern the conditions under which carcinomas induce an angiogenic phenotype.

The results from the studies described in this chapter were obtained using immunoblot analysis of cells cultured under serum starved conditions. Each result described is representative of at least four immunoblots performed for each

experiment. Results from immunoblots were all similar to the blots depicted in this section.

## I: Expression signatures in epithelial cells

Given the fact that angiogenesis is essential for carcinoma progression beyond the *in situ* stage (Ch.1 section I), I decided to confirm earlier reports demonstrating the basal level of expression of genes commonly mutated in human carcinomas. I chose to examine the expression levels of p53, Myc and Tsp-1 in human epithelial cells derived from mammary and kidney tissues. Tsp-1 has been demonstrated to be negatively regulated by Myc and positively regulated by p53, this places Tsp-1 at the nexus of the transformation process (Ch. 1 section III). However, as determined by immunoblot analysis, human mammary and renal epithelial cell lines express detectable levels of Myc, p53 and Tsp-1 (Fig. 1A and 1B). Taken together, these observations suggest that in epithelial cells Tsp-1 levels are regulated by a balance of both Myc and p53 expression. These results, however, do not rule out the possibility that the expression levels of Tsp-1, Myc and p53 are not a function of direct transcriptional regulation of Myc and/or p53.

## II: Effects of SV40 large T antigen on epithelial cells

Having established the basal levels of Tsp-1, Myc and p53 expression, I sought to determine whether these expression levels are modified during epithelial cell transformation. To test this hypothesis I used SV40 large T antigen as the first step in a model for cellular transformation (Hahn et al. 1999). I transduced human mammary and renal epithelial cells with a retroviral construct specifying the cDNA for

the LT gene (LT). LT differs from the genomic version in that it only encodes the SV40 large T antigen and not the small T antigen (Chen et al. 2004). The lack of small T antigen expression therefore simplifies interpretation of results with LT expression. Thus, the primary effect of LT expression is loss of p53 and pRb activity (Ch.1 section VII). As confirmation of LT activity, I observed that both LT-containing epithelial cell lines express elevated levels of p53 compared to wild-type control cells (Fig. 2A). It should also be noted that expression of LT in epithelial cells conferred a phenotype similar to previously described cell lines expressing LT (Shein and Enders 1962). This LT phenotype included an increase in the rate of cell growth and a decrease in the dependence on growth factors for cell growth (data not shown).

I then determined the levels of Tsp-1 and Myc expression in the LT containing cells using immunoblot analysis. Expression of LT represses Myc expression in epithelial cells (Figure 2A). LT also increases the level of Tsp-1 expression in both cell lines, though the increase is more dramatic in the renal epithelial cells (Fig. 2B). Thus, it appears that in epithelial cells expression of LT results in the repression of Myc and stimulation of Tsp-1 expression. Given that one of the targets of SV40 LT inactivation is p53, it would seem that in epithelial cells inactivation of Myc and p53 leads to the induction of Tsp-1 expression. However, an alternative interpretation of these results is that LT-mediated induction of Tsp-1 is due to other transformative effects of LT not related to p53 and Myc. For example, it is possible that LT-mediated effects on cell cycle progression may also be mediating an increase in the levels of Tsp-1. If these effects on Tsp-1 expression are mediated by p53 and Myc, then these results suggest that Myc is a more potent effector of Tsp-1 expression than is p53.

Furthermore, the potent effects of Myc on Tsp-1 expression could explain why expression of RasV12 in conjunction with LT expression is needed to repress Tsp-1 levels in epithelial cells (Watnick et al. 2003).

## III: The effects of p53 deficiency on Myc and Tsp-1 expression in epithelial cells

The results of LT expression in epithelial cells were striking because they suggest that in these epithelial cells, inactivation of p53 may lead to the repression of Myc. These observations are contrary to published reports that Myc is a target of p53 transrepression (Moberg et al. 1992; Levy et al. 1993; Ragimov et al. 1993; Ho et al. 2005). However, LT not only inactivates p53, but pRb as well. It is also important to note that pRb activity has been shown to repress Myc expression (Murphy et al. 1991; Salcedo et al. 1995; Zhao and Day 2001). Therefore, to ascertain whether loss of p53 activity is mediating the repression of Myc expression in epithelial cells, I transduced the human mammary epithelial cell line with a lentiviral construct specifying a previously published sequence for an shRNA against p53 (shp53) (Brummelkamp et al. 2002). In order to demonstrate that the shp53 construct was functional in these cells, I used the DNA damaging agent etoposide to stimulate p53 activity. I then determined the levels of p53 and p21 expression using immunoblot analysis (Nair et al. 2005). As expected, epithelial cells expressed higher levels of both p53 and p21 in response to etoposide treatment (el-Deiry et al. 1994) (Fig. 3C). In cells containing the shp53 construct, however, there were no detectable levels of p53 expression and no induction of p21 following treatment with etoposide (Fig. 3C).

Immunoblot analysis revealed that silencing p53 in mammary epithelial cells is sufficient to repress Myc. (Fig.3A). This observation is consistent with the results obtained in epithelial cells expressing LT, and demonstrates that p53, not pRb, is correlated with the loss of Myc expression. Also consistent with LT-expressing epithelial cells, Tsp-1 expression is stimulated in p53-deficient epithelial cells (Fig. 3B). It is unresolved whether these results are due to direct effects of p53-deficiency. Furthermore, these results do not clearly demonstrate whether the effects of p53 deficiency are due to changes in signaling cascades that regulate Myc and Tsp-1 expression or indirect effects caused by changes in cell cycle regulation due to the loss of p53 activity. However, these results are contrary to the published report that in fibroblast cell lines derived from Li-Fraumeni patients, loss of p53 activity leads to the repression of Tsp-1 expression (Dameron et al. 1994). These contrasting results suggest that modulation of Tsp-1 expression is cell-type-specific.

The results obtained with p53-deficient epithelial cells also suggest that p53 is upstream of Myc in a signaling pathway leading to Tsp-1 repression. To test this hypothesis, I used a retroviral construct that constitutively expresses Myc to transduce the p53-deficient human mammary epithelial cells. This retroviral transduction yielded an epithelial cell line overexpressing Myc but deficient for p53. In order to demonstrate the activity of the Myc overexpression construct, I used immunoblot analysis to determine the levels of Myc expression in epithelial cells containing only the shp53 construct, and cells expressing both the shp53 and Myc overexpression constructs. As expected, p53-deficient cells containing the Myc construct expressed higher levels of Myc than did control cells (Fig. 4).

To test whether p53 and Myc are part of the same pathway that stimulates Tsp-1 expression in epithelial cells, I used immunoblot analysis to determine the level of Tsp-1 expression in p53-deficient epithelial cells overexpressing Myc. The p53-deficient epithelial cells containing the Myc construct expressed lower levels of Tsp-1 than did cells expressing only the shp53 construct or wild-type control cells (Fig. 4). The ability of Myc to inhibit the stimulation of Tsp-1 in p53-deficient cells demonstrates that not only is Myc part of the same signaling pathway as p53, but that Myc is downstream of p53. These results suggest that in epithelial cells Myc is a target of p53 induction (Fig. 5). Additionally, the observation that overexpression of Myc, even in a p53-deficient background, is able to repress Tsp-1 expression is consistent with published reports that Myc activity inhibits Tsp-1 expression (Watnick et al. 2003; Dews et al. 2006). However, it is also possible that Myc overexpression is potent enough to overcome the Tsp-1 stimulatory effects of p53-deficiency and repress Tsp-1 expression independent of p53 activity.

## IV: Ras stimulates Myc expression through the MAPK p38 in epithelial cells

A previously published report demonstrates that a Ras-mediated pathway that activates PI3K, Rho, and ROCK can repress Tsp-1 expression in human embryonic kidney and human mammary epithelial cells (Watnick et al. 2003). Activation of ROCK leads to phosphorylation of Myc, which represses Tsp-1 expression (Watnick et al. 2003). I sought to confirm these results in my experimental system and therefore decided to determine the levels of Tsp-1 and Myc expression in human

mammary epithelial cells expressing both SV40 LT and oncogenic H-rasV12 (Elenbaas et al. 2001). Immunoblot analysis revealed that expression of LT and Ras in human mammary epithelial cells leads to the repression of Tsp-1 (Fig. 6). However, the Ras-expressing epithelial cells that I tested not only show an increase in Myc phosphorylation, but also show an increase in the total amount of Myc protein (Fig. 6). This is in contrast to Watnick et al. where there were no changes in Myc protein levels when Ras was expressed (Watnick et al. 2003). This difference in Myc expression could be due to differences in culture conditions or differences in the number of cells seeded.

The study by Watnick et al. also left unanswered the question of whether ROCK directly phosphorylates Myc or if another kinase downstream of ROCK activates Myc (Watnick et al. 2003). In an effort to further elucidate this pathway, I investigated the possible downstream targets of ROCK. It has been shown that Ras-transformed cells can activate p38 through NOTCH signaling, and that Ras-induced breast tumor cell invasion is mediated through p38 (Weijzen et al. 2002; Kim et al. 2003). Additionally, it has been shown that p38 activates intra-ribosomal-mediated Myc translation during apoptosis, and a recent report has demonstrated that Myc-mediated rat aortic smooth muscle cell proliferation is mediated by p38 (Stoneley et al. 2000; Chen et al. 2006).

Based on the p38 findings mentioned above, I chose to study p38 signaling in human embryonic kidney epithelial cells expressing SV40 LT and oncogenic Ras. To test the role of p38 in these epithelial cells, I used the p38 inhibitor SB203580 (Lee et al. 1999). This inhibitor is specific for both p38 MAPK$\alpha$ and p38 MAPK$\beta$ (Lee et al.

1999). SB203580 inhibits the catalytic domain of p38 and does not inhibit p38 phosphorylation, meaning p38 can be activated but its catalytic function remains inhibited (Lee et al. 1999). To test the functionality of SB203580 treatment, I used immunoblot analysis to determine the levels of phosphorylated p38 (phospho-p38) in control cells and Ras-expressing cells treated with SB203580. As expected, cells not expressing Ras display undetectable levels of phospho-p38 and cells expressing high levels of Ras display high levels of phospho-p38 (Fig. 6). In cells expressing high levels of Ras and treated with SB203580, the levels of phospho-p38 increases, which is characteristic of active p38 bound to SB203580 (Fig. 7).

To test whether p38 influences Tsp-1 expression, I determined the levels of Tsp-1 expression using immunoblot analysis. In embryonic kidney epithelial cells, the Ras-mediated repression of Tsp-1 was abrogated by treatment with SB203580 (Fig. 7). In fact the levels of Tsp-1 expression were equivalent to those in cells not expressing Ras (Fig. 7). These results suggest that p38 acts downstream of Ras in the repression of Tsp-1. To test whether p38 is downstream of ROCK, I treated the high Ras expressing cells with the ROCK inhibitor Y27632 and determined the level of phospho-p38 expression (Uehata et al. 1997). Treatment with Y27632 inhibited the phosphorylation of p38 to the levels observed in cells not expressing Ras (Fig. 7). This result indicates that the ROCK inhibitor also inhibits activation of p38.

Taken together, the results with the p38 and ROCK inhibitors suggest that p38 is in the Ras-Rho-ROCK-Myc pathway that represses Tsp-1, and that it is upstream of Tsp-1 repression but downstream of ROCK activation (Fig. 8). Unfortunately I was not able to test levels of Myc and phospho-Myc so I could not determine whether p38

activation effected Myc activation status. However, based on the above results, I would predict that inhibition of p38 represses Myc activity. Furthermore, it may be that p38 is the kinase that phosphorylates Myc in the Ras-mediated signaling cascade that represses Tsp-1.

## V: Preliminary experiments determining the role of p63 in Tsp-1 expression

The published report by Watnick et al. suggests that the role of Ras in Tsp-1 repression is to activate Myc (Watnick et al. 2003). However, this report did not address the role of SV40 LT in the repression of Tsp-1 expression. Based on the results described above, p53 does not appear to play an independent role, since loss of p53 leads to Myc repression (Fig. 3A). I decided to test proteins that are related to p53 but are inhibited by active p53. One attractive candidate is the p53 family member p63. The p63 protein has been implicated in various human malignancies (Hibi et al. 2000; Park et al. 2000; Massion et al. 2003). Although p63 does not bind to SV40 LT, it has been shown that when p53 is inactivated MDM-2 can trans-activate p63 (Roth and Dobbelstein 1999; Kojima et al. 2001; Calabro et al. 2002). Furthermore a recent study has shown that p63 can induce expression of adhesion molecules in epithelial cells (Carroll et al. 2006). Given the fact that Tsp-1 is also an adhesion molecule (Ch.1 section III), and that expression of SV40 LT inhibits p53 activity, I decided to determine the role, if any, of p63 in Tsp-1 regulation in human mammary epithelial cells.

I first sought to determine the effects of loss of p63 activity in mammary epithelial cells. In preliminary experiments, I transiently expressed an shRNA lenti-viral construct specific for p63 (shp63b) in human mammary epithelial cells, and using immunoblot analysis determined the levels of p63 expression (Fig. 9A). The levels of p63 did not decrease in the cells transiently expressing the shp63b construct (Fig. 9A). However, the amount of protein used for these immunoblots was not equal. There is much more protein present in the cells containing shp63b than there is in the control cells. Therefore, it is hard to determine whether there was any decrease in the amount of p63 activity in cells expressing shp63b (Fig. 9A). If p63 activity influences Tsp-1 expression, then the levels of Tsp-1 expression suggest that p63 levels were repressed in the cells expressing the shp63 construct (Fig. 9A). Cells expressing the shp63 construct have lower levels of Tsp-1 protein than do the control cells, despite the fact that there is approximately two-fold less total protein present in the control cells, as determined by the GAPDH loading control (Fig. 9A). Although inconclusive, these results suggest that even slight inhibition of p63 activity may be enough to repress Tsp-1 expression.

I then tested the role of p63 in epithelial cells expressing SV40 LT (LT) in order to determine whether LT stimulates Tsp-1 expression via p63 activity. Using lentiviral constructs specifying 2 different shRNA sequences specific for p63 (shp63a and shp63b), I transduced human mammary epithelial cells expressing LT (Rubinson et al. 2003). I tested the activity of the shp63 constructs using immunoblot analysis to determine the levels of p63 expression. As in the previous experiment, cells that were infected with the shp63 constructs did not display decreased levels of p63 (Fig.

9B). In fact, levels of p63 were higher in the cells containing the shp63a construct (Fig. 9B). The lack of decreased p63 expression in this experiment cannot be explained by loading error, because the total amount of protein used was equivalent between control cells and cells expressing the shp63 constructs. However, cells expressing the shp63b construct display reduced levels of Tsp-1, which suggests that perhaps even a slight reduction in the levels of p63 is sufficient to affect Tsp-1 expression (Fig. 9B). It is also worth noting that the cells containing both LT and oncogenic Ras expressed higher levels of p63 than cells containing only LT (Fig. 9B). Consistent with results from the previous experiment, cells expressing shp63b repressed Tsp-1 expression, although the level of repression was not as low as in cells expressing Ras (Fig. 9B). If p63 is indeed being inhibited in these cells, then this result suggests that loss of p53 activity may induce p63 expression.

The results obtained with cells containing SV40 LT suggest that p63 is activated by loss of p53 activity. To test this hypothesis I transiently expressed the shp63b construct in human mammary epithelial cells deficient for p53 (via stable expression of the shp53 construct), and determined the levels of p63, p53 and Tsp-1 expression using immunoblot analysis. The shp63b construct did not reduce the levels of p63 expression in control cells expressing p53; however, it did reduce the levels of p63 in p53-deficient cells (Fig. 10). Furthermore, expression of the shp63b construct in cells deficient for p53 led to a decrease in Tsp-1 expression (Fig. 10). The level of Tsp-1 expression in p53-deficient cells expressing the shp63 construct was equivalent to the level of Tsp-1 expression in control cells, suggesting that loss of p63 activity leads to a decrease in Tsp-1 expression. These results, then, also

suggest that loss of p53 activity leads to the activation of p63. It is also worth noting that p53-deficient cells express higher levels of p63 compared to control cells, which suggests that p53 activity inhibits p63 expression (Fig. 10). Taken together, these results suggest that p53 regulates p63 expression.

These pilot studies aimed at determining the role of p63 in the regulation of Tsp-1 expression are promising but are by no means complete. Technical difficulties have hampered efforts to conclusively show that p63 expression is inhibited in cells that contain the shp63b construct. These studies also fail to address the difference in p63 expression between human mammary epithelial cells that do not express SV40 LT and those that do. I also have not demonstrated what effect loss of p63 has on Myc expression. Additionally, these pilot studies do not address the effect of ectopic expression of p63 on human mammary epithelial cells. However, these studies do reveal a potential mechanism by which Tsp-1 repression occurs in the absence of an oncogenic signal.

## VI: Summary of Results

In this chapter I have shown the results of experiments designed to determine how p53 affects Myc and Tsp-1 expression in human epithelial cells, as well as how p53 may interact with p63 to regulate Tsp-1 expression. I also performed experiments aimed at further elucidating the Ras-induced signaling pathway that leads to Tsp-1 repression in epithelial cells.

