Investigation on the Effect of Menin on the TGF-β Pathway

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Abstract

TGF-β plays a dual role in cancer: In pre-malignant cells TGF-β inhibits tumor growth, but in malignant tumor cells it helps tumor progression. One of the most important effects of TGF-β is the induction of EMT, which allows epithelial cells to acquire mesenchymal traits, allowing tumors of epithelial origin to become malignant. Menin is a ubiquitous tumour suppressor protein that interacts with Smad3 of the TGF-β pathway, suggesting a role in the TGF-β pathway. There is a possibility that Menin may act as a factor for progression of cancer by participating in TGF-β signalling. To assess its effect in the TGF-β pathway, Menin was knocked down using specific siRNA and TGF-β function was tested. Results suggest knocking-down Menin decreases N-cadherin expression and alter its localization. The absence of Menin also affected TGF-β-induced migration, suggesting Menin may play an important role in TGF-β-induced tumour progression.

Résumé

TGF-ß joue un double role dans la progression du cancer; dans les cellules pré-malignes, TGF-ß inhibe la croissance du cancer, cependant, dans les lignées plus invasives, TGF-ß promouvoit la progression du cancer. Un des effets les plus importants du TGF-ß est l'induction de la transition épithéliale à mésenchymale, permettant aux tumeurs d'origine épithéliale de devenir malignes. Menin est un suppresseur de tumeur omniprésent qui interagit avec Smad3, une protéine nécéssaire à la voix de signalisation du TGF-ß. Il est cependant possible que Menin agisse en tant que promoteur du cancer lorsqu'il s'intègre à la voix de signalisation du TGF-ß. Pour évaluer ses effets, l'expression de Menin a été réduite en utilisant des siRNA. Les résultats suggèrent que l'absence de Menin reduit l'expression de N-cadherine and altère sa localization. L'absence de Menin affecte aussi la migration induite par TGF-ß, suggérent que cette protéine joue un rôle important dans la progression tumorale induite par la cytokine.

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I thank Dr. Jean-Jacques Lebrun for his help and guidance throughout the years I was pursuing this project. I would also like to thank my thesis committee members, Dr. Christian E. Rocheleau, Dr. Geoffrey N. Hendy, and Dr. Louis Larose who provided guidance and valuable discussions. Thanks to Dr. Stephen Laporte and Dr. Christian E. Rocheleau for being generous and sharing their valuable equipments such as microscopes and cell culture hoods. I cannot thank enough my colleagues on the floor for their mental support and help regarding laboratory techniques and in life in general. Special thanks goes to Nadege Fils-Aime, who provided me her scratch assay figures and who is such a wonderful friend to have around, and to Eugenie Goupil, Jimin Guo, Meiou Dai, Juliana Korah, Brandon Zimmerman and Marc-Andre Sylvain, who was always there to help me, guide me, and help find answers to all the problems I encountered in the lab.

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List of Abbreviations

AMH (Anti-Muellerian Hormone)

APC (Antigen Presenting Cells)

CBP (CREB Binding Protein)

CDK (Cyclin Dependent Kinase)

Co-Smad (Common partner Smads)

CTL (Cytotoxic T Lymphocytes)

DPC4 (Deleted in Pancreatic Cancer- Locus 4)

ECM (Extracellular Matrix)

EMT (Epithelial to Mesenchymal Transition)

GFAP (Glial Fibrillary Acidic Protein)

HRP (Horseradish Peroxidase)

IL-2 (Interleukin 2)

I-Smads (Inhibitory Smads)

LAP (Latency associated peptide)

LLC (Large latent complex)

LTBPs (Latent TGF-β Binding Proteins)

MAD (Mothers Against Decapentaplegic)

MAPK (Mitogen Activated Protein Kinase)

MEF (Mouse Embryo Fibroblasts)

MEN1 (Multiple Endocrine Neoplasia 1)

MH1 (Mad Homology 1)

MH2 (Mad Homology 2)

MLL (Myeloid/lymphoid or mixed-lineage leukemia)

MMPs (Matrix-Metalloproteinases)

NES (Nuclear Export Signal)

NK (Natural Killer)

PAI-1 (Plasminogen Activator Inhibitor 1)

PTH (Parathyroid hormone)

q-RT-PCR (Quantitative Real Time Polymerase Chain Reaction)

ROS (Reactive oxygen species)

R-Smads (Receptor regulated Smads)

SAD (Smad Activating Domain)

SARA (Smad Anchor for Receptor Activation)

SBE (Smad-Binding Element)

SHIP (Src Homology 2 domain-containing 5' inositol phosphatase)

SIP-1 (Smad Interacting Protein 1)

SKI (Sloan Kettering Institute)

SLC (Small Latent Complex)

Smurf (Smad Ubiquitination Regulatory Factor)

TFs (Trascription Factors)

TGF-β (Transforming Growth Factor beta)

TNF (Tumor Necrosis Factor)

TRE (TPA Responsive Elements)

UTR (Untranslated region)

Cancer is a genetic disease that results from combinations of mutations in the genome. About 25% of the population in the developed world die of cancer (Jemal et al., 2008), despite the efforts made to cure cancer patients. Among various cancers a human being may expect to develop during their lifetime, breast cancer holds special (Derynck and Akhurst, 2007) significance for women; it is the most diagnosed cancer and it ranks second in the list for the most numbers of deaths (Jemal et al., 2008). Although it still claims many lives progress continues to bring down those numbers, and breast cancer rates have shown a drastic decline in response to improvements in diagnostic tools and therapeutics. An even deeper understanding of the mechanisms that cause breast cancer and its progression would be necessary to bring down the breast cancer rates further and prevent unnecessary deaths.