In the first two sections, I demonstrated that expression of large T antigen (LT) leads to the repression of Myc expression and the stimulation of Tsp-1 expression in

epithelial cells (Fig. 2). I then demonstrated that loss of p53 expression is sufficient to repress Myc expression and stimulate Tsp-1 expression in epithelial cells (Fig. 3). These results are consistent with results observed in epithelial cells expressing large T antigen. Furthermore, loss of p53 activity may explain how large T antigen is able to repress Myc expression and stimulate Tsp-1 expression in epithelial cells. I also demonstrated that Myc is downstream of p53 in a pathway that leads to the stimulation of Tsp-1 expression (Fig. 4).

Preliminary results also demonstrated that p53-dependent p63 expression may stimulate Tsp-1 expression in epithelial cells (Fig. 9 and Fig. 10). I also demonstrated that the p38 MAP kinase is downstream of ROCK kinase in the Ras-mediated signal transduction pathway that leads to Myc phosphorylation in epithelial cells (Fig. 8).

## VII: Materials and Methods

### Cell lines and constructs

The retroviral constructs pBabeZeo-$LT_c$, pBabeBlast-Mn, pBabeHygro-hTERT and the shRNA vector pLK0.1-shp53 were generous gifts from Prof. Robert Weinberg, Whitehead Institute, Cambridge, MA.

The immortalization of human mammary epithelial cells (HME) was described previously (Elenbaas et al. 2001). Human renal epithelial cells (HRE) (Cambrex, Walkersville, MD) were immortalized via transduction of the retroviral vector pBabeHygro-hTERT. HME and HRE cell lines expressing the SV40 large T antigen protein (LT) were generated by retroviral transduction using the pBabe vector described above. Retroviruses were produced as previously described (Elenbaas et

al. 2001). Human mammary epithelial cells (HME) expressing the lentiviral shRNA construct described above were generated via lentiviral transduction as previously described (Brummelkamp et al. 2002; Rubinson et al. 2003). The two lentiviral shRNA vectors pLK0.1-shp63a and pLK0.1-shp63b were purchased from Sigma-Aldrich and contained the following sequences: shp63a: CCG GCC GTT TCG TCA GAA CAC ACA TCT CGA GAT GTG TGT TCT GAC GAA ACG GTT TTT and shp63b: CCG GCC CAG CTC ATT TCT CTT GGA ACT CGA GTT CCA AGA GAA ATG AGC TGG GTT TTT.

Both HME and HRE cell lines were cultured in a 1:1 mixture of F-12 Nutrient Mixture and Dulbecco's Modified Eagle Medium (DMEM) (GIBCO, Carlsbad, CA) and supplemented with 5% fetal bovine serum (GIBCO) 1μg/ml hydrocortisone, 10ng/ml EGF, and 10mg/ml insulin (Sigma Chemicals, St. Louis, MO). Human embryonic kidney cells with hTERT (HA1E) were generated as described previously, and were cultured in MEMα medium containing 10% FBS (GIBCO, Carlsbad, CA) (Hahn et al. 1999; Elenbaas et al. 2001).

**Immunoblot analysis**

For immunoblot analysis, mammary epithelial cells (HME) and renal epithelial cells (HRE) were transferred from normal culture conditions mentioned above to a 1:40 dilution of the enriched media in a 1:1 F-12 and DMEM mixture and cultured for 14 hours (GIBCO). The human embryonic kidney cell lines (HA1E) were switched from MEMα medium with 10% FBS to MEMα medium containing 0.1% FBS and cultured for 14 hours (GIBCO).

Cells were lysed in 50mM Tris-HCl (pH 7.5), 150mM NaCl, 1% Sodium deoxycholate, 1% NP-40, 0.1% SDS, 2nM DTT, and Complete Mini protease inhibitor cocktail (Roche, Mannheim, Germany). Immunoblots were performed as previously described (Watnick et al, 2003) using the following antibodies: c-Myc (rabbit pAb, Cell Signaling Technologies, Danvers, MA), Tsp-1 (Ab-11 cocktail Lab Vision , Fremont, CA), p53 (rabbit pAb, Cell Signaling Technologies), p63 (), phosphor-p38 ( rabbit pAb, Cell Signaling Technologies, Danvers, MA), Ras (c-20 Santa Cruz Biotechnology) β-actin (Abcam, Cambridge, United Kingdom) and GAPDH (rabbit pAb, Trevigen, Gaithersburg, MD).

All figures are representative of immunoblots performed at least four times. In each case, all immunoblots gave similar results to those depicted as the representative blot chosen for each figure.

**p53 activity assay**

For the p53 activity assay mammary epithelial cells were grown in the same complete medium described above. 500,000 cells were plated and cultured overnight, then medium was replaced and 50μM etoposide (Calbiochem, San Diego, CA) was added to the medium. Cells were incubated with etoposide for 8 hours then were harvested and lysates were prepared for western blots (as described above)

**Treatment with chemical inhibitors**

For the p38 and ROCK inhibitor assays, cells were grown in the same complete medium described above. 500,000 cells were plated and cultured overnight. The next morning the cells were serum starved for 4 hours (using MEMα with 0.1% FBS), then 10μM SB203580 or 10μM Y27632 was added to the cells and

incubated for 8 hours. Cells were then harvested and lysates were prepared for immunoblots (as described above). Both of these inhibitors were purchased from Calbiochem, San Diego, CA.

**Statement of data contribution**

All the experiments in these studies were performed and analyzed by Roberto K. Rodriguez and Prof. Randolph S. Watnick.

**Figure 1**

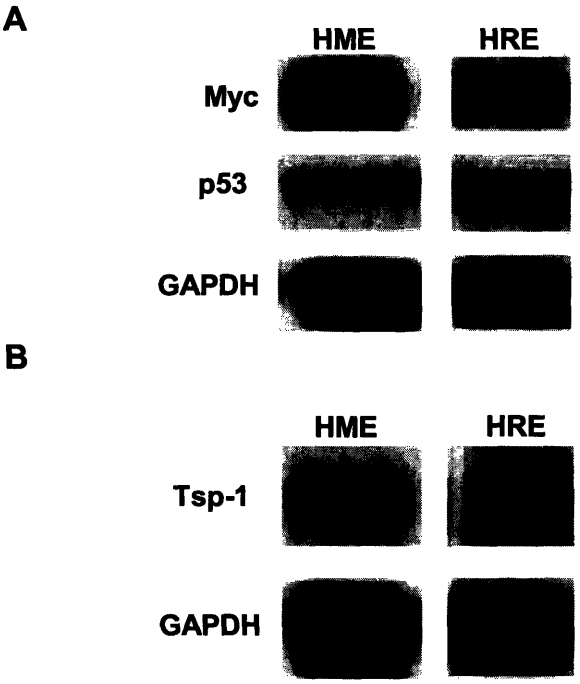



Figure 1.

(A) Immunoblot analysis of Myc, p53 and GAPDH proteins expressed by human mammary and renal epithelial cells expressing hTERT alone (HME and HRE).

(B) Immunoblot analysis of Tsp-1 and GAPDH proteins expressed by the same cells .

**Figure 2**

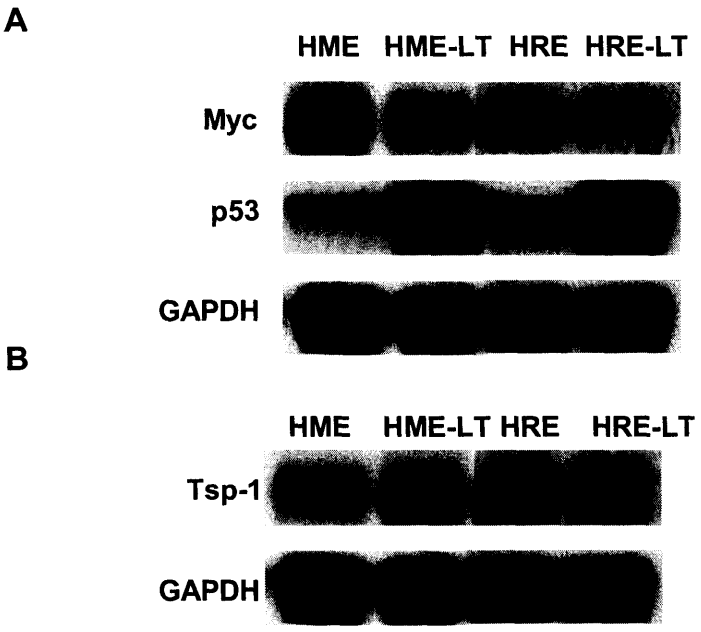



Figure 2.

(A) Immunoblot analysis of Myc, p53 and GAPDH proteins expressed by human mammary and renal epithelial cells expressing hTERT alone (HME, HRE) or with SV40 Large T (LT).

(B) Immunoblot analysis of Tsp-1 and GAPDH proteins expressed by the same cells .

Figure 3

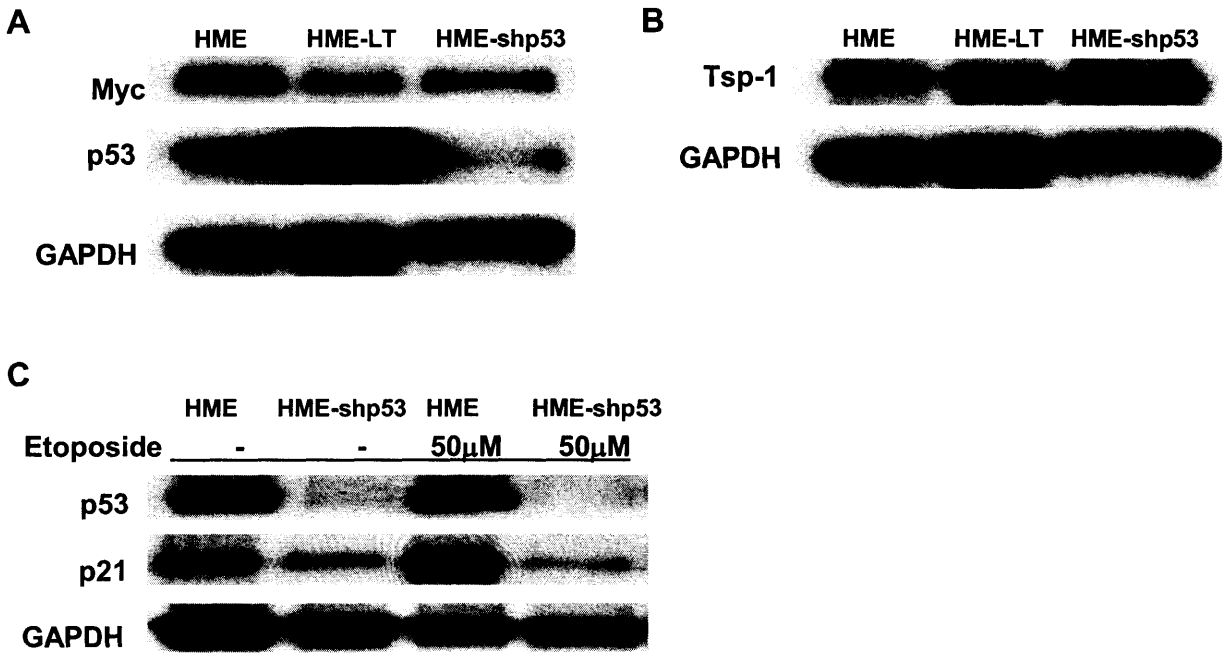



Figure 3.

(A) Immunoblot analysis of Myc, p53 and GAPDH proteins expressed by human mammary epithelial cells expressing hTERT (HME), hTERT and LT (LT), and hTERT and shp53 (shp53)

(B) Immunoblot analysis of Tsp-1 and GAPDH proteins expressed by the same cell lines used above

(C) Immunoblot analysis of p53, p21 and GAPDH proteins expressed by human mammary epithelial cells expressing hTERT (HME) and hTERT and shp53 (shp53) that were untreated (-) or treated with 50mM etoposide for 8 hours

**Figure 4**

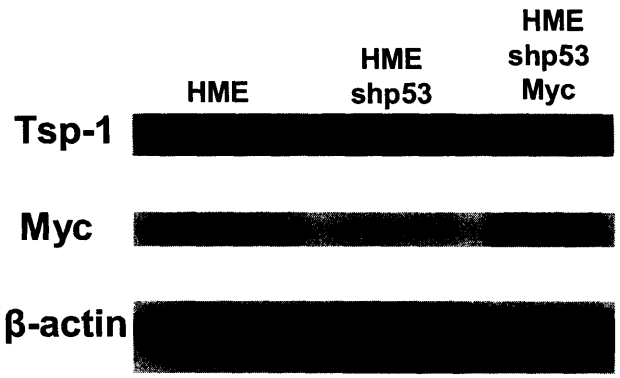



Figure 4.

Immunoblot analysis of Tsp-1, Myc and β-actin proteins expressed by human mammary epithelial cells expressing hTERT alone (HME), hTERT and shp53 (shp53) and hTERT, shp53, and pWZLNeo-Myc (Myc)

**Figure 5**

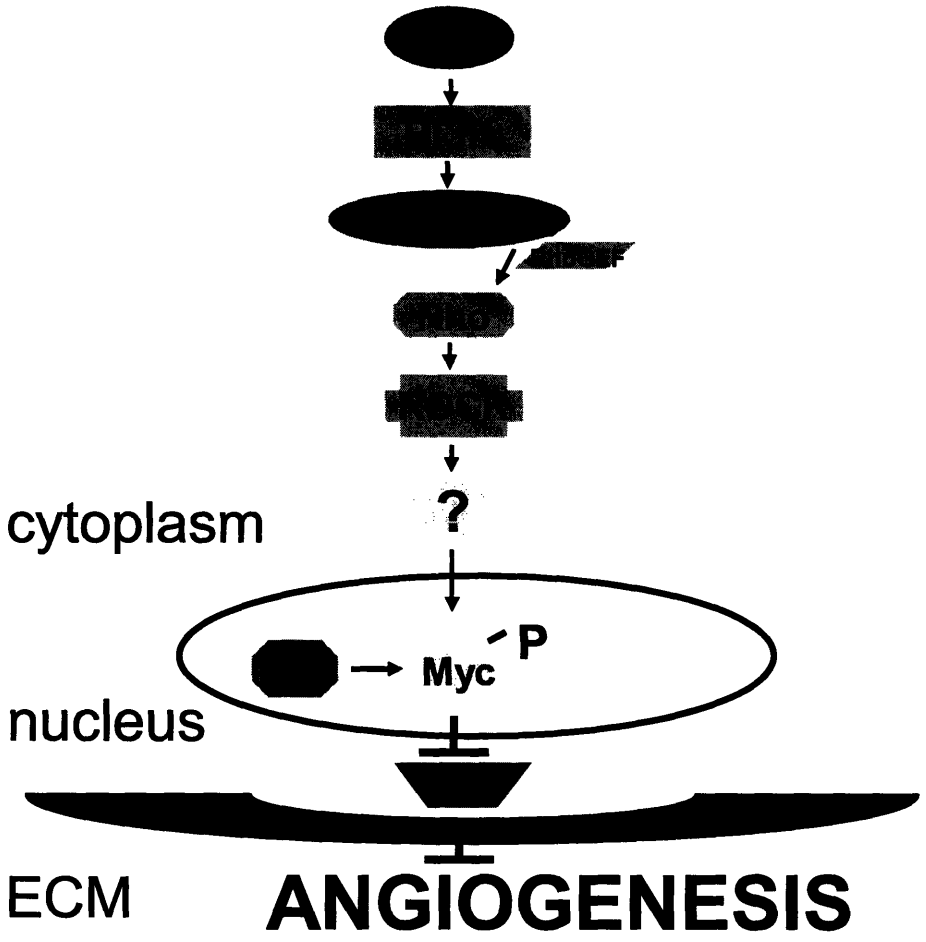



Figure 5.

Schematic diagram of the Ras-mediated signaling pathway that promotes angiogenesis in the extra cellular matrix (ECM) via repression of Tsp-1

**Figure 6**

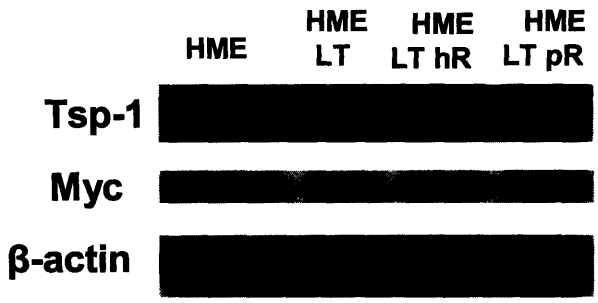



Figure 6

Immunoblot analysis of Tsp-1, Myc, and β-actin proteins expressed by human mammary epithelial cells expressing hTERT alone (HME), hTERT and SV40 LT (LT), hTERT, SV40 LT and low Ras (LT hR), and hTERT, SV40 LT, and high Ras (LT pR)

**Figure 7**

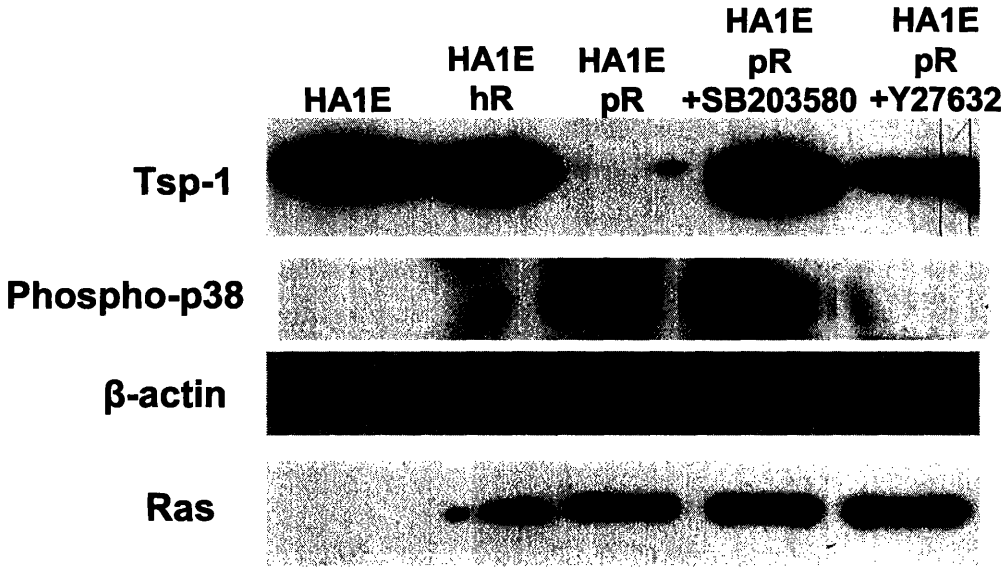



Figure 7

Immunoblot analysis of Tsp-1, phospho-p38, β-actin, and Ras proteins expressed by embryonic human kidney epithelial cells expressing hTERT alone (HA1E), hTERT and and low Ras (hR), hTERT and high Ras (pR), hTERT and high Ras with the p38 inhibitor SB203580 (pR +SB203580), and hTERT and high Ras with the ROCK inhibitor Y27632 (pR +Y27632)

**Figure 8**

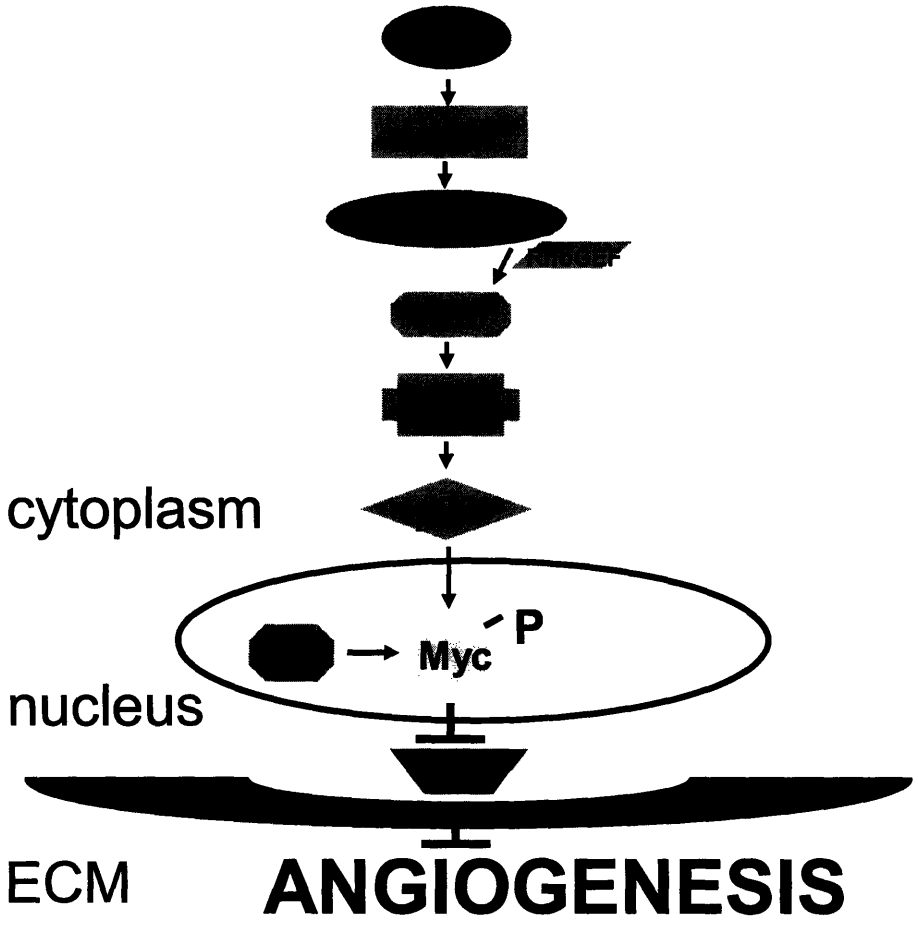



Figure 8

Schematic diagram of the Ras-mediated signaling pathway, including p38, that promotes angiogenesis in the extra cellular matrix (ECM) via repression of Tsp-1.

Figure 9

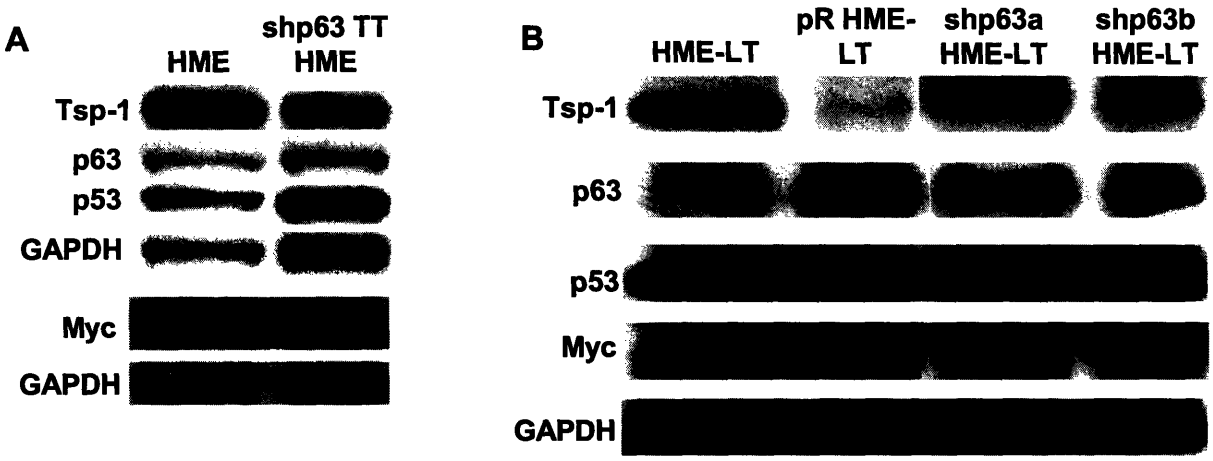



Figure 9

(A) Immunoblot analysis of Tsp-1, p63, p53, GAPDH, Myc and GAPDH proteins expressed by human mammary epithelial cells expressing hTERT alone (HME), or with hTERT and transiently expressing the shp63 construct (shp63 TT).

(B) Immunoblot analysis of Tsp-1, p63, p53, Myc, and GAPDH proteins expressed by human mammary epithelial cells expressing SV40 LT alone (LT), LT and high Ras (pR LT), LT and stably expressing the shp63a construct (shp63a), and LT and stably expressing the shp63b construct (shp63b).

**Figure 10**

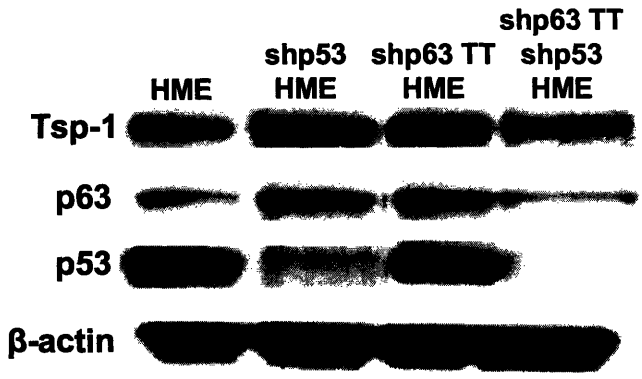



Figure 10

Immunoblot analysis of Tsp-1, p63, p53, and β-actin proteins expressed by human mammary epithelial cells expressing hTERT alone (HME), hTERT and the shp53 construct (shp53), hTERT and transiently expressing the shp63b construct (shp63 TT), and hTERT, shp53 and transiently expressing shp63b (shp63 TT shp53)

# Chapter 3. Regulation of Thrombospondin-1 Expression in Fibroblasts

Work from the Weinberg laboratory has demonstrated that ectopic expression of H-RasV12, SV40 Large T antigen, and the catalytic subunit of telomerase (hTERT) is sufficient to transform both epithelial cells and fibroblasts (mesenchymal cells) (Hahn et al. 1999; Elenbaas et al. 2001). Despite these studies, however, the precise genetic events that regulate subsequent steps in the tumor progression of mesenchymal cells (i.e. angiogenesis and metastasis) are not clearly understood. It has also been demonstrated that fibroblasts existing in the tumor-associated stroma, termed CAFs (cancer-associated fibroblasts) can have a growth promoting role in tumor progression (Olumi et al. 1999). Furthermore, it has recently been demonstrated that the tumor-promoting property of CAFs can be partially mediated by the cells' ability to promote angiogenesis (Orimo et al. 2005). However, little is known about the regulation of endogenous angiogenesis inhibitors, such as Tsp-1, in the tumor-associated stroma (Kalas et al. 2005). It is also unclear how disruption of p53 activity in fibroblasts compares to loss of p53 activity in epithelial cells in the regulation of molecular events that promote tumorigenesis. Additionally, the signal transduction pathways activated in CAFs by tumor-secreted proteins that regulate invasion and angiogenesis have yet to be elucidated.

Given the previous results describing p53 signaling in epithelial cells (Ch. 2), I decided to investigate the role of p53 in regulating Myc and Tsp-1 expression in fibroblasts. In much the same manner as previously described, I have made use of

both the SV40 large T antigen (LT) and a short hairpin RNA directed against p53. Based on previously published reports (Dameron et al. 1994; Dews et al. 2006), it seems likely that loss of p53 should stimulate Myc and repress Tsp-1 expression in fibroblasts. However, the results obtained with epithelial cells cast doubt on these assumptions. Thus, disrupting p53 in fibroblasts should yield novel insight into the molecular events induced by epithelial tumors in tumor-associated fibroblasts during tumor invasion, angiogenesis and metastasis.

The results from the studies described in this chapter were obtained using immunoblot analysis of cells cultured under serum starved conditions. Each result described is representative of at least four immunoblots performed for each experiment. Results from all immunoblots were similar to those depicted in the figures of this section.

## I: Expression signature in fibroblasts

Having established in epithelial cells the interaction of several proteins commonly altered in cancer, I decided to determine the basal level of expression of the same cancer-related proteins in fibroblasts. I examined the expression levels of p53, Myc and Tsp-1 in human fibroblasts derived from dermal and lung tissue. As determined by immunoblot analysis, human dermal fibroblasts and lung fibroblasts express detectable levels of Myc and Tsp-1 (Fig. 11). However, unlike epithelial cells, these fibroblasts express very low levels of p53 under non-stress conditions (Fig. 11A). It is also worth noting that fibroblasts express levels of Myc that are lower than the basal levels of Myc expressed in epithelial cells (Fig. 1A and 11A). These

observations are striking because these fibroblasts express comparatively high levels of Myc and low levels of p53, yet the levels of Tsp-1 are also high (Fig. 11A and 11B). This is in contrast to epithelial cells, which express comparatively high levels of Tsp-1 accompanied by high levels of Myc and p53 expression (Fig 1).

Given the high levels of Tsp-1 expression in fibroblasts, these results suggest that Myc is not sufficient to repress Tsp-1 in these fibroblasts. Alternatively, it may be that there is a threshold level of Myc required for Tsp-1 repression, or that there is a cofactor that is not expressed under these conditions. Additionally it may be that Myc phosphorylation plays a role in Tsp-1 repression. Under conditions that induce the repression of Tsp-1 in fibroblasts, it may be that an increase in the level of phosphorylated Myc is enough to repress Tsp-1 expression. It is also a formal possibility that Myc plays no role in regulating Tsp-1 expression under the culture conditions used or in the fibroblast cell lines used. However, it may be that a protein other than p53 is positively regulating Tsp-1 expression to counter-balance the inhibitory effects of Myc (Salnikow et al. 1994), or that the cells utilize a Myc-independent and p53-independent method of Tsp-1 expression.

## II: Effects of SV40 large T antigen on fibroblasts

Fibroblasts associated with the tumor stroma, and fibroblasts undergoing cellular transformation often display an angiogenic phenotype (Ch.1 section II). Therefore, I wanted to determine how the basal levels of Tsp-1, Myc and p53 change under conditions of cellular transformation in fibroblasts. These conditions should provide insight into how p53, Myc and Tsp-1 expression patterns are modulated in

fibroblasts associated with the tumor stroma. To test fibroblasts under these conditions, I used SV40 large T antigen (LT) as the first step in a model for cellular transformation (Hahn et al. 1999). I transduced human dermal and lung fibroblasts with a retroviral construct specifying the cDNA for the LT gene ($LT_C$). $LT_C$ differs from the genomic version in that it only encodes the SV40 large T antigen and not the small T antigen (Chen et al. 2004). The lack of small T antigen expression therefore simplifies interpretation of results with $LT_c$ expression. Thus, the primary effect of $LT_c$ expression is loss of p53 and pRb activity (Ch.1 section VII). Confirming $LT_C$ activity, both human dermal and lung fibroblasts that express $LT_C$ display elevated levels of p53 compared to control cells (Ch.2 sec. II and Fig. 12A). It should also be noted that expression of $LT_c$ in fibroblasts conferred a phenotype similar to previously described cell lines expressing $LT_c$ (Shein and Enders 1962). This $LT_c$ phenotype included an increase in the rate of cell growth and a decrease in the dependence on growth factors for cell growth (data not shown).

I then determined the levels of Myc and Tsp-1 expression in the $LT_C$-expressing fibroblasts using immunoblot analysis. Human fibroblasts expressing $LT_C$, display elevated levels of Myc and repressed levels of Tsp-1 (Fig. 12A and 12B). This is consistent with the hypothesis that Myc stimulation represses Tsp-1 activity (Watnick et al. 2003). However, this result is striking because the activity of $LT_C$ alone is sufficient to repress Tsp-1 expression (Fig. 12B). As was reported by Watnick et al., epithelial cells require the expression of high levels of oncogenic Ras in addition to SV40 LT in order to repress Tsp-1 expression. The above results

suggest that fibroblasts require fewer genetic modifications to modulate Tsp-1 expression than do epithelial cells.

The results with fibroblasts expressing $LT_c$ also demonstrate that these cells express higher levels of Myc compared to control cells (Fig. 12A). This is contrary to the case in epithelial cells containing LT, which express lower levels of Myc compared to control cells (Ch.2 section II and Fig. 2A). Considering the fact that p53 activity is inhibited in cells containing $LT_C$, these results are consistent with published reports indicating that p53 is a trans-repressor of Myc. (Moberg et al. 1992; Levy et al. 1993; Ragimov et al. 1993; Ho et al. 2005). However, these results do not rule out the possibility that a p53-independent activity of $LT_c$ is responsible for the stimulation of Myc activity. It may be that loss of pRb activity stimulated Myc expression in these $LT_c$-expressing fibroblasts. It is also worth noting that these results suggest that under non-stressed conditions, fibroblasts do not express sufficiently high levels of Myc to repress Tsp-1 expression.

In epithelial cells, p38 is part of the Ras-Rho-ROCK pathway that leads to Myc activation and Tsp-1 repression (Fig. 8). In order to determine whether this Ras-mediated pathway has any role in the repression of Tsp-1 in fibroblasts, I treated human dermal fibroblasts expressing $LT_C$ or both $LT_C$ and H-RasV12 with the p38 inhibitor SB203580 (Lee et al. 1999). I then determined the levels of Myc and Tsp-1 expression in the SB203580 treated cells using immunoblot analysis (Fig. 13). Treatment of fibroblasts expressing hTERT, $LT_c$ and Ras with SB203580 resulted in the repression of both Myc and Tsp-1 expression (Fig. 13). Furthermore, treatment of fibroblasts expressing only hTERT with SB203580 resulted in the repression of Tsp-1

but the stimulation of Myc (Fig. 13). Finally, treatment of fibroblasts expressing $LT_c$ with SB203580 resulted in the repression of Tsp-1, but had no effect on Myc levels (Fig. 13). These results suggest that under certain conditions p38 does not affect Myc expression in fibroblasts, and imply that even the pathway(s) downstream of Ras involved in Tsp-1 repression differs between fibroblasts and epithelial cells.