Cytokines, an essential component of cellular signalling, plays very important roles in cancer biology. One of the most important cytokines that affect breast cancer, as well as all other cancers, is a cytokine named TGF-β (Transforming Growth Factor beta). TGF-β is a multi-functional cytokine that is expressed in most cell types. It is a critical factor in development and homeostasis, as it plays major roles in cell growth, differentiation, proliferation, angiogenesis, EMT (Epithelial to Mesenchymal Transition), migration, ECM (Extracellular Matrix) protein production and regulation of the immune system (Massague, 2008).

Despite the critical roles played by TGF-β in homeostasis, excessive TGF-β expression has been linked to many diseases and cancer related deaths. TGF-β is associated with increased death in pancreatic or colon cancer patients (Watanabe et al., 2001) and is known to facilitate cancer metastasis in breast cancer patients (Muraoka-Cook et al., 2004; Siegel et al., 2003; Yin et

al., 1999). Paradoxically, over-expression of TGF-β or TGF-β receptors was shown to impede tumour growth in numerous *in vivo* and *in vitro* studies (Cui et al., 1996; Siegel and Massague, 2003; Siegel et al., 2003). These observations are just a few examples that support the fact that TGF-β plays a dual role in tumour pathogenesis and can be used as an advantage to cancer cells when the pathway components or the players that keep the pathway under control are compromised.

TGF-β, in the early stages of tumour development, can limit the level of growth and proliferation, playing a protective role against cancer. However, in the late stages of cancer development, tumour cells selectively lose some components of the pathway, disabling the cytostatic arm of TGF-β. In addition to losing the cytostatic arm of the pathway, the remaining function of TGF-β pathway can be used to provide the cancer cells with several advantages. Cancer cells can become even more aggressive by going through EMT, a process that allows cells to acquire mesenchymal characteristics and gain motility, allowing them to spread to nearby areas or to metastasize to distant organs. In addition to promoting EMT and motility, tumour-derived TGF-β plays an important role in hiding the tumour cells from the immune system and rendering the immune system ineffective at recognizing the tumour, providing a perfect environment for the tumour cells to nest and grow.

A deeper understanding will undoubtedly further contribute to developing novel therapies to treat breast cancer and increase the survival rate of those patients with aggressive invasive tumours.

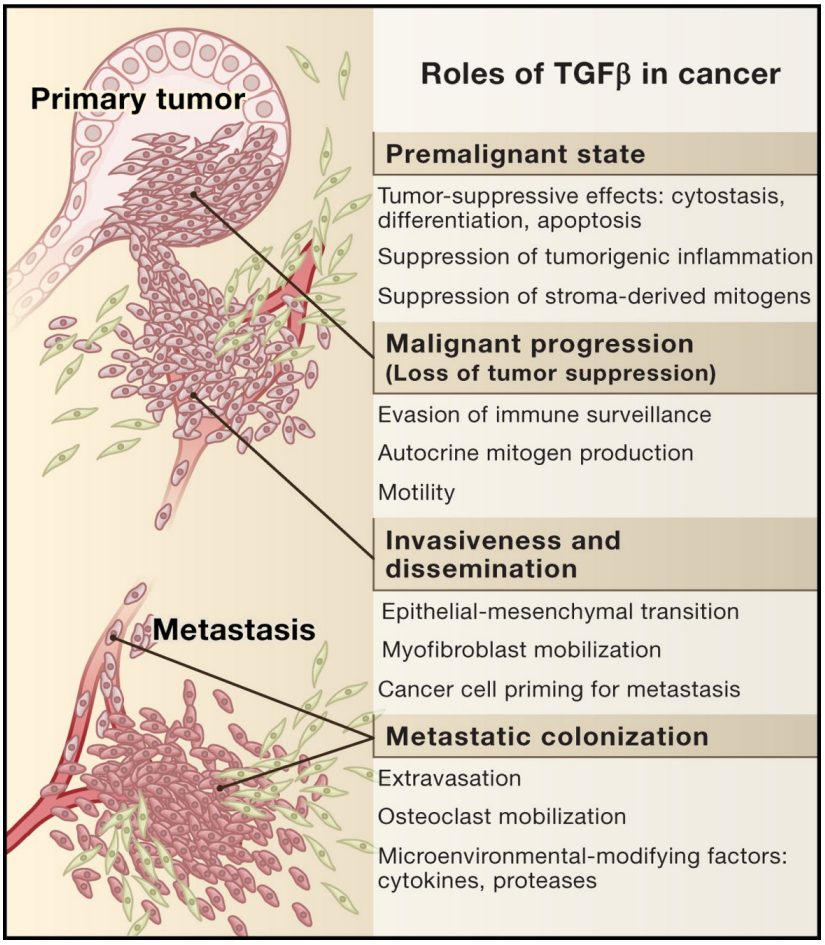


Figure 1.1: The dual role of TGF-β in cancer. TGF-β can exert different effects depending on the state of the cancer cells. In the premalignant state, TGF-β suppresses growth of the tumour by affecting the tumour cells directly or by affecting the environment, preserving homeostasis and protecting the host from tumour. Mutations in the pathway results in loss of the beneficial effects of, and TGF-β becomes a factor promoting invasion and metastatic colonization. Adapted from (Massague, 2008).