## III: The effects of p53 deficiency on Myc and Tsp-1 expression in fibroblasts

The results obtained with fibroblasts containing $LT_C$ suggest that loss of p53 activity leads to the stimulation of Myc expression (Fig 12). To verify that loss of p53 activity is sufficient to stimulate Myc expression in fibroblasts, I transduced human dermal fibroblasts with a lentiviral construct specifying a previously published sequence for an shRNA against p53 (shp53) (Brummelkamp et al. 2002). In order to demonstrate that the shp53 construct was functional in these fibroblasts, I used a DNA damaging agent (etoposide) to stimulate p53 activity and determined p53 and p21 levels via immunoblot analysis (Nair et al. 2005). As expected, control fibroblasts express high levels of both p53 and p21 in response to etoposide (Fig. 14C) (el-Deiry et al. 1994). In fibroblasts containing the shp53 construct, however, there is no detectable p53 expression and no induction of p21 following treatment with etoposide (Fig. 14C).

Immunoblot analysis revealed that silencing p53 in fibroblasts is sufficient to stimulate Myc expression (Fig.14A). This observation is consistent with the results observed in cells expressing $LT_C$. However, unlike fibroblasts containing $LT_C$, p53-

deficient fibroblasts do not repress Tsp-1 expression (Fig. 14B). This is contrary to the published report demonstrating that ectopic expression of Myc in fibroblasts is sufficient to repress Tsp-1 expression (Tikhonenko et al. 1996). My results suggest that the stimulation of Myc resulting from the loss of p53 expression is not sufficient to repress Tsp-1 expression in fibroblasts (Fig. 14A and 14B). One possible explanation for the lack of Tsp-1 repression by Myc could be that loss of p53 does not stimulate sufficient levels of Myc to repress Tsp-1 expression. It could also be due to differences in fibroblast cell lines used or culture conditions. It is worth noting that these results do not demonstrate how loss of p53 activity leads to the stimulation of Myc expression in fibroblasts. The stimulation of Myc could be due the derepression of p53 in a signaling pathway, or it could be due to changes in cell cycle regulation caused by the loss of p53 activity.

The results of Myc stimulation by loss of p53 activity are consistent with the results obtained in fibroblasts containing $LT_C$. However, in contrast to the effects of $LT_C$, Tsp-1 expression did not vary significantly between control fibroblasts and p53-deficient fibroblasts (Fig. 14B). While these results run contrary to published reports that loss of p53 expression in human diploid fibroblasts is sufficient to repress Tsp-1 activity, it is worth noting that the previous results were obtained using a fibroblast cell line derived from Li Fraumeni patients (Dameron et al. 1994). It is possible that when these cells became nullizygous for p53 there was a collaborating event that resulted in the repression of Tsp-1 expression. In this work, these confounding variables are avoided by specifically silencing p53 expression via an shRNA construct in normal human diploid fibroblasts. Therefore, these results suggest that loss of p53 may be

necessary, but not sufficient to repress Tsp-1 expression in human fibroblasts. Furthermore, it seems that induction of an angiogenic phenotype in human fibroblasts requires fewer genetic, or epigenetic, events than in epithelial cells.

## IV: The effects of SV40 large T antigen mutants on p53, Myc and Tsp-1 expression in fibroblasts

Loss of p53 expression in fibroblasts leads to the stimulation of Myc expression, but does not lead to Tsp-1 repression (Fig 14). This suggests that there is a collaborating event induced by SV40 $LT_c$ that is not present in p53-deficient fibroblasts. Alternatively, expression of SV40 $LT_C$ in fibroblasts could cause the repression of Tsp-1 expression through a p53-Myc-independent mechanism. It has been noted, however, that the large T antigen of SV40 interacts with other proteins beside p53 (Ch.1 section VII). Therefore, I wanted to determine which other target(s) of $LT_C$ is responsible for the repression of Tsp-1 expression. Accordingly, I transduced human dermal fibroblasts with retroviral constructs specifying mutant forms of $LT_C$ and determined their effects on Myc, p53, and Tsp-1 expression using immunoblot analysis.

As expected, expression of the LT-K1 mutant, which inhibits the p53 pathway but lacks a functional Rb binding motif, stimulates Myc expression to similar levels as wild-type $LT_C$ (Fig. 15A and Fig. 15B). However, unlike wild-type $LT_C$, the LT-K1 mutant fails to repress the expression of Tsp-1 (Fig. 15B). This result is consistent with the results from p53-deficient fibroblasts (Fig. 14B). I then examined other variants of $LT_C$ which contain mutations in different regions of the protein (Fig. 15A).

The J domain binding-region mutant LT-H42Q and the Cul7 binding-domain mutant LT-Δ69-83 both possess normal p53 and pRb inhibiting activity and have elevated levels of Myc (Fig. 15B). Consistent with wild-type $LT_C$, these mutants are also able to repress Tsp-1 expression (Fig. 15B). However, in $LT_{350}$ and LT-D402H, two LT mutants that are able to inhibit pRb but not p53 activity, Tsp-1 expression is not repressed (Fig. 15B). Also, Myc is not stimulated in the LT-D402H mutant, although it is stimulated in the $LT_{350}$ mutant (Fig. 15B). The $LT_{350}$ mutant is a deletion mutation that only contains the first 350 amino acids of $LT_C$, and this region contains the pRb binding domain but not the p53 binding domains (Fig. 15A). It could be that within the 350 amino acids is another functional region that aids in the stimulation of Myc expression, which would explain the Myc results observed with the $LT_{350}$ mutant. However, neither of these pRb activity inhibitors represses Tsp-1 expression. Taken together, these results suggest that loss of p53 activity is sufficient to stimulate Myc expression in fibroblasts, but that repression of Tsp-1 expression requires the additional loss of pRb activity. However, these experiments do not rule out the possibility that expression of these $LT_c$ mutants cause unpredictable phenotypic changes within the fibroblast that result in alterations of Myc and Tsp-1 expression that are independent of the signaling pathways under investigation.

## V: The combined loss of p53 and pRb activity represses Tsp-1 expression in fibroblasts

The results obtained from using $LT_C$ mutants suggest that loss of both p53 and pRb is required to repress Tsp-1 expression in fibroblasts. To test this hypothesis, I

performed complementation studies to demonstrate whether the combined loss of p53 and pRb activity leads to the repression of Tsp-1 expression. I transduced fibroblasts containing either $LT_{350}$ or LT-D402H, $LT_c$ mutants which inhibit pRb but not p53 activity, with the shp53 lentiviral construct described above. Consistent with the results from above, Tsp-1 expression was repressed in fibroblasts where both pRb and p53 activities are inhibited (Fig. 16). For example, Tsp-1 was repressed in p53-deficient fibroblasts expressing LT-D402H, an $LT_C$ mutant containing a point mutation in the p53 binding domain, whereas expression of shp53 or LT-D402H alone was not sufficient to repress Tsp-1 expression (Fig. 16). Similarly, Tsp-1 was repressed in p53-deficient fibroblasts expressing $LT_{350}$, (Fig. 16). These results further suggest that Tsp-1 repression in human fibroblasts requires the inactivation of both the p53 and the pRb pathways. Since these experiments do not directly test the loss of pRb activity, it can be argued that there is a different $LT_c$ activity that combines with the loss of p53 activity to repress Tsp-1 expression. However, it should be noted that these $LT_c$ mutants have lost pRb activity through changes in different regions of the $LT_c$ protein, thereby strengthening that argument that it is the loss of pRb activity that synergizes with the loss of p53 activity to repress Tsp-1 expression in fibroblast.

## VI: The role of E2F1 in the modulation of Tsp-1 expression

In order to determine the role of pRb in the regulation of Tsp-1 expression, I examined the effects of loss of E2F1, a direct target of pRB inhibition, in human dermal fibroblasts (Ch.1 section VI). E2F1 has established roles in cell cycle regulation as well as apoptosis (Dimmova and Dyson, 2002). Additionally, a recently

published report has demonstrated that E2F1 inhibits expression of the angiogenesis stimulator VEGF (Qin et al. 2006). However, no link has been demonstrated between E2F1 and the angiogenesis inhibitor Tsp-1. Therefore, I wanted to determine whether modulation of E2F1 expression affects the levels of Tsp-1 expression.

To test the effects of E2F1 deficiency in human dermal fibroblasts, I transduced the fibroblasts with lentiviral constructs specifying 2 different shRNA sequences against E2F1 (shE2F1) (Rubinson et al. 2003). Using immunoblot analysis, I determined that cells transduced with the shE2F1 constructs express lower levels of E2F1 than do control cells (Fig. 17A). Loss of E2F1 expression in fibroblasts results in the stimulation of Tsp-1 expression (Fig. 17A). This result suggests that E2F1 can affect Tsp-1 expression.

Having determined that loss of E2F1 expression stimulates Tsp-1 expression in fibroblasts, I decided to test whether ectopic expression of E2F1 can repress Tsp-1 expression. I transiently expressed E2F1 in normal human dermal fibroblasts and in p53-deficient human dermal fibroblasts (Helin et al. 1993). Immunoblot analysis was used to verify that cells containing the E2F1 expression vector displayed elevated levels of E2F1 compared to control cells (Fig 17B). Overexpression of E2F1 resulted in the modest repression of Tsp-1 in normal dermal fibroblasts, though not to the same level induced by $LT_c$ (Figure 17B). These results indicate that ectopic expression of E2F1can lead to a partial repression of Tsp-1 expression in fibroblasts, and implies that regulation of E2F1 expression is the mechanism by which pRb modulates Tsp-1 expression in fibroblasts.

Given that ectopic expression of E2F1 can partially repress Tsp-1 expression in fibroblasts, I hypothesized that perhaps E2F1 expression in combination with loss of p53 activity can repress Tsp-1 expression to the same extent as $LT_c$. To test this hypothesis, I ectopically expressed E2F1 in p53-deficient dermal fibroblasts (Fig. 17C). Cells ectopically expressing E2F1 displayed higher levels of E2F1 than control cells (Fig. 17C). Compared to cells only deficient for p53, fibroblasts expressing E2F1 and deficient for p53 show a marked reduction in Tsp-1 expression (Fig. 17C). Furthermore, the repression of Tsp-1 in p53-deficient fibroblasts expressing E2F1 is comparable to that induced by $LT_C$ (Fig. 17C). It is also worth noting that fibroblasts containing $LT_C$ express higher levels of E2F1 than control cells, which is consistent with the idea that $LT_C$ represses Tsp-1 by inhibiting p53 activity and stimulating E2F1 expression. In the context of Tsp-1 repression, these results demonstrate a novel synergy between p53, Myc and E2F1 in the regulation of angiogenesis (Fig. 18). These results, however, do not suggest a mechanism by which pRb and E2F1 modulate Tsp-1 expression in fibroblasts. It may be that repression of pRb and/or overexpression of E2F1 induce changes in the cell cycle regulatory machinery of fibroblasts that lead to an as yet unexplained alteration of Tsp-1 expression. Alternatively, it may be through its effects on the apoptotic machinery that E2F1 induces the changes in Tsp-1 expression. Whatever the mechanism of action, these results argue that E2F1 collaborates with p53 and Myc to repress Tsp-1 expression in fibroblasts (Fig. 18).

The above results demonstrate how E2F1 can inhibit the expression of an angiogenesis inhibitor, and there is evidence that E2F1 can also inhibit pro-

angiogenic factors. A recently published report demonstrates that E2F1 inhibits the transcriptional expression of VEGF (Qin et al. 2006). Furthermore, this study demonstrates that E2F1 induces p53, and that it is p53 that inhibits VEGF transcription (Qin et al. 2006). It is clear that E2F1-mediated induction of p53 has no role in the repression of Tsp-1 in fibroblasts, but it is interesting that E2F1 plays a role in repressing both angiogenic stimulators and repressors.

## VII: Preliminary observations implicating topoisomerase inhibitors in the regulation of Tsp-1 expression

In the process of testing the functionality of the shp53 constructs in both epithelial cells and fibroblasts, I observed that etoposide treatment repressed Tsp-1 expression. To verify this observation I treated normal human dermal fibroblasts, human dermal fibroblasts containing $LT_c$, and p53-deficient human dermal fibroblasts with 50μM etoposide and use immunoblot analysis to measure the level of Tsp-1 expression (Fig. 19). I also measured levels of p53 and p21 expression to verify that etoposide was having an effect on the fibroblasts. In both normal and $LT_c$-containing fibroblasts treatment with etoposide resulted in the repression of Tsp-1 expression (Fig. 19A). Cells treated with etoposide also expressed higher levels of p53 and p21, which is the normal cellular response to a DNA damaging agent (Nair et al. 2005) (Fig. 19A). In p53-deficient fibroblasts, however, treatment with etoposide did not result in the repression of Tsp-1 expression (Fig. 19B). This result is striking because it suggests that etoposide-mediated repression of Tsp-1 involves p53 activity. Furthermore, p53 activity is inhibited in $LT_c$-containing cells even though etoposide

treatment represses Tsp-1 expression. These results also suggest that a downstream effector of p53 is modulated by SV40 $LT_c$ and allows etoposide to repress Tsp-1 expression in the absence of p53 activity.

To verify the etoposide results in epithelial cells, I treated normal and p53-deficient human mammary epithelial cells with 50μM etoposide, and using immunoblot analysis determined the levels of p53, p21 and Tsp-1 expression. Similar to results obtained with fibroblasts, normal epithelial cells display an increase in the levels of p53 and p21 expression upon treatment with etoposide (Fig. 19C). Epithelial cells treated with etoposide also display a decrease in the level of Tsp-1 expression (Fig. 19C). Additionally, p53-deficient epithelial cells do not display a decrease in the level of Tsp-1 expression upon treatment with etoposide (Fig. 19C). As was the case in fibroblasts, it appears that etoposide-mediated repression of Tsp-1 in epithelial cells also requires p53 activity.

To determine whether the effect of etoposide on Tsp-1 expression is dose-dependent, I treated $LT_c$-expressing and normal human dermal fibroblasts with 10μM, 50μM, 100μM, or 200μM etoposide and measured the levels of Tsp-1 expression using immunoblot analysis (Fig. 20A and 20B). In normal fibroblasts the decrease in the levels of Tsp-1 correlates with increased etoposide concentration up to 50μM, above which Tsp-1 levels increase slightly (Fig. 20A). At concentrations between 100μM and 200μM etoposide, the levels of Tsp-1 expression are steady but still lower than in cells not treated with etoposide (Fig. 20A). This suggests that the cellular response to etoposide becomes saturated and no further repression of Tsp-1 is possible. A similar effect is seen in fibroblasts expressing $LT_c$. However, at a

concentration of 10 μM, etoposide does not appear to have much of an effect on Tsp-1 levels in $LT_c$-expressing cells (Fig. 20B). Furthermore, the saturation point of etoposide activity is reached at a higher concentration in fibroblasts expression $LT_c$, since the level of Tsp-1 expression increases between 100μM and 200μM etoposide (Fig. 20B). Taken together, these results suggest that etoposide, in a dose-dependent manner, represses Tsp-1 by inducing p53 activity. Furthermore, etoposide seems to induce the activity of another SV40 LT target to repress Tsp-1 in $LT_c$-expressing cells.

Etoposide is a topoisomerase II inhibitor that stabilizes the DNA-topo II complex causing permanent DNA breaks in chromosomes, which eventually lead to cell death (HANDE 1998). Topoisomerases are essential enzymes that make transient breaks in DNA strands in order to manipulate the geometry of DNA (Chen and Liu 1994). Topoisomerases are needed during DNA replication, recombination, chromosomal segregation, and transcription (Chen and Liu 1994). There are two primary classes of topoisomerases, topoisomerase I and topoisomerase II, which either make single-strand cuts or double-strand breaks in DNA (Chen and Liu 1994).

Knowing that one topoisomerase inhibitor, etoposide, is able to repress the expression of Tsp-1, I tested the effects of other topoisomerase inhibitors on Tsp-1 expression. I treated human dermal fibroblasts with a series of topoisomerase inhibitors in a preliminary study to test their effects on Tsp-1 expression (Fig. 21).

Daunorubicin, a topo-poison that effects DNA relegation, inhibits both topoisomerase I and II (Denny and Baguley 2003). Fibroblasts treated with daunorubicin stimulated the expression of not only p53, but Tsp-1 as well (Fig. 21A).

Merbarone, which inhibits the catalytic activity of topoisomerase I, also stimulated the expression of Tsp-1, though it did not induce p53 expression in fibroblasts (Brewer et al. 1985; Khelifa and Beck 1999) (Fig. 21A).

Ellipticine, a topoisomerase II inhibitor that intercalates DNA and stimulates DNA breakage, represses Tsp-1 and modestly increases in the levels of p53 (Pommier et al. 1985; Froelich-Ammon et al. 1995) (Fig. 21A). β-Lapachone, an inhibitor of topoisomerase I activity, also represses Tsp-1 with only a modest increase in the levels of p53 expression (Li et al. 1993; Li et al. 1995) (Fig. 21A).

Unlike other topoisomerase inhibitors, doxorubicin, a topoisomerase II inhibitor that intercalates DNA and affects DNA relegation, stimulates the expression of p53 but does not repress Tsp-1 expression (Triton and Yee 1982; A'Hern and Gore 1995) (Fig. 21B). In fact, doxorubicin stimulates Tsp-1 expression (Fig. 21B).

Taken together these varied results demonstrate that topoisomerase inhibitors that lead to DNA damage repress Tsp-1, while those that intercalate DNA or inhibit the catalytic activity of topoisomerases do not repress Tsp-1. This suggests that signaling pathways induced by the DNA damage response lead to the repression of Tsp-1. Furthermore, the observation that Tsp-1 can be repressed without the induction of p53 suggests that both p53-dependent and p53-independent response pathways can repress Tsp-1 expression.

## VIII: Summary of Results

In this chapter I have shown the results of experiments designed to determine how p53 and pRb affect Tsp-1 expression in human fibroblasts. I also performed

experiments to elucidate how topoisomerase inhibitors may regulate Tsp-1 expression.

In the first two sections I demonstrated that fibroblasts express low basal levels of p53 compared to epithelial cells and that expression of $LT_c$, in fibroblasts is sufficient to repress Tsp-1 expression. These results were in contrast to results in epithelial cells where repression of Tsp-1 required the expression of both LT and oncogenic Ras (Watnick et al. 2003).

I then demonstrated how expression of $LT_c$ leads to the repression of Tsp-1 in fibroblasts. In fibroblasts, loss of p53 stimulated Myc expression (Fig. 14A). This is in contrast to epithelial cells, where loss of p53 activity results in the repression of Myc expression (Fig. 3A). I also demonstrated that, unlike $LT_c$, loss of p53 does not repress Tsp-1 expression in fibroblasts (Fig. 14B). Using $LT_c$ mutants, I determined what other $LT_c$ activity contributes to the repression of Tsp-1 expression in fibroblasts (Fig. 15). These results suggested that loss of pRb activity synergizes with loss of p53 activity to repress Tsp-1 expression (Fig.16). To further elucidate the mechanism by which loss of pRb activity leads to the repression of Tsp-1 expression, I repressed and ectopically expressed E2F1 expression in fibroblasts (Fig. 17). These results demonstrated that expression of E2F1 combined with the loss of p53 expression induces the repression of Tsp-1 expression in fibroblasts. Furthermore, the repressed levels of Tsp-1 expression are similar to the levels seen in fibroblasts expressing $LT_c$ (Fig. 17C)

Finally, in preliminary experiments, I demonstrated that topoisomerase inhibitors that cause DNA damage can repress Tsp-1 expression, and topoisomerase inhibitors that do not directly damage DNA do not repress Tsp-1 expression (Fig. 21).

## IX: Materials and Methods

### Cell lines and constructs

The retroviral constructs pBabeZeo-$LT_c$ and pBabePuro-LTK1 and the lentiviral shRNA vector pLK0.1-shp53 were a generous gift from Prof. Robert Weinberg, Whitehead Institute, Cambridge, MA. The retroviral SV40 large T antigen mutant constructs, pBabePuro-LT-Δ69-83, pBabeNeo-$LT_{350}$, pBabeNeo-LT-D402H, and pBabeNeo-LT-H42Q were a generous gift from Dr. James DeCaprio, Dana Farber Cancer Institute, Boston, MA. The two lentiviral shRNA vectors pLK0.1-E2F1a and pLK0.1-E2F1b were purchased from Sigma-Aldrich and contained the following sequences: shE2F1a: CCG GCA GGA TGG ATA TGA GAT GGG ACT CGA GTC CCA TCT CAT ATC CAT CCT GTT TTT G and shE2F1b: CCG GCG CTA TGA GAC CTC ACT GAA TCT CGA GAT TCA GTG AGG TCT CAT AGC GTT TTT G. The pCMV and pCMV-E2F1 vectors were a generous gift from Prof. Jacqueline Lees, Center for Cancer Research, Cambridge, MA.

Human dermal fibroblasts (hDF) and lung fibroblasts (MRC5) were obtained from ATCC and immortalized via retroviral transduction with pBabeHygro-hTERT (a generous gift from Prof. Robert Weinberg, Whitehead Institute, Cambridge, MA) to yield hFhT and MRC5-hT. The hFhT and MRC5-hT cell lines expressing the wild-type SV40 large T antigen protein and the SV40 LT mutants were generated by

retroviral transduction with the pBabe vectors described above. Retroviruses were produced as previously described (Elenbaas et al. 2001). Human dermal fibroblasts expressing pCMV and pCMV-E2F1 were produced through transient transfections as previously described (Helin et al. 1993). Human dermal fibroblasts (hFhT) expressing the lentiviral shRNA constructs described above were generated via lentiviral transduction as previously described (Brummelkamp et al. 2002; Rubinson et al. 2003).

All hFhT cell lines were cultured in DMEM (GIBCO) supplemented with 10% FBS. MRC5 cell lines were cultured in Minimum Essential Medium-α (GIBCO) supplemented with 10% FBS.

**Immunoblot analysis**

For immunoblot analysis lung fibroblast (MRC5) cell lines were switched to MEMα medium containing 0.1% FBS and cultured for 14 hours, and all dermal fibroblast cell lines were switched to DMEM medium containing 0.1% FBS and cultured for 14 hours.

Cells were lysed in 50mM Tris-HCl (pH 7.5), 150mM NaCl, 1% Sodium deoxycholate, 1% NP-40, 0.1% SDS, 2nM DTT, and Complete Mini protease inhibitor cocktail (Roche, Mannheim, Germany). Immunoblots were performed as previously described (Watnick et al, 2003) using the following antibodies: c-Myc (rabbit pAb, Cell Signaling Technologies, Danvers, MA), Tsp-1 (Ab-11 cocktail Lab Vision, Fremont, CA), p53 (rabbit pAb, Cell Signaling Technologies), p63 (clone 4A4, Abcam Cambridge, MA), E2F1 (C-20, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), β-

actin (Abcam, Cambridge, United Kingdom) and GAPDH (rabbit pAb, Trevigen, Gaithersburg, MD).

All figures are representative of western blots performed at least four times. In each case, all immunoblots gave similar results and a representative blot was chosen for each figure.

**p53 activity assay**

For the p53 activity assay, human dermal fibroblasts were grown in DMEM with 10% FBS. 500,000 cells were plated and cultured overnight, then medium was replaced and 10μM, 50μM, 100μM, or 200μM etoposide (Calbiochem, San Diego, CA) was added to fresh medium. Cells were incubated with etoposide for 8 hours and then harvested. Lysates were prepared from the harvested cells for immunoblot analysis (as described above).

**Treatment with chemical inhibitors**

For the chemical inhibitor assays, cells were grown in DMEM with 10% FBS. For the p38 inhibitor, 500,000 cells were plated and cultured overnight, the next morning cells were serum starved for 4 hours (using DMEM with 0.1% FBS), then 25μM SB203580 was added to the cells and incubated for 8 hours. For the topoisomerase inhibitors, 300μM 5-fluorouracil (5-FU), 50μM β-lapachone, 25μM daunorubicin, 10μM doxorubicin, 50μM ellipticine, 50μM merbarone, or 55μM methotrexate were added to the culturing medium of the cells and incubated for 8 hours. Cells were then harvested and lysates prepared for immunoblot analysis (as described above). All chemical inhibitors were purchased from Calbiochem, San Diego, CA.

**Statement of data contribution**

The experiments in these studies were performed and analyzed by Roberto K. Rodriguez and Prof. Randolph S. Watnick, with technical assistance from Adam Johnston.

**Figure 11**

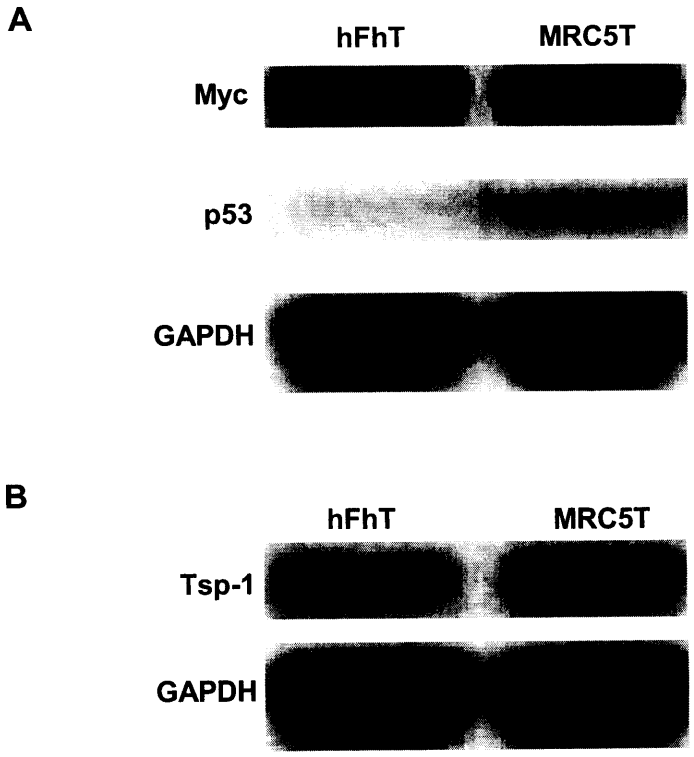



Figure 11.

(A) Immunoblot analysis of Myc, p53 and GAPDH proteins expressed by human dermal and lung fibroblasts expressing hTERT

(B) Immunoblot analysis of Tsp-1 and GAPDH proteins expressed by the same cells.

**Figure 12**

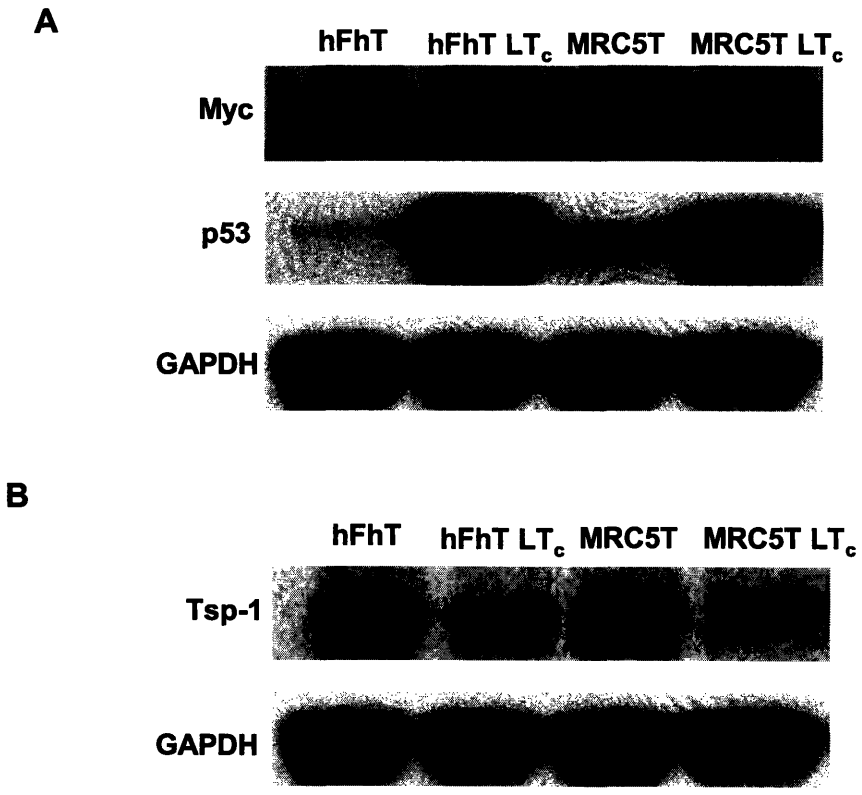



Figure 12.

(A) Immunoblot analysis of Myc, p53 and GAPDH proteins expressed by human dermal and lung fibroblasts expressing hTERT alone (hFhT, MRC5T) or with SV40 Large T ($LT_c$)

(B) Immunoblot analysis of Tsp-1 and GAPDH proteins expressed by the same cells.

**Figure 13**

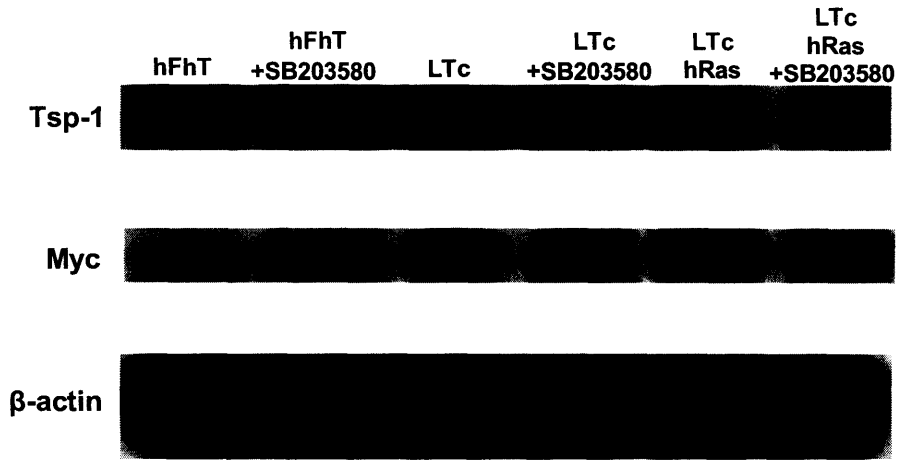



Figure 13.

Immunoblot analysis of Tsp-1, Myc and β-actin proteins expressed by human dermal fibroblasts expressing hTERT alone (hFhT); hTERT and 25μM SB203580; hTERT and $LT_c$; hTERT, $LT_c$ and 25μM SB203580; hTERT, $LT_c$ and Ras; or hTERT, $LT_c$, Ras and 25μM SB203580.

**Figure 14**

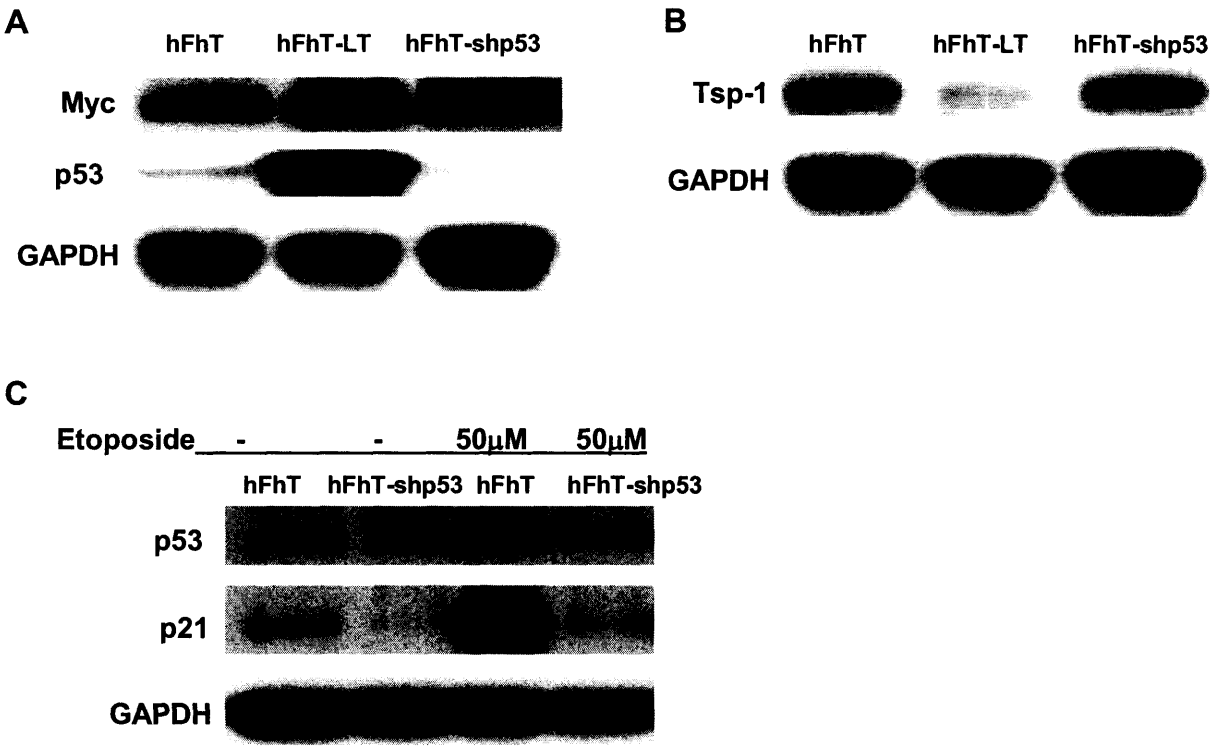



Figure 14.

(A) Immunoblot analysis of Myc, p53 and GAPDH proteins expressed by human dermal fibroblasts expressing hTERT (hFhT), hTERT and LT, and hTERT ad shp53

(B) Immunoblot analysis of Tsp-1 and GAPDH proteins expressed by the same cells as in above.

(C) Immunoblot analysis of p53, p21 and GAPDH proteins expressed by hFhT cells expressing hTERT alone, hTERT and LT, and hTERT and shp53 that were untreated (-) or treated with 50mM etoposide for 8 hours.