In the following section, an overview of TGF-β will be given, followed by an overview of Menin, a protein that was shown to participate in the TGF-β pathway (Kaji et al., 2001; Naito et al., 2005; Sowa et al., 2003) and play an important role in the development of a life-threatening disease, MEN1 (Marx et al., 1999a). The focus of this project is to shed light on the

role of Menin as a component in the TGF-β pathway, either participating in the tumour suppressive arm or the tumour promoting aspect of TGF-β, in attempt to better understand this pathway.

1.1 Overview of the TGF-β super family

TGF-β is a member of the TGF-β family of cytokines, a large family that includes numerous factors with some more than 50 members (Yan et al., 2002), divided into two large subfamilies, one containing Activin and TGF-β and the other containing Bone Morphogenic Proteins and growth and differentiation factors (Kang et al., 2003; Shi and Massague, 2003). All the family members share structural similarities characterized by their six conserved cystein residues (Lander et al., 2001). The TGF-β family is known to be the main regulator of cell growth and differentiation, selection of cell lineage and progression along that lineage, and organ development (Derynck and Akhurst, 2007).

TGF-β family are peptide dimers that interact with a hetero-tetramer of trans-membrane receptor complex that has serine/threonine kinase activity at their cytoplasmic tail (Massague, 2008). The receptor consists of type I and II receptors, with type II being the constitutively active one that binds the ligand first, in the case of the TGF-β receptor, then recruiting the other and activating it by phosphorylation on the highly conserved GS domain (Yan et al., 2002). While this entire family of cytokines is named after TGF-β, TGF-β may not necessarily represent the entire family; this is especially so when mechanisms of ligand-receptor interactions are compared (Groppe et al., 2008). In contrast to the other members of the TGF-β family who

promiscuously share receptors, TGF-β interacts exclusively with only two receptors, TGF-β Receptor-I and II (Groppe et al., 2008).

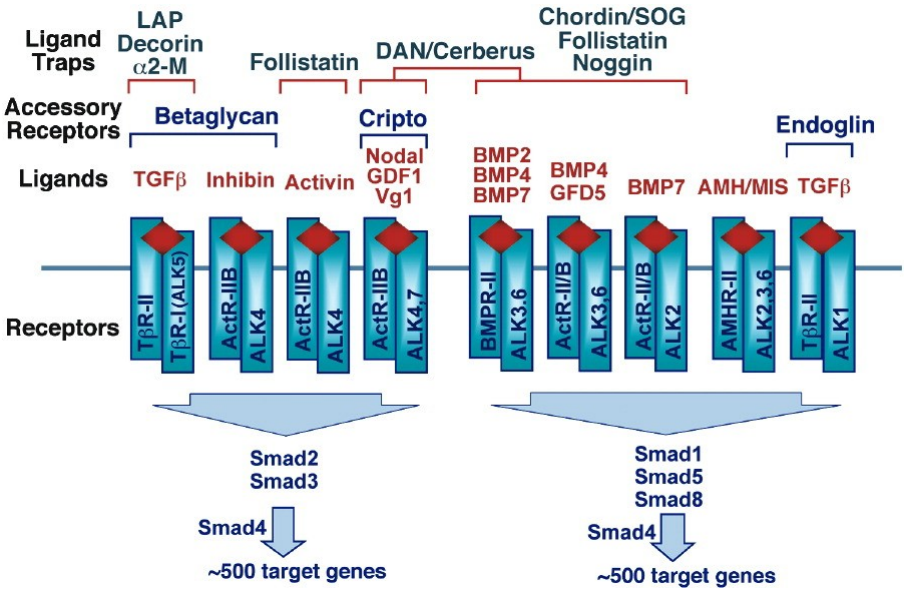


Figure 1.2: TGF-β family divided into their two subfamilies. The relationships of the ligands, ligand traps, accessory receptors, receptors, and intracellular interactors are described. Adapted from (Shi and Massague, 2003).

1.2 Review of the literature

1.2.1 The discovery of TGF-β

TGF-β was discovered decades ago, in the late 1970's, when researchers were trying to identify growth factors that caused 'transformed,' cancer-like phenotypes on 'normal' cells (Roberts et al., 1980). The "growth factor" was isolated from the experimentally transformed cells and was named the transforming growth factor beta (TGF-β) (Sporn, 1999). As the name indicates, Transforming Growth Factor-beta was initially considered as a growth factor, until 1984, when the growth inhibitory effects of the cytokine were discovered (Tucker et al., 1984).

In the next year, another group found that TGF-β can have mixed effects even on the same cell line (Roberts et al., 1985). Also, it became apparent that the cytokine was not only present around transformed cells, but found generally in various tissues in normal, healthy circumstances.

Since the duality of TGF-β was discovered, many researchers have devoted their lives trying to elucidate the 'switch' mechanism that made TGF-β so unique in character. Although the exact mechanism behind the switch still remains elusive, some extraordinary progresses were made in the field, including elucidation of the main or 'canonical' pathway of TGF-β signalling that involves Smad proteins, and discovery of the effect of TGF-β on the immune system.

There are three homologous isoforms of TGF-β: TGF-β1, TGF-β2, and TGF-β3. Each serves a distinct function and are regulated by unique regulatory elements (Sporn, 1999). Some of their functions overlap, and other subtle differences exist (Jenkins, 2008) that still need more investigation. All three are needed for proper development. Despite similarities observed *in vitro*, *in vivo* effects are notably different among TGF-β isoforms (Annes et al., 2003); the isoforms exhibit distinct tissue localization and null mutant mice of each die due to strikingly different causes, suggesting one cannot substitute the other and roles *in vivo* might differ significantly (Jenkins, 2008), the dissimilarity possibly being a product of differential regulation in each tissue type. Although all three seems to play a role to a certain point, TGF-β1 isoform is the most relevant one in the context of cancer, and this specific isoform is the one that is involved in most researches with TGF-β. Therefore, whenever TGF-β is mentioned in this thesis, it should be noted that TGF-β is referring to TGF-β1 and not the others unless specified.