**Figure 15**

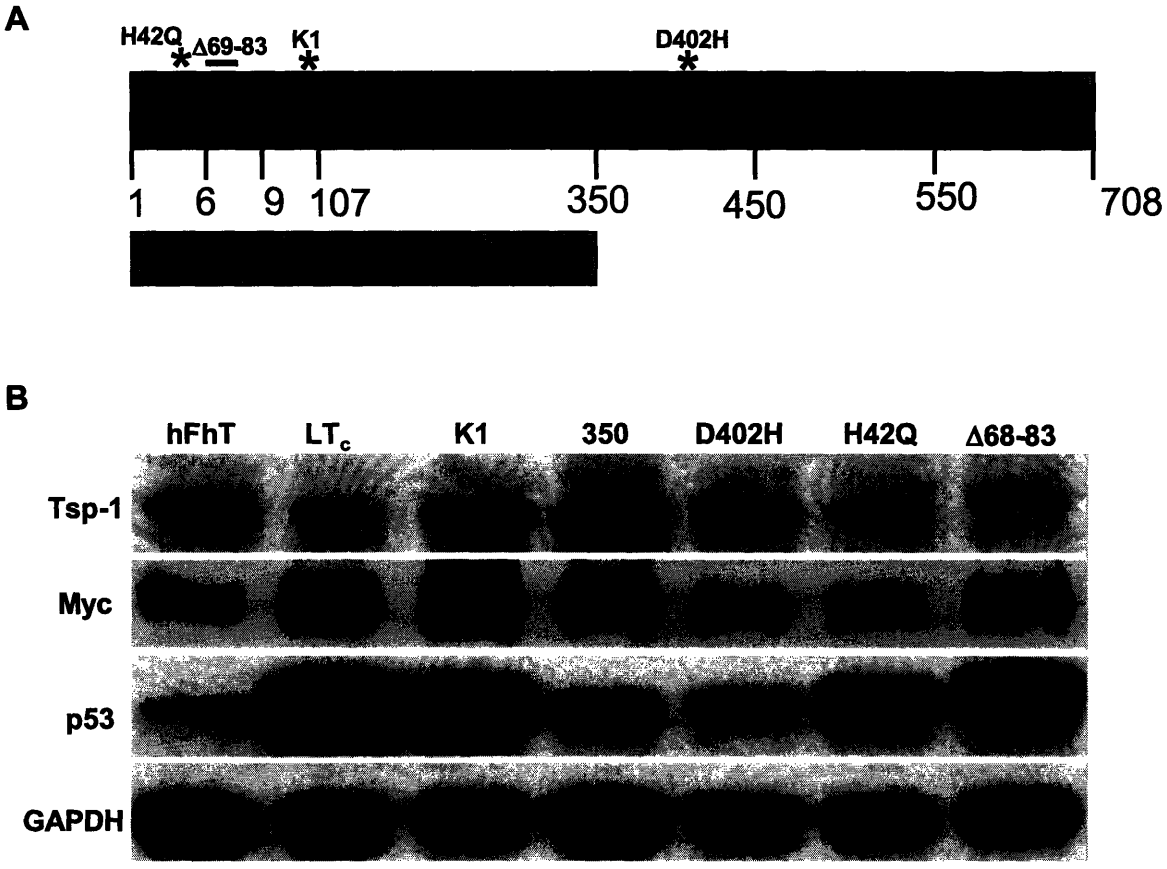



Figure 15.

(A) Schematic depiction of the domain structure of the SV40 large T antigen ($LT_c$) protein

(B) Immunoblot analysis of Tsp-1, Myc, p53, and GAPDH proteins expressed by immortalized human dermal fibroblasts expressing the Large T mutants depicted in figure 15a.

**Figure 16**

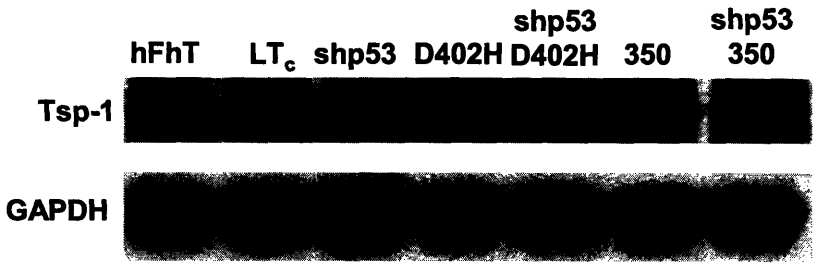



Figure 16.

Immunoblot analysis of Tsp-1 and GAPDH proteins expressed by human dermal fibroblasts expressing hTERT (hFhT) alone or in combination with: Large T (LT), shp53 (shp53), LTD402H, shp53 and LTD402H, LT350, and shp53 and LT350

Figure 17

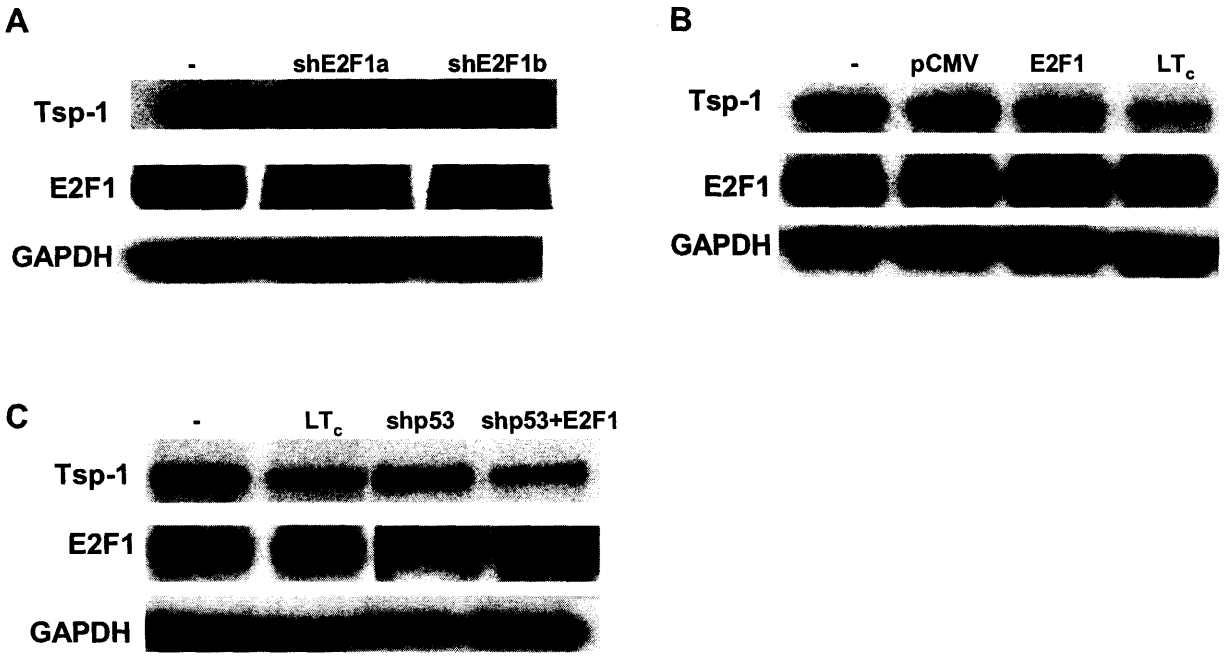



Figure 17.

(A) Immunoblot analysis of Tsp-1, E2F1 and GAPDH proteins expressed by human dermal fibroblasts expressing hTERT alone (hFhT) or in combination with shRNA specific for E2F1 (shE2F1a and shE2F1b)

(B) Immunoblot analysis of Tsp-1, E2F1 and GAPDH proteins expressed by human dermal fibroblasts expressing hTERT alone (hFhT), or in combination with pCMV, pCMVE2F1 (E2F1) or Large T ($LT_c$)

(C) Immunoblot analysis of Tsp-1, E2F1 and GAPDH proteins expressed by human dermal fibroblasts expressing hTERT alone (hFhT), or in combination with Large T ($LT_c$), shp53, and shp53 plus pCMVE2F1 (E2F1);

**Figure 18**

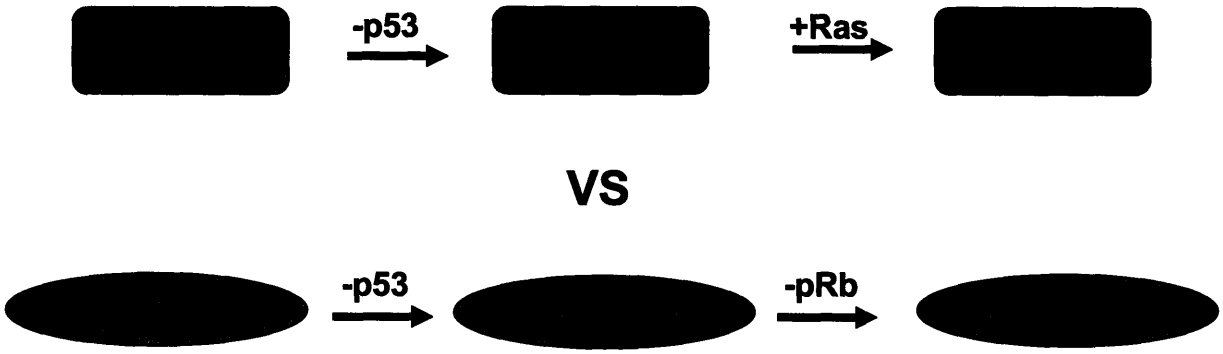



Figure 18.

Schematic diagram of Tsp-1 and Myc regulation in fibroblasts and epithelial cells.

Figure 19

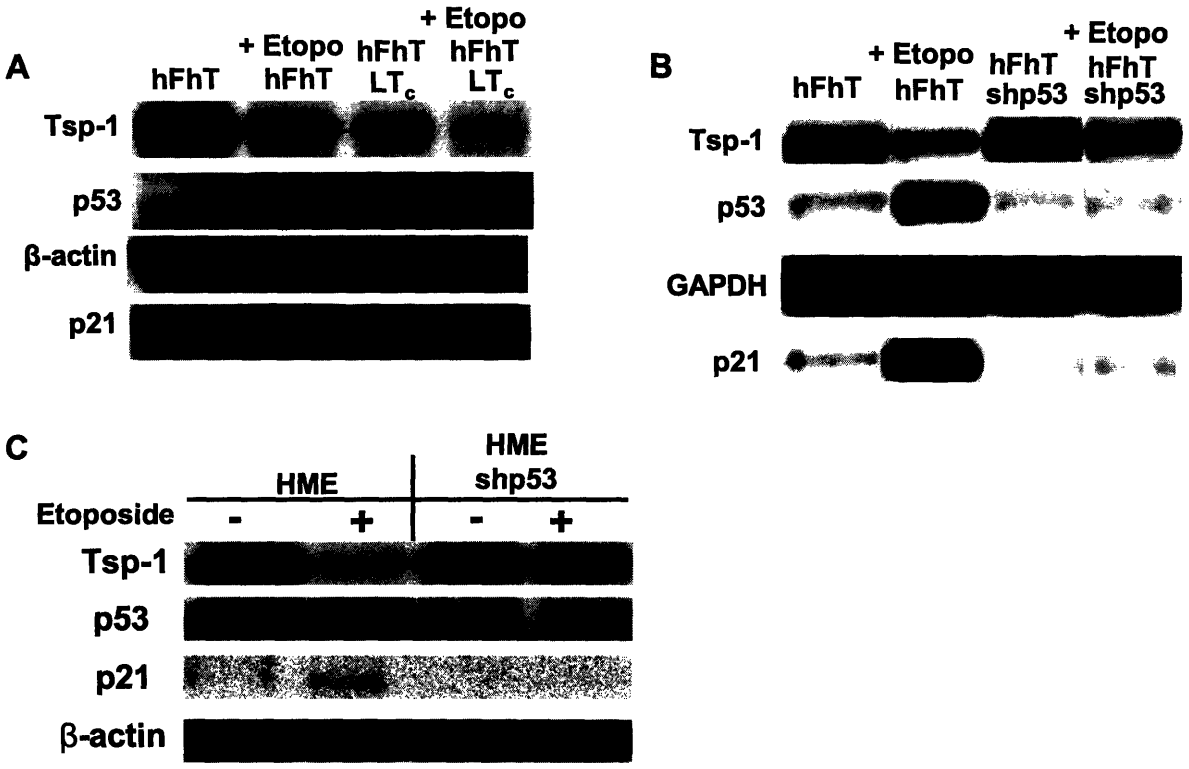



Figure 19.

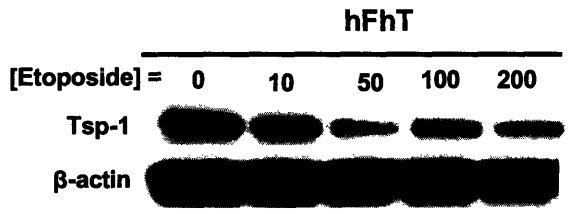
(A) Immunoblot analysis of Tsp-1, p53, β-actin, and p21 proteins expressed by human dermal fibroblasts expressing hTERT alone (hFhT) or in combination with LTc and untreated or treated with 50μM etoposide.

(B) Immunoblot analysis of Tsp-1, p53, GAPDH, and p21 proteins expressed by human dermal fibroblasts expressing hTERT alone (hFhT) or in combination withshp53 and untreated or treated with 50μM etoposide.

(C) Immunoblot analysis of Tsp-1, p53, β-actin, and p21 proteins expressed by human mammary epithelial cells expressing hTERT alone (HMET) or in combination with shp53 and untreated (-) or treated (+) with 50μM etoposide.

**Figure 20**

**A**




**B**

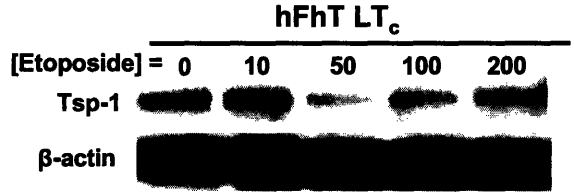



Figure 20.

(A) Immunoblot analysis of Tsp-1and β-actin proteins expressed by human dermal fibroblasts expressing hTERT and treated with 0μM, 10μM, 50μM, 100μM, or 200μM etoposide.

(B) Immunoblot analysis of Tsp-1and β-actin proteins expressed by human dermal fibroblasts expressing hTERT in combination with $LT_c$ and treated with 0μM, 10μM, 50μM, 100μM, or 200μM etoposide.

**Figure 21**

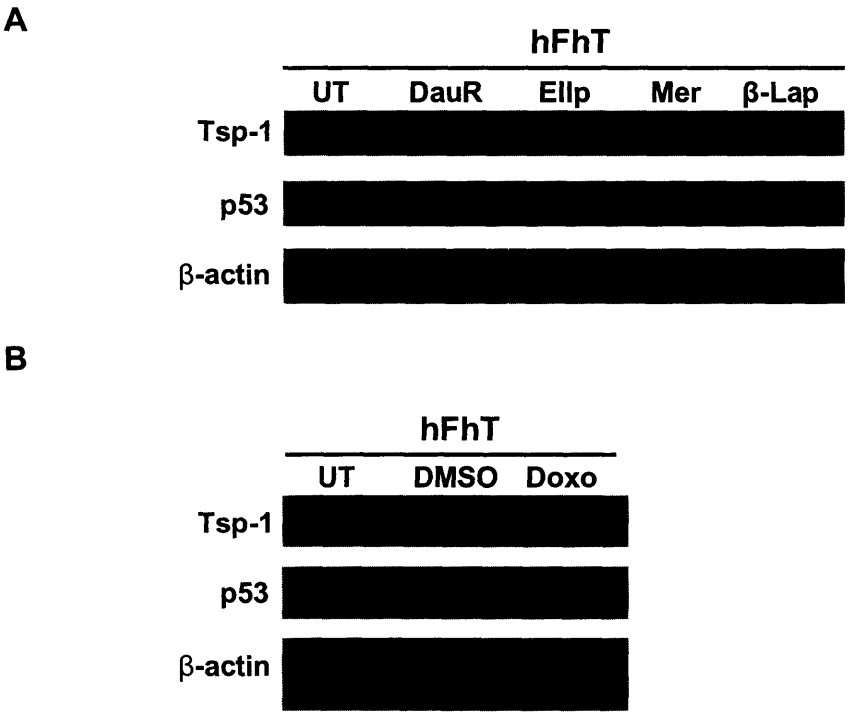



Figure 21.

(A) Immunoblot analysis of Tsp-1, p53, and β-actin proteins expressed by human dermal fibroblasts expressing hTERT and treated with 25μM daunorubicin, 50μM ellipticine, 50μM merbarone, 50μM β-Lapachone or untreated (UT).

(B) Immunoblot analysis of Tsp-1, p53, and β-actin proteins expressed by human dermal fibroblasts expressing hTERT and treated with 50μM DMSO, 10μM doxorubicin, or untreated.

# Chapter 4. Discussion

## I: Summary

In this report I have used human epithelial cells and fibroblasts to study the impact of a subset of genes commonly mutated in human cancers on the regulation of the angiogenesis inhibitor Tsp-1 (Ch. 2 and Ch. 3). I have demonstrated that expression of Myc and Tsp-1 is regulated differentially in human fibroblasts as compared to human epithelial cells. Previous studies have demonstrated that the tumor suppressor p53 stimulates the expression of Tsp-1 and inhibits the expression of Myc in human fibroblasts. My work confirms these findings in fibroblasts with respect to Myc but not to Tsp-1. My results demonstrate that loss of p53 in human fibroblasts does not affect the levels of Tsp-1 expression (Fig. 14).