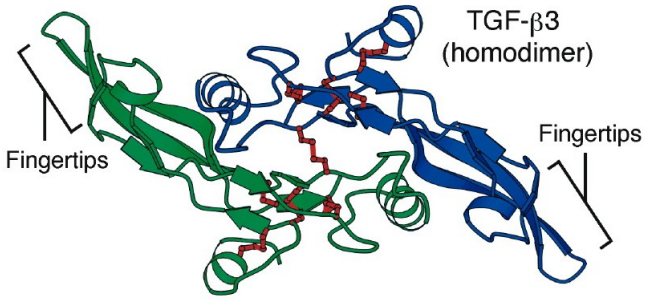


Figure 1.3: TGF-β3 ligand structure. TGF-β3 homodimer is shown, each monomer being depicted blue or green. The monomer consists of several antiparallel β sheets and α helices that are locked into place by disulfide bridges (depicted red) and hydrophobic interactions. The pro-peptide fragments that bind non-covalently to the mature molecule to prevent unwanted interactions are not shown in this diagram. Adapted from (Shi and Massague, 2003).

1.2.2 TGF-β and Regulation of TGF-β

TGF-β is a multifunctional cytokine, that controls virtually all mammalian cell types and tissues and it is known to be essential for development (Massague, 2008). It is a relatively new addition to the TGF-β family in terms of evolution since it "made its debut with the rise of the vertebrates (Massague, 2008)," and this fact alludes to their highly sophisticated effects suited for regulation of more complex organisms. It is involved in the maintenance of homeostasis in tissues and in organ systems, regulation of the immune system, tissue repair, and development (Wrzesinski et al., 2007). The effects of TGF-β is known to be highly contextual in different cell types and in cells immersed in different environments, such as the state of the cell and the state of the surrounding cells (Kang et al., 2003; Massague, 2008).

Owing to their importance, TGF-β molecules are tightly regulated from the moment they are synthesized. The active ligand is a dimer stabilized by hydrophobic interactions and a disulfide bridge (see figure 1.3), and to prevent uncontrolled TGF-β stimulation in the cell, they are synthesized as proproteins.

In the trans-golgi apparatus where the propeptide is cleaved by furin-type enzymes, the cleaved and dimerized mature TGF-β binds back non-covalently to the cleaved fragments, which is also called a LAP (latency associated peptide), to result in the formation of a small complex known as the SLC (Small Latent Complex) (Jenkins, 2008). In addition to this safety mechanism, the SLC can also be further bound to the LTBPs (Latent TGF-β Binding Proteins), further preventing any unregulated signalling (De Robertis and Kuroda, 2004; Jenkins, 2008). LTBPs are extracellular glycoproteins that sequester and tether the SLC to a LLC (large latent complex) which is further bound to the ECM to await for activating cues for TGF-β under normal conditions (Annes et al., 2003).

Synthesis of TGF-β normally occurs in excess, and the latent TGF-β complex is abundantly tethered to the ECM as LLC, where it awaits activation by enzymes such as furins or other convertases in the vicinity (De Robertis and Kuroda, 2004; Rifkin, 2005). It has also been shown that the dissociation and activation of TGF-β can occur by certain stress conditions such as heat, acid, ROS (Reactive Oxygen Species) and so forth (Jenkins, 2008). These activation mechanisms are a critical step in TGF-β regulation because they prevent TGF-β activation until it is needed. Uncontrolled activation is especially dangerous, since once the pathway is activated, TGF-β can further cause amplification of TGF-β availability by up-regulating its own activating enzymes in a feed-forward fashion (Jenkins, 2008). In addition to the previously mentioned activation mechanisms, evidence suggest that the integrin mediated activation is another

important method, perhaps more important than just protease dependent activation mechanism alone (Yang et al., 2007), suggesting a cooperation between the two mechanisms.

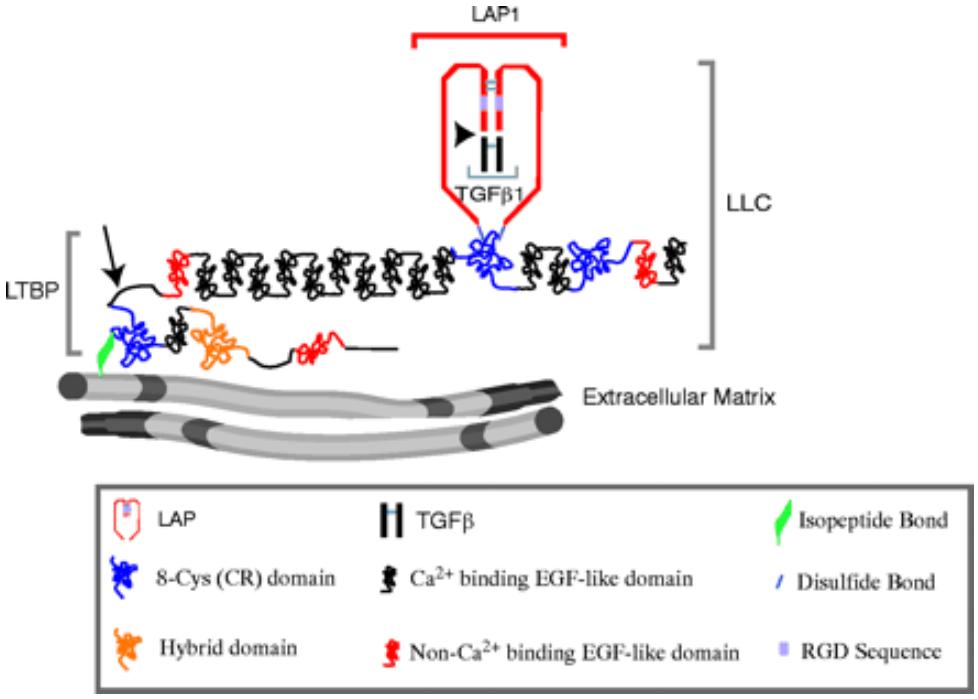


Figure 1.4: Latent TGF-β complex awaits activation in the ECM as a LLC. TGF-β dimer is first bound to the latency associated protein, which further binds to the latent TGF-β binding protein that is tethered to the extracellular matrix by an isopeptide bond (covalent bond). The whole complex is named the large latent complex, and in this form TGF-β awaits for activation. This is a critical regulatory mechanism for TGF-β. The arrows indicate cleavage sites. Adapted from (Annes et al., 2003).

1.2.3 TGF-β Signalling

1.2.3.1 TGF-β Receptors

TGF-β signals are transduced from the cell surface to the nucleus mainly by a well established 'canonical' pathway. The ligand detectors of the TGF-β pathway are the TGF-β

receptor type I and TGF-β receptor type II which are glycoproteins that have similar structure and function. The receptors are part of a set of transmembrane serine/threonine kinases that are paired in different combinations (see figure 1.2) to detect TGF-β family members like BMP and Activin. TGF-β is unique in the sense that it does not share receptors like other family members do so promiscuously.

The receptors reside on the cellular membrane to detect any active TGF-β dimers in the vicinity. Without the ligand, the receptors remain as homodimers, but after the latent TGF-β from the LLC is cleaved and activated, the TGF-βR type II dimers bind to the active ligand dimer. This binding results in a conformational change, which then recruits the TGF-βR type I dimer, resulting in a receptor heterotetramer interacting with the ligand (see figure 1.4) (Shi and Massague, 2003). TGF-βR type II dimers are constitutively active serine/threonine kinases that recruites and phosphorylates TGF-β receptor type I dimers. TGF-β receptor type I differs from receptor type II by a 30 amino-acid Glycine-Serine rich region that is called the "GS region" which is a conserved juxtamembrane segment just upstream of the kinase domain. Following TGF-β stimulation, the recruited TGF-βR type I dimer are phosphorylated on the GS region and this phosphorylation of the type I receptor dimer results in the activation of the kinase domain of receptor type I, allowing phosphorylation of its substrates.

TGF-β type III receptors are accessory receptors that are not directly involved in signalling but rather concentrating the ligand near the type I and II receptors, allowing efficient signalling (Esparza-Lopez et al., 2001). Also known as β-glycan, this receptor is a membrane anchored proteoglycan with a short cytoplasmic domain with no function (Cheifetz et al., 1992).

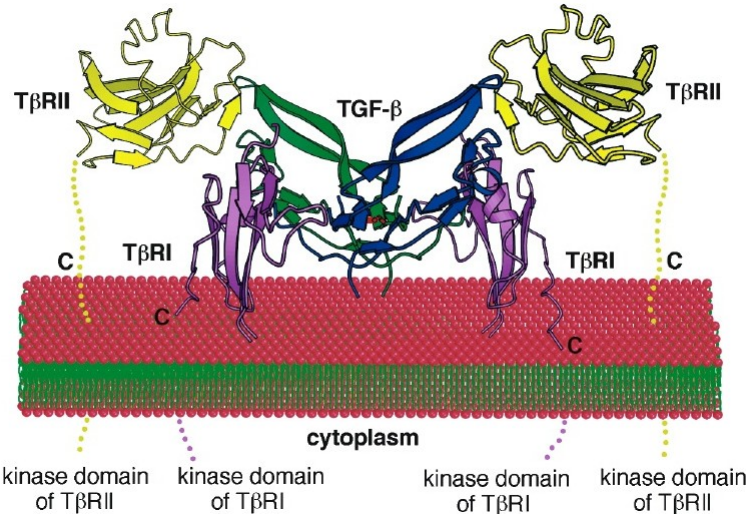


Figure 1.5: Active TGF-β molecule docking on its receptor heterotetramer. The active TGF-β molecule sits in the binding pocket created by the receptors. Adapted from (Shi and Massague, 2003).

1.2.3.2 The Smad Proteins

Smad Proteins

Major signal transducers of the TGF-β pathway are the Smad proteins. These are intracellular proteins that play an essential role in transducing the signal from the receptor to the nucleus, where specific target genes are modulated as a result. The Smads are a structurally distinct group of proteins that measure around 400 to 500 amino acids in length, composed of two conserved domains linked by a non-conserved linker region (Attisano and Lee-Hoeflich, 2001). They are divided into three classes; R-Smads (Receptor regulated Smads) that are phosphorylated by TGFBR-I, Co-Smad (Common Partner Smads) that interacts with the R-Smads to relay the signal to the nucleus, and I-smads (Inhibitory Smads) that play a role in shutting off, or diminishing the signal (Shi and Massague, 2003).