These results also unexpectedly reveal that loss of p53 has different consequences in human epithelial cells as compared to fibroblasts. Specifically, I demonstrate that the loss of p53 activity in epithelial cells results in the repression of Myc and the stimulation of Tsp-1 expression, in contrast to fibroblasts where loss of p53 results in the stimulation of Myc and no repression of Tsp-1 (Fig. 22). The studies in epithelial cells also provide strong evidence suggesting that Myc is downstream of p53 in a pathway that regulates Tsp-1 expression. Furthermore, these results suggest that the reason why SV40 LT alone cannot repress Tsp-1 expression in epithelial cells is that Myc expression is inhibited, therefore another Myc stimulator like oncogenic Ras is required (Watnick et al. 2003).

While I have demonstrated here that ectopic expression of Myc can overcome the inhibitory effects of losing p53, I have not formally demonstrated that expression

of oncogenic Ras alone is sufficient to repress Tsp-1 expression in epithelial cells that have lost p53 activity. However, it seems likely that ectopic expression of oncogenic Ras in p53-deficient epithelial cells induces enough active Myc expression to bypass the inhibitory effects of losing p53. This prediction implies that carcinomas (tumors of an epithelial origin) can induce an angiogenic phenotype via oncogenic signaling (i.e. Ras or Myc) even in the presence of an anti-angiogenic stimulus, such as loss of p53. Given the frequent rate of Myc activation and p53 inactivation seen in carcinomas, it is clear that Myc provides a more potent signal that tips the balance of angiogenesis stimulators and inhibitors towards the induction of angiogenesis (Spencer and Groudine 1991; Levine 1993; Greenblatt et al. 1994; Nesbit et al. 1999).

In fibroblasts, loss of p53 activity in fibroblasts is necessary but not sufficient to repress Tsp-1 expression (Fig. 22). These results run contrary to studies that show ectopic expression of Myc can repress Tsp-1 expression in fibroblasts (Tikhonenko et al. 1996). An explanation for this discrepancy could be that the level of Myc stimulation induced by the loss of p53 is insufficient to repress Tsp-1 expression. However ectopic expression of Myc, beyond the levels induced by the loss of p53, may lead to a level of Myc activity that is so high it can override other cellular mechanisms that maintain an elevated level of Tsp-1. It might also be the case that loss of p53 not only activates Myc, but modulates the expression of a Tsp-1 stimulator. To further verify my results with p53-deficient fibroblasts, it would be helpful to create fibroblasts deficient for both p53 and Myc. If my hypothesis is correct, then these doubly deficient fibroblasts would show the same levels of Tsp-1 expression as the p53-deficient fibroblasts. Additionally, it is unclear whether Myc

stimulation via loss of p53 activity is necessary for $LT_c$-mediated Tsp-1 repression. Therefore, it would be useful to generate Myc-deficient fibroblasts that express $LT_c$ to determine if Myc is necessary for the repression of Tsp-1. In any event, it appears that in fibroblasts loss of p53 uncouples Myc from Tsp-1 expression.

In both epithelial cells and fibroblasts, Myc activation leads to the repression of Tsp-1 expression. A recent report has demonstrated that in K-Ras-expressing p53-deficient tumor-derived mouse epithelial cells, Myc mediates Tsp-1 repression through a series of micro-RNAs (Dews et al. 2006). However, it has not been demonstrated whether these Myc-induced microRNAs also repress Tsp-1 in fibroblasts. To test this idea, I would extract RNA from fibroblasts which display both elevated levels of Myc and repressed levels of Tsp-1 (i.e. $LT_c$-expressing fibroblasts and p53-deficient fibroblasts expressing LT-D402H) and probe the RNA for elevated levels of the micro-RNAs. If Myc induces the micro-RNAs in cells that repress Tsp-1, then Northern blot analysis should reveal elevated levels of micro-RNAs. This experiment would be a first step in determining how Myc mediates the repression of Tsp-1 expression in fibroblasts.

The results in human dermal fibroblasts reveal that repression of Tsp-1 requires not only the repression of p53, but also the repression of pRb. These studies demonstrate that pRb inhibition leads to the E2F1-mediated repression of Tsp-1. Repression of E2F1 stimulates Tsp-1 expression and ectopic expression of E2F1 represses Tsp-1 in human fibroblasts. When taken together with the p53 studies, these results indicate that the observed repression of Tsp-1 in human dermal fibroblasts is Ras-independent and is mediated by the concomitant activation of Myc

and E2F1 (Fig. 22). While I do not directly test this conclusion in my studies, a clear way to validate this hypothesis is to generate fibroblasts that ectopically express both Myc and E2F1, then test for the levels of Tsp-1 expression. If this hypothesis is correct, then these fibroblasts should repress Tsp-1 expression to the same extent that $LT_c$-expressing fibroblasts do.

The findings of this study suggest that mutations that deregulate cell cycle control in fibroblasts have the added consequence of increasing the cell's angiogenic potential. In this way fibroblasts differ from epithelial cells, where it has previously been demonstrated that Tsp-1 repression requires the expression of LT in combination with hyper-physiologic levels of oncogenic Ras (Fig. 22). These striking differences between fibroblasts and epithelial cells suggest that both cellular transformation and acquisition of the angiogenic phenotype may be differentially regulated in these two cell types.

The results presented here have increased our understanding of the regulation of Tsp-1 expression in epithelial cells and fibroblasts. However, these results raise new questions that are not addressed in this work. Some of the unanswered questions pertain to how particular proteins mediate their effect. For example, how does p38 mediate Myc phosphorylation in epithelial cells (Ch.2 section IV)? Or, does p53 directly activate the transcription of Myc in epithelial cells and directly repress the transcription of Myc in fibroblasts? These and other similar questions are elaborated upon below.

## II: Future work

### Does Ras-induced p38 directly phosphorylate Myc in epithelial cells?

The MAPK p38 is a downstream effector of the Ras-Rho-ROCK-Myc pathway that represses Tsp-1 in epithelial cells (Ch.2 section IV). In the Ras-mediated pathway, p38 is downstream of the kinase ROCK but upstream of Myc. The role of the Ras-mediated pathway in the repression of Tsp-1 is to phosphorylate Myc. However, it is not known which kinase in the Ras-mediated pathway is responsible for the phosphorylation of Myc. The MAP kinase p38 is a good candidate given that it is downstream of ROCK.

The first step in determining the role of p38 in the phosphorylation of Myc would be to determine the levels of phospho-Myc in human mammary epithelial cells treated with the p38 inhibitor SB203580 (Lee et al. 1999). If the levels of phospho-Myc decrease in the presence of SB203580, this would suggest that p38 has a role in Myc phosphorylation. Given the increased levels of total Myc in epithelial cells expressing Ras (Fig. 6), I would also test the levels of total Myc in epithelial cells treated with SB203580. If the levels of total Myc do not change in the presence of the p38 inhibitor, this would confirm that the role of p38 is in the phosphorylation of Myc. If, however, the levels of total Myc decrease in the presence of the inhibitor, this would suggest that p38 is promoting the transcriptional activation of Myc via activation of a Myc transcription factor. It has been demonstrated that phosphorylation affects the stability of Myc, which implies that affecting Myc phosphorylation could also affect protein levels (Sears et al. 2000). Therefore, if both the levels of total Myc and

phospho-Myc decrease in the presence of the inhibitor, it could be that p38 is affecting Myc stability.

I would then genetically verify the results obtained with the p38 inhibitor. I would create lentiviral constructs containing shRNA sequences specific for p38, and transduce oncogenic Ras-expressing epithelial cells with these constructs (Rubinson et al. 2003). If p38 mediates the transcriptional activation of Myc in these cells, then I would predict that loss of p38 results in decreased levels of Myc expression. However, if p38 mediates Myc phosphorylation in these cells, then I would expect no change in the total levels of Myc, but a decrease in the levels of phospho-Myc.

To verify that the effects of SB203580 are specific to p38, I would treat the p38-deficient cells with SB203580. The presence of the p38 inhibitor should have no added effects on the levels of Myc and phospho-Myc expression in cells that are deficient for p38. Additionally, I would ectopically express p38 in normal mammary epithelial cells and test the levels of Myc and phospho-Myc expression (Tessner et al. 2004). Depending on its mode of action, ectopic expression of p38 should increase the levels of either Myc or phospho-Myc, and treatment with SB203580 should abrogate this effect.

If the results suggest that p38 affects Myc phosphorylation, I would determine whether p38 directly phosphorylates Myc. I would first determine if p38 or phospho-p38 associates with Myc in the Ras-expressing epithelial cells. I would immunoprecipitate p38 and phospho-p38 and test for co-precipitation of Myc and phospho-Myc (Kim et al. 2002a). If Myc and phospho-Myc are observed in the p38 or phospho-p38 immunoprecipitation, this would suggest that p38 physically associates

with Myc. I would also repeat this experiment in the presence of SB203580 to verify the specificity of the phospho-Myc co-precipitation. I would then test the ability of phospho-p38 to directly phosphorylate Myc by performing an *in vitro* kinase assay (Kim et al. 2002a). To do this, I would immunoprecipitate phospho-p38 (the active form of p38) from Ras-expressing epithelial cells and test its ability to phosphorylate a Myc substrate. I would confirm Myc phosphorylation by immunoblot analysis of phospho-Myc levels. If active p38 can phosphorylate Myc, then the immunoblot should show the presence of phospho-Myc.

Taken together, these experiments should elucidate the role of p38 in Ras-induced Myc expression. Activation of p38 should either transcriptionally activate Myc expression or phosphorylate Myc. These experiments should give a better understanding of how expression of oncogenic Ras can lead to the repression of Tsp-1 expression.

**How does p63 effect Tsp-1 expression in epithelial cells?**

Preliminary experiments in human mammary epithelial cells demonstrate that loss of p63 represses Tsp-1 expression in the absence of oncogenic Ras (Ch.2 section V and Fig. 9). These experiments also demonstrate that expression of oncogenic Ras or the loss of p53 can stimulate p63 expression. This would seem to link Ras and p53 to p63 expression, but I have also demonstrated that Ras expression and p53 deficiency have opposite effects on Tsp-1 expression in epithelial cells (Fig. 3 and 6). These preliminary results, however, leave several unanswered questions that could help resolve the seemingly contradictory results of p63 activity.

One key issue that has not yet been addressed is how p63 affects Myc expression. To determine how Myc expression is affected, I would first generate a human mammary epithelial cell line that is unambiguously deficient for p63. Then I would use immunoblot analysis to compare the levels of Myc expression in both p63-deficient cells and normal control cells. If the levels of Myc increase in p63-deficient cells, this would imply that p63 represses Myc and that loss of p63 leads to Myc-mediated Tsp-1 repression. This would be consistent with the results demonstrating that p63-deficient cells repress Tsp-1 (Fig. 9). However, if loss of p63 does not change the levels of Myc expression, this would imply that loss of p63 represses Tsp-1 via a Myc-independent mechanism.

Another related question is how oncogenic H-RasV12 is able to repress Tsp-1 expression while also activating p63 in epithelial cells. A simple answer may be that the preliminary data is in error and Ras does not in fact stimulate p63 expression. However, a recent study using mice with an inducible oncogenic K-Ras found that inducing K-Ras generated salivary gland tumors that have elevated levels of p63 expression compared to normal tissue surrounding the tumor (Raimondi et al. 2006). It is worth noting that the tumors expressed higher levels of the ΔNp63 splice variant of p63, which has been shown to transcriptionally repress p53 activity (Raimondi et al. 2006). This suggests that it is at least possible that H-RasV12 is capable of stimulating p63 expression. This hypothesis can be tested by ectopically expressing H-RasV12 in human mammary epithelial cells and then measuring the levels of p63 expression. If it is the case that oncogenic Ras promotes p63 expression, then the

activity of Myc induced by Ras must be sufficient to overwhelm any p63-mediated stimulatory effects on Tsp-1 expression.

It is also unclear from these experiments if p63 actually stimulates Tsp-1 expression. The experiments using p53-deficient epithelial cells show an increase in the levels of p63 and Tsp-1, and epithelial cells expressing shp63b show decreased levels of Tsp-1, but this is not direct evidence that increased p63 levels stimulate Tsp-1 expression (Fig. 10). To directly test this result, I would ectopically express p63 in normal human mammary epithelial cells and then measure the level of Tsp-1 expression in these cells using immunoblot analysis (Nishi et al. 2001). If the levels of Tsp-1 expression increase, this would suggest that p63 is mediating Tsp-1 stimulation in epithelial cells. Stimulation of Tsp-1 by p63 would also imply that Ras expression is able to overcome the effects of p63 to repress Tsp-1 expression. If the levels of Tsp-1 expression do not increase, this would suggest that p63 does not regulate Tsp-1 expression.

These proposed experiments should answer some of the questions posed by my preliminary experiments. The role of p63 in Myc expression is of particular interest since Myc expression is a major determinant of Tsp-1 repression in epithelial cells. It would be very interesting if p63 is able to repress Tsp-1 expression independent of Myc in epithelial cells.

**Does p53 activate Myc in epithelial cells and repress Myc in fibroblasts?**

One of the primary distinctions between epithelial cells and fibroblasts is how p53 influences Myc expression in each cell type. In epithelial cells loss of p53 leads

to the repression of Myc; but in fibroblasts loss of p53 stimulates Myc expression (Ch.2 section III and Ch.3 section III). These results imply that p53 stimulates Myc expression in epithelial cells and represses Myc expression in fibroblasts. Given the fact that p53 is a transcription factor, it seems likely that p53 is modulating Myc transcription in both cases (Ch.1 section IV).

To demonstrate that p53 is affecting Myc expression in both epithelial cells and fibroblasts, I would ectopically express p53 in both cell types and expect results consistent with the loss of function data. Ectopic expression of p53 in the epithelial cells should stimulate the levels of Myc expression. Conversely, ectopic expression of p53 in fibroblasts should repress the levels of Myc expression.

I would then test whether p53 is a transcriptional activator of Myc in human mammary epithelial cells. I would create a luciferase reporter gene driven by the Myc promoter and transiently transfect it along with a construct expressing wild-type p53 into epithelial cells. I would also repeat this experiment using a loss-of-function p53 mutant construct to control for p53 specificity. I would then measure the amount of luciferase emitted by the cells to determine whether p53 is activating the Myc-driven luciferase gene. If p53 trans-activates Myc, I would expect to see increased luciferase activity in the epithelial cells expressing both the reporter gene and wild-type p53. With mutant p53, I would not expect to see an increase in the amount of luciferase. As an additional control, I would create truncated versions of the Myc promoter construct to determine the minimal p53 binding element and test them with wild-type and mutant p53. I would not expect to see any luciferase activity in truncation mutants that do not contain the p53 binding element. I would repeat these

experiments in fibroblasts using the same Myc promoter luciferase constructs and both wild-type and mutant p53 expression constructs. In fibroblasts, I would expect to see a decrease in luciferase activity in cells expressing the Myc promoter construct and wild-type p53.

If p53 modulates Myc transcription in both epithelial cells and fibroblasts, I would determine whether p53 binds different regions of the Myc promoter in epithelial cells compared to fibroblasts. To test this I would perform chromatin immunoprecipitation (ChIP) on both epithelial cells and fibroblasts. I would express wild-type and mutant p53 in both cell types, cross-link proteins to DNA, and shear all free DNA. I would then immunoprecipitate using an antibody against p53, reverse the cross-linking, and treat with proteinases to degrade all protein. Finally I would PCR amplify the DNA that was bound to p53 using Myc-specific primers and sequence the PCR products. In this way I would be able to analyze what regions of the Myc locus p53 binds to in both epithelial cells and fibroblasts. If transcriptional activation and repression are mediated by p53 binding to different regions of the Myc locus, then the regions amplified by the ChIP assay would be different in epithelial cells and fibroblasts. These experiments should demonstrate the ability of p53 to act as a trans-activator in epithelial cells and as a trans-repressor in fibroblasts.

If transcriptional expression of Myc is not affected by p53, I would determine whether p53 is directly affecting Myc protein levels in either epithelial cells or fibroblasts. I would determine the effects of p53 on Myc protein levels by testing if p53 physically associates with Myc in either cell type. I would accomplish this by immunoprecipitating Myc from epithelial cells and fibroblasts, and then using

immunoblot analysis to probe for p53. If p53 physically associates with Myc, I would expect to be able to use immunoblot analysis to identify the presence of p53 in the Myc immunoprecipitate of both epithelial cells and fibroblasts. If I do not detect p53 in the immunoprecipitate from either cell type, this would imply that p53 modulates Myc expression indirectly. It is also possible that p53 interacts with Myc in epithelial cells but not fibroblasts. In epithelial cells, p53 may target Myc for degradation through a third protein that is not expressed in fibroblasts. To identity the third protein, I would run the p53 immunoprecipitates from both epithelial cells and fibroblasts on a 2-dimensional gel and identifying spots that are present in epithelial cells but not fibroblasts. I would then excise the spots from the gel and use mass spectrometry to sequence them.