MAD was first discovered in 1995 in Drosophila (Sekelsky et al., 1995) and in subsequent years, Smads were discovered in other species including humans. There are eight family members in mammals, encoded on 4 different chromosomes: Smad 2, 4, 7, are found closely clustered on Chromosome 18; Smad 3, 5, 6 are on Chromosome 15; Smad1 is on Chromosome 4; Smad 8 is on Chromosome 13 (Attisano and Lee-Hoeflich, 2001). Mammals have only one Co-Smad, Smad4 (Wrana and Attisano, 2000). Therefore, Smad4 serves as the common Co-Smad for all the members of the TGF-β family.

The R-Smads are phosphorylated by the activated receptor hetero-tetramer and subsequently forms a homo-trimer of R-Smads. TGF-β signal-transducing R-Smads are Smad2 and Smad3, and they also propagate signals for Activin and Nodal (see figure 1.2) receptors. Smad1, Smad5, and Smad8 are also R-Smads but these transduce signals initiated by BMP and AMH (Anti-Muellerian) receptors (see figure 1.2). R-Smads can be recruited near the receptors on the membrane and early endosomes by SARA (Smad Anchor for Receptor Activation), an adaptor protein for Smad2 and Smad3 retention in the cytosol, to facilitate signalling (Tsukazaki et al., 1998).

Co-Smad, Smad4 is also known as DPC4 (Deleted in Pancreatic Cancer-Locus 4), alluding to its importance as a safeguard against tumours. Smad4 mutations in pancreatic cancer and colorectal cancer are seen during the transition of the adenoma to carcinoma (Jaffee et al., 2002), although more experiments are needed to determine whether the mutation caused the transition or the progression of cancer caused mutation, it does seem to suggest an important connection. There is only one Co-Smad for mammals and thus Smad4 serves as the common Co-Smad for the signal transduction of all TGF-β family members (see figure 1.2) (Kang, 2006), connecting both branches of the TGF-β family to the nucleus. Following the homotrimerization

of R-Smads after their phosphorylation, one R-Smad is replaced with a Co-Smad to form a heterotrimer, and the heterotrimer work together to relay the signal to the nucleus with the help from cofactors (Chen et al., 2007). In the nucleus the Smad heterotrimer complex exerts its effect by modulating gene transcription in cooperation with various DNA binding cofactors, depending on the state of the cell (Massague, 2008).

There are two I-Smads that function as negative regulators in the pathway, Smad6 and Smad7. I-Smads inhibit the process through interfering with Smad-receptor interactions by competing with the R-smads for receptor binding or by recruiting Smurf, an E3 ubiquitin ligase, to the activated receptors and target them for degradation (Attisano and Lee-Hoeflich, 2001).

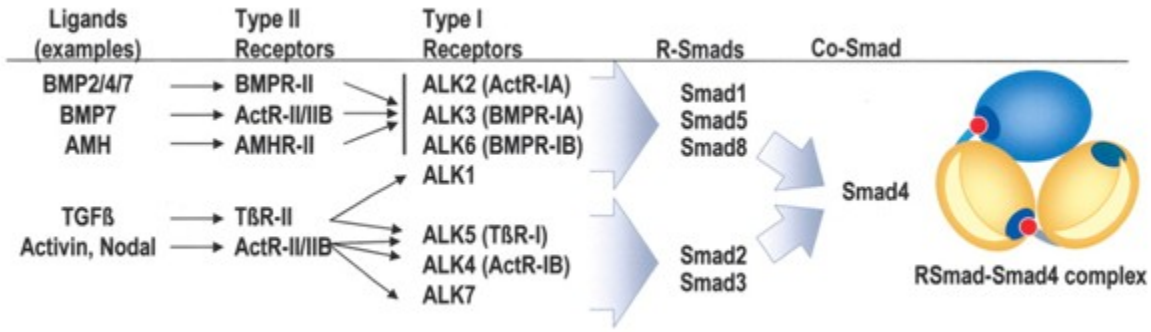


Figure 1.6: Relationship of TGF-β family members, their receptors, and Smad proteins. Smad4 is the common component of all the TGF-β super family members. After type I receptors phosphorylate the R-Smads, the R-Smads form a trimer. Upon interacting with Smad4, one R-Smad is replaced by Smad4 to form a heterotrimer, which travels to the nucleus to regulate target genes. Adapted from (Massague et al., 2005).

Structure of Smads

R-smads and Co-Smad (Smad4) are composed of two domains; the conserved MH1 (Mad Homology 1) and MH2 (Mad Homology 2) domains with a non-conserved linker region connecting the two, which also serves important functions (Attisano and Lee-Hoeflich, 2001; Shi and Massague, 2003). I-Smads, Smad 6 and 7, are an exception because they have a divergent N' termini replacing an MH1 domain (Attisano and Lee-Hoeflich, 2001). MH1 domain found in R-Smads and Co-Smad is involved in DNA binding, whereas MH2 is responsible for protein-protein interactions (Attisano and Lee-Hoeflich, 2001; Shi and Massague, 2003). Smad MH1 domain binds to DNA at the SBE (Smad-Binding Element), the GNCN repeats, but the affinity is too weak to be efficient, hence requiring cooperation from other DNA binding TFs (Transcription Factors) such as Jun and Sp1 (Attisano and Lee-Hoeflich, 2001; Gomis et al., 2006).