These sets of experiments would provide strong evidence to support the idea that p53 regulates Myc differently in epithelial cells than it does in fibroblasts. These experiments would also demonstrate how the same protein (i.e. p53) can be utilized by different mechanisms to give different results in the process of angiogenesis regulation. Furthermore, this difference in the interaction between p53 and Myc in epithelial cells and fibroblasts demonstrates how epithelial-derived tumors can influence the phenotype of stroma associated fibroblasts without affecting changes in their own phenotype.

**How does activation of E2F1 mediate the repression of Tsp-1?**

In fibroblasts, Tsp-1 repression requires not only the activation of Myc but also the activation of E2F1 (Ch.3 section VI). However, these results do not describe the

mechanism by which E2F1 induces the repression of Tsp-1. Even though it has been demonstrated that E2F1 can indirectly inhibit VEGF expression, there is little evidence that E2F1 can act directly as a transcriptional repressor, therefore it seems likely that a target of E2F1 induces the repression of Tsp-1 (Qin et al. 2006).

Given the multitude of E2F1 targets, a genomic approach might help in the search for candidate genes that repress Tsp-1 expression in human dermal fibroblasts. To find candidate targets that repress Tsp-1, I would use microarray analysis to compare the expression patterns of fibroblast cell lines that express wild-type levels of E2F1,overexpress E2F1, or are deficient for the expression of E2F1, to cells expressing SV40 $LT_c$. I would then identify all the genes that are up-regulated in the fibroblast cell lines that repress Tsp-1 (i.e. cells ectopically expressing $LT_c$ or E2F1). From that set of genes, I would identify those that are implicated in the modulation of Tsp-1 expression.

I would then test whether the candidate genes can be stimulated by E2F1, and whether they can repress Tsp-1 expression in p53-deficient fibroblasts. To test the responsiveness of these candidate genes to E2F1, I would use immunoblot analysis to test their levels of expression in human dermal fibroblasts deficient for E2F1 and in cells ectopically expressing E2F1 (Ch.3 section VI). If these genes are responsive to E2F1, their expression should decrease in E2F1-deficient cells and increase in cells overexpressing E2F1. To test the ability of the candidate genes to substitute for loss of pRb activity in the repression of Tsp-1 expression, I would ectopically express each candidate gene in p53-deficient human dermal fibroblasts. If indeed one of the candidate genes is the target of E2F1 in the repression of Tsp-1 expression, then

ectopic expression of this gene should synergize with loss of p53 to fully repress Tsp-1 in fibroblasts.

These experiments should yield insight into the target(s) of E2F1 involved in repressing Tsp-1 in fibroblasts. These experiments should also give us a better understanding of the underlying mechanisms controlling Tsp-1 expression in normal human fibroblasts. Furthermore, a greater understanding of how angiogenesis regulatory proteins like Tsp-1 are regulated in normal fibroblasts should give us insight into how angiogenic genes are dysregulated in carcinoma-associated fibroblasts.

**How do topoisomerase inhibitors modulate Tsp-1 expression?**

Experiments using the topoisomerase II inhibitor etoposide revealed that Tsp-1 is repressed in fibroblasts and epithelial cells treated with etoposide (Ch.3 section VII). I subsequently determined that etoposide represses Tsp-1 in a dose dependent manner, and that repression of Tsp-1 requires p53 activity. The effect of other topoisomerase inhibitors on Tsp-1 expression was also tested. The results demonstrate that not all inhibitors repress Tsp-1 expression. Additionally, some inhibitors repress Tsp-1 expression without inducing p53 expression. From these preliminary experiments it seems likely that only topoisomerase inhibitors that promote DNA damage repress Tsp-1, and that this repression is mediated by both p53-dependent and p53-independent pathways. However, these experiments did not address the mechanisms by which topoisomerase inhibitors repress Tsp-1 expression.

To further characterize the role of topoisomerase inhibitors in the repression of Tsp-1, I would use etoposide to test the downstream effectors of p53 involved in repressing Tsp-1. I would first determine the levels of Myc and E2F1 in human dermal fibroblasts treated with etoposide. Given the fact that p53 inhibits Myc expression in fibroblasts (Fig. 22), it is important to determine if etoposide has an effect on Myc expression independent of p53. This would be achieved by treating p53-deficient fibroblasts with etoposide and then testing the levels of Myc expression using immunoblot analysis. If the levels of Myc increase upon treatment with etoposide, this would suggest that the etoposide effect on Tsp-1 is mediated through Myc. I would perform the same type of analysis with E2F1. If the levels of E2F1 increase upon etoposide treatment, this suggests that etoposide also mediates Tsp-1 repression through the combined activities of Myc and E2F1. However, if the levels of E2F1 expression decrease or do not change, this would suggest that etoposide is not mediating Tsp-1 repression through E2F1.

If the levels of Myc do not change or decrease in either normal fibroblasts or p53-deficient fibroblasts treated with etoposide, this would suggest that the effect of etoposide on Tsp-1 expression is not mediated through Myc expression If this were the case, I would test the Ras-PI3K-Rho-ROCK pathway to determine whether it is involved in etoposide mediated Tsp-1 expression (Watnick et al. 2003). The idea that the Ras-MAPK pathway might be involved in etoposide-mediated Tsp-1 repression is supported by a published report that demonstrates normal human diploid fibroblasts irradiated with UV or treated with mitomycin C induce p53 expression, which subsequently induces the Ras-MAPK signaling cascade (Lee et al. 2000). This study

suggests that the Ras-MAPK pathway is most likely induced in fibroblasts treated with etoposide. I would test this idea by determining the levels of Ras and phospho-p38 in wild-type and p53-deficient fibroblast treated with etoposide. If Ras and active p38 levels increase in the presence of etoposide, then Tsp-1 repression is most likely mediated through the p53-induced activation of Ras. However, it may be that Ras inhibits Tsp-1 expression independent of p38. Ras signaling activates other pathways including the ERK pathway and the PI3K-Akt pathway. It may be that one of these other pathways mediates the repression of Tsp-1 through a mechanism that has yet to be characterized. It could very well be that all topoisomerase inhibitors that inhibit Tsp-1 do so by activating Ras-mediated signaling pathways.

The findings that topoisomerase inhibitors can effect Tsp-1 expression highlight the increasingly complex role of Tsp-1 in both epithelial cells and fibroblasts. Apart from the accepted to roles in angiogenesis, cell migration and cell adhesion, it appears that Tsp-1 may also be involved in stress response. To verify these results, however, I would first need to conclusively show that not only topoisomerase inhibitors, but also genotoxic stress represses Tsp-1 expression. Published reports demonstrating that ultra violet radiation induces angiogenesis through the stimulation of VEGF and the repression of Tsp-1 provide more evidence to support the role of Tsp-1 in DNA damage response (Howell et al. 2004; Yano et al. 2004; Yano et al. 2005). It has also been shown that mice transgenically expressing Tsp-1 in the skin are resistant to UV-induced angiogenesis (Yano et al. 2002). Furthermore, a recent study has demonstrated that ultraviolet radiation represses Tsp-1 expression via the PI3K-Akt pathway (Kim et al. 2006). Given these recent findings, it seems likely that

some topoisomerase inhibitors repress Tsp-1 expression through Ras-induced activation of the PI3K-Akt pathway.

## III: Perspectives

The conclusions drawn from these data must be viewed with certain caveats. One caveat is that these studies were all performed at the protein level using immunoblot analysis; therefore it is unclear whether the observed regulation occurs at the transcriptional, post-transcriptional or translational level. In fact there is evidence that p53 can repress Myc expression by directly binding to the Myc promoter (Ho et al. 2005). It is clear that further studies need to be done to address the question of how p53 influences the levels of Myc protein in human epithelial cells and fibroblasts. Another caveat is that these experiments do not differentiate between intracellular levels of Tsp-1 and secreted levels of Tsp-1. Previous work has shown that while absolute levels of Tsp-1 differ between cell lysates and secreted Tsp-1 in media samples, the regulation of Tsp-1 is not mediated at the level of secretion (Kang, SK and Watnick, RS, unpublished results). Despite these caveats, the results in these studies strongly support the interpretation that p53 can influence Myc and Tsp-1 levels in human epithelial cells and fibroblasts.

One of the goals of this study was to determine how fibroblasts repress Tsp-1 expression. This determination would then give us a better understanding of how carcinoma-associates fibroblasts (CAFs) acquire an angiogenic phenotype. In this work I have demonstrated that fibroblasts, as compared to epithelial cells, have fewer requirements for the repression of Tsp-1. This result is interesting because it has

been shown that CAFs contribute to the induction of oncogenic and angiogenic phenotypes in carcinomas (Ch.1 section II). Thus it is possible that neoplastic epithelial cells convert normal stromal fibroblasts into activated fibroblasts, and in turn the activated fibroblasts aid in the progression of neoplastic epithelial cells to fully metastatic carcinomas.

Another intriguing aspect of the interdependent relationship between stromal fibroblasts and neoplastic epithelial cells is whether the neoplastic epithelial cells repress Tsp-1 expression in fibroblasts as part of the process of fibroblast activation. The results of this work mechanistically demonstrate how epithelial cells might induce repression of Tsp-1 expression, but the question of timing is not addressed. Do epithelial cells induce the repression of Tsp-1 in fibroblasts only during the early stages of tumor progression, or are advanced metastatic carcinoma cells also able to repress Tsp-1 in CAFs? One study has shown that metastatic tumor cells can secrete factors that activate fibroblasts *in vitro* (Olaso et al. 1997). While this study doesn't address the question of Tsp-1 expression, it does demonstrate that metastatic cells can induce changes in fibroblasts. Additionally, work from the Watnick laboratory has demonstrated that secreted factor(s) from metastatic carcinoma cells can repress Tsp-1 expression in normal fibroblasts derived from tissues at the sight of metastasis (manuscript in review). It would seem likely then, that carcinoma cells are able to repress Tsp-1 expression at different stages of carcinoma progression.

The results presented in this work also highlight the differences in gene regulation between two basic cell types. For example, my results dealing with loss of p53 in epithelial cells and fibroblasts suggest that at least one of the many functions

of p53 differs between epithelial cells and fibroblasts. These results also underscore the potency of p53 inactivation in fibroblasts. Not only does this event result in the loss of a tumor suppressor in fibroblasts, but in the upregulation of an oncogenic transcription factor. In the case of epithelial cells, however, loss of p53 activity still leaves a second barrier to overcome. Specifically, epithelial cells must independently upregulate Myc expression and/or activity subsequent to the loss of p53 activity. One of the implications of these results is how epithelial-derived tumors (carcinomas) can arise under certain circumstances, while mesenchymal-derived tumors (sarcomas) arise under different circumstances.

Carcinomas account for approximately 90% of all solid tumors in the adult population (Parkin et al. 2001; Parkin et al. 2005). In sharp contrast, sarcomas account for approximately 90% of all childhood solid tumors (Parkin et al. 2001; Parkin et al. 2005). Molecular studies of both carcinomas and sarcomas reveal that these two types of tumors share common genetic events involved in cellular transformation (Merlino and Helman 1999; Ye et al. 2004). For example, the vast majority of both carcinomas and sarcomas contain mutations in the tumor suppressor p53 (Wang and Harris 1997). Interestingly, two different mouse models have demonstrated that mice harboring a mutation in the *p53* gene develop a different tumor spectrum than mice that contain a homozygous deletion of the gene (Donehower et al. 1992; Li et al. 1998; Olive et al. 2004). The former develop predominantly carcinomas, while the latter develop predominantly sarcomas and lymphomas.

One possible explanation for this phenomenon might be differences in the mechanisms by which carcinomas and sarcomas regulate angiogenesis. Angiogenesis allows solid tumors to grow beyond the microscopic size (<~2mm diameter) imposed by the diffusion limit of oxygen and nutrients (Gimbrone et al. 1972). It has been previously shown that one mechanism by which tumors induce angiogenesis is the repression of the endogenous angiogenesis inhibitor Tsp-1 (Good et al. 1990; Lawler 2002). Significantly, I demonstrate here that the repression of Tsp-1 in fibroblasts requires the loss of p53 and pRb activity. Furthermore, in epithelial cells, loss of p53 stimulates Tsp-1, and repression of Tsp-1 requires the stimulation of both oncogenic Ras and Myc.

Despite the presence of commonly mutated tumor suppressors and oncogenes among carcinomas and sarcomas, there must be some underlying mechanism(s) to account for the predisposition of one tumor type over the other in childhood versus adulthood. The results presented here suggest a possible explanation for this discrepancy, namely that deregulation of the cell cycle has a more profound effect on the subsequent steps involved in tumorigenesis in fibroblasts than in epithelial cells.

The results also suggest that mutations in p53 and pRb have a greater transformative effect in fibroblasts than in epithelial cells. However, this alone cannot explain why children are more prone to sarcomas, while adults are more prone to carcinomas. It could be that differences in the replication rates of fibroblasts and epithelial cells in childhood and adulthood can explain this phenomenon. Fibroblasts in childhood are dividing at far greater rates than in adulthood, when tissues have become fully developed (Moore et al. 2003). Thus, mutations in fibroblasts are

theoretically less likely to occur in adults given the reduced rate of cell division and DNA replication. However, epithelial cells, which form the linings of all the ducts in the body, are subject to greater exposure to insult or injury and so throughout life must continuously undergo cell division and DNA replication (Otto 2002). While the probability of developing these mutations during childhood is equivalent in fibroblasts and epithelial cells, these mutations have a greater transformative potential in fibroblasts due to their effects on Myc and Tsp-1 expression. Therefore, my results suggest that transformation of epithelial cells requires a greater number of collaborating mutations than does transformation of mesenchymal cells.

Angiogenesis is an essential step in the progression of all tumors to the malignant state, and it is clear that induction of angiogenesis depends on the balance of angiogenic stimulators and inhibitors. The results I have presented here give us a better understanding of the mechanisms governing the regulation of the angiogenesis inhibitor Tsp-1 in different cells types. These results add to our understanding of how the balance between angiogenic stimulators and inhibitors is maintained, and how it can become unbalanced under pathologic conditions. A better understanding of the mechanisms controlling Tsp-1 expression will also help in the development of new treatments for cancer. By knowing which pathways to inhibit (like the Ras-p38-Myc pathway in epithelial cells) or to stimulate (like the p53 and pRb pathways in fibroblasts), Tsp-1 expression can be selectively stimulated thus tipping the balance and inhibiting angiogenesis in malignant tumors.

**Figure 22**

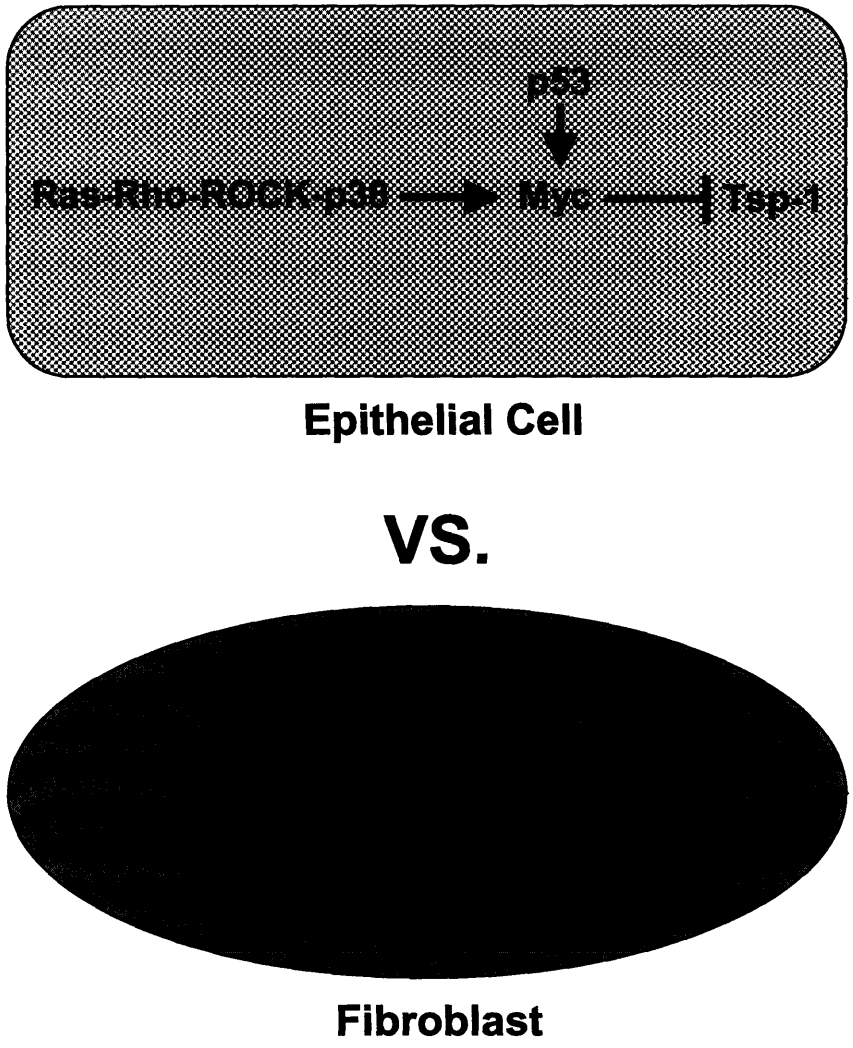



Figure 22.

Schematic diagram of pathways leading to the repression of Tsp-1 in epithelial cells and fibrobalsts.

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