The MH2 domain is a multifunctional region critical for mediating several different interactions (Attisano and Lee-Hoeflich, 2001). The carboxy-terminal tail located in the MH2 domain contains the two essential serine residues from a "SSXS" motif that can be directly phosphorylated by TGFBR-I, resulting in activation of R-Smads (Abdollah et al., 1997; Attisano and Lee-Hoeflich, 2001). MH2 domain is also important for binding to SARA (Smad Anchor for Receptor Activation), which recruits Smads to the TGF-β receptors for a more efficient signalling process (Attisano and Lee-Hoeflich, 2001).

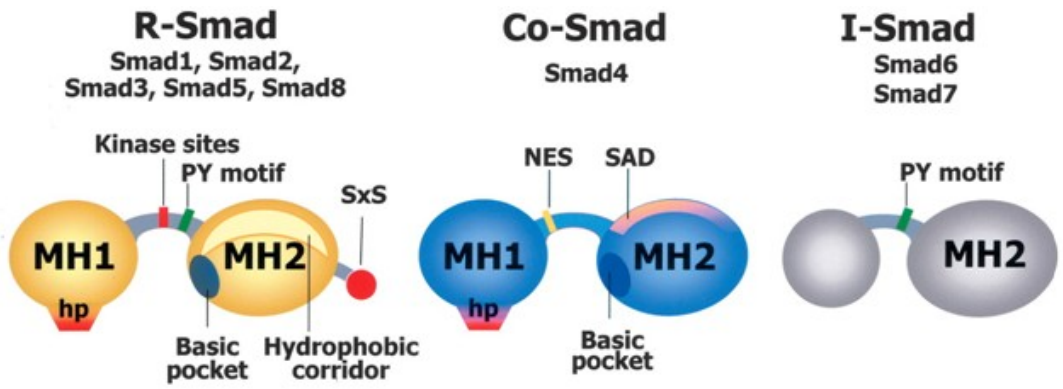


Figure 1.7: The structure and relationship of different Smads. The Smads consist of 3 distinct regions. The two conserved globular domains, MH1 region and MH2 region, are separated by a non-conserved linker. MH1 domain is generally involved in DNA binding, whereas MH2 domain is involved protein-protein interaction. The R-Smads have the SSXS motif at their carboxy terminal of MH2 region, which can be phosphorylated by type I receptors. Co-Smad carries a NES (Nuclear Export Signal) and a SAD (Smad Activating Domain). Both types of Smads mentioned possess a basic pocket, which allows them to bind to from trimers. I-Smads differ from the other two types by showing only a weak homology to the MH1 domain, but it still has the MH2 domain without the SSXS motif at the carboxy end. Adapted from (Massague et al., 2005)

The linker regions also serve several purposes. The linker region of R-Smads contains potential phosphorylation sites that may possibly be phosphorylated by MAPK (Mitogen-Activated Protein Kinase) (Attisano and Wrana, 2000; Ho et al., 2005; Massague et al., 2000; ten Dijke et al., 2000; Zhang and Derynck, 1999) this phosphorylation blocks normal function of R-Smads, suggesting that a significant role is played by the linker region for signal regulation (Ho et al., 2005). The linker region of R-Smads and I-Smads carry a proline-tyrosine (PY) motif that mediates interaction with Smurf (Smad Ubiquitination Regulatory Factor), which is an ubiquitin ligase (Bonni et al., 2001; Kavsak et al., 2000; Zhu et al., 1999). Also, the linker domain of Co-

Smad, Smad 4, carries a NES (Nuclear Export Signal) and a SAD (Smad activation domain) (Attisano and Lee-Hoeflich, 2001; Kurisaki et al., 2001; Pierreux et al., 2000; Watanabe et al., 2000).

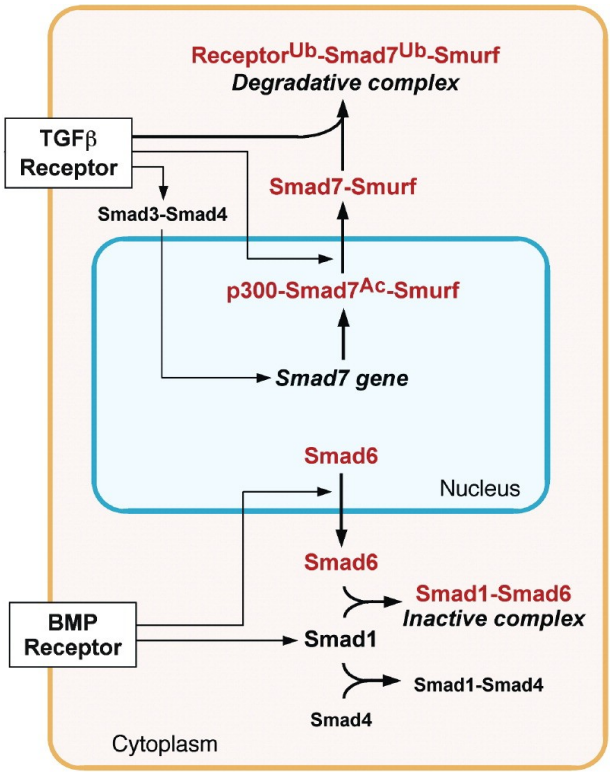


Figure 1.8: Inhibitory Smads and Their Mechanism of Function. There are two I-Smads that negatively regulate TGF-β signalling. Smad6 and Smad7 do not have the conserved MH1 globular domain that binds to DNA. Rather, the I-Smads compete with the R-Smads and inhibit signalling by competition. Smad6 can interact with Smad1 to render Smad1 inactive, and Smad7 can trigger degradation of TGF-β receptors by actively recruiting Smurf ubiquitin ligase. Adapted from (Shi and Massague, 2003).

I-Smads, Smad6 and Smad7, show only a weak homology to the MH1 domain, but still possess a conserved MH2 domain present in R-Smads without the serine residues at the carboxy terminal (the SSXS motif), which would still allow protein-protein interaction (Attisano and Lee-Hoeflich, 2001; Shi and Massague, 2003). They stably bind activated receptor complexes to compete and interfere with R-Smad binding to the receptor, therefore diminishing the overall signal (Attisano and Lee-Hoeflich, 2001). In addition, I-Smads can interact with R-Smads to render R-Smads inactive (Shi and Massague, 2003). To even further decrease the signal, Smad7 can recruit Smurfs, which are ubiquitin ligases, to degrade the active receptor complexes

(Ebisawa et al., 2001; Kavsak et al., 2000). Smad7 normally resides in the nucleus, and upon ligand stimulation, Smad7 is exported out of the nucleus to perform its function (Attisano and Lee-Hoeflich, 2001).

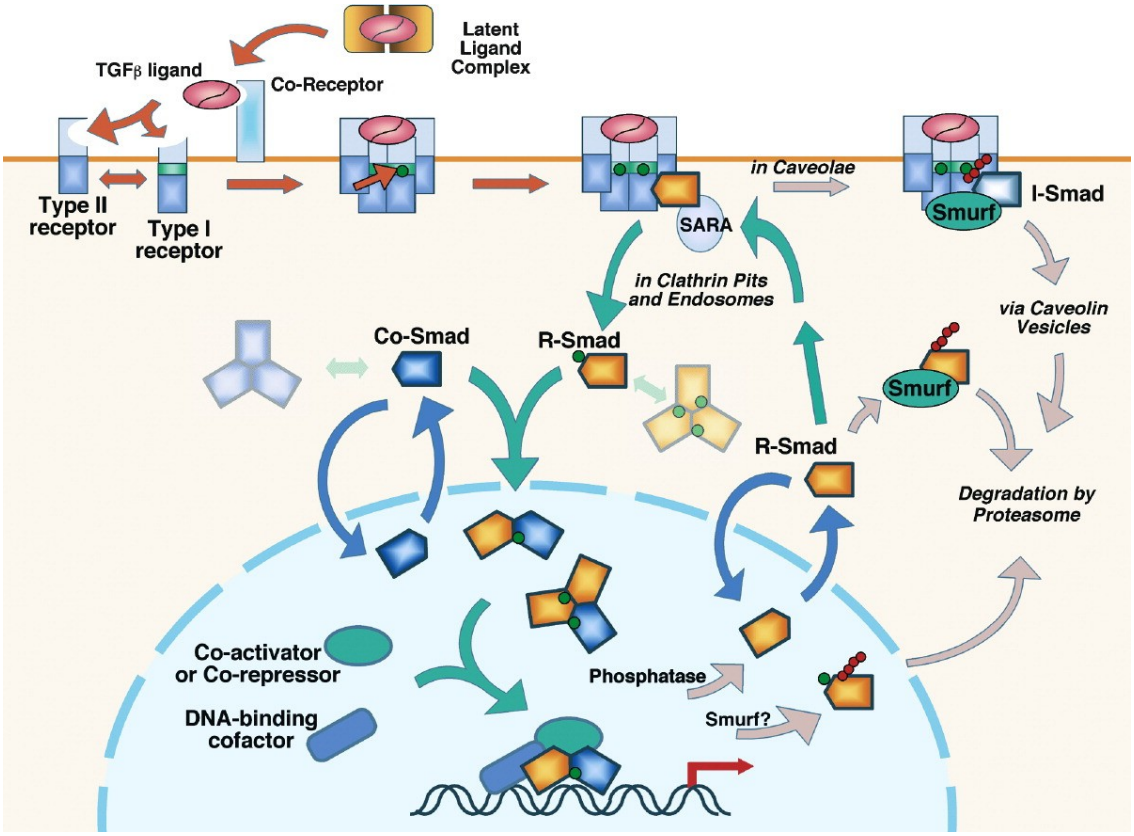


Figure 1.9: A simplified outline of the TGF-β signalling pathway. The active TGF-β homodimer interacts with the receptor heterotetramer, causing activation of TGFBR-I by TGFBR-II, which is a constitutively active serine kinase. The activation of TGFBR-I results in R-Smad phosphorylation at the C terminal serine residues, the SSXS motif. R-Smads are held in the cytosol by SARA, an adaptor protein. The activated R-Smad homotrimer then interacts with a Co-Smad, forming a heterotrimer which translocates to the nucleus, where it interacts with other cofactors to assist DNA binding. Phosphatases can dephosphorylate the activated R-Smad Co-Smad complex and Smurf marks the activated receptor complex by ubiquitination, targeting them for degradation. Adapted from (Shi and Massague, 2003).

Gene Regulation by Smads

The wide spectrum of response that TGF-β may elicit is likely to be caused by combinations of Smads and DNA binding cofactors; cofactor expression pattern at a given time and location which would reflect the state of the cell and its environment is postulated to be the source of variety in this relatively simplistic pathway (Chen et al., 2007). A proposed mechanism involves Ski and CBP (CREB Binding Protein), cofactors for smads with relatively equal binding affinities but with opposite outcomes; CBP activates transcription of TGF-β responsive genes but Ski represses their transcription (Chen et al., 2007).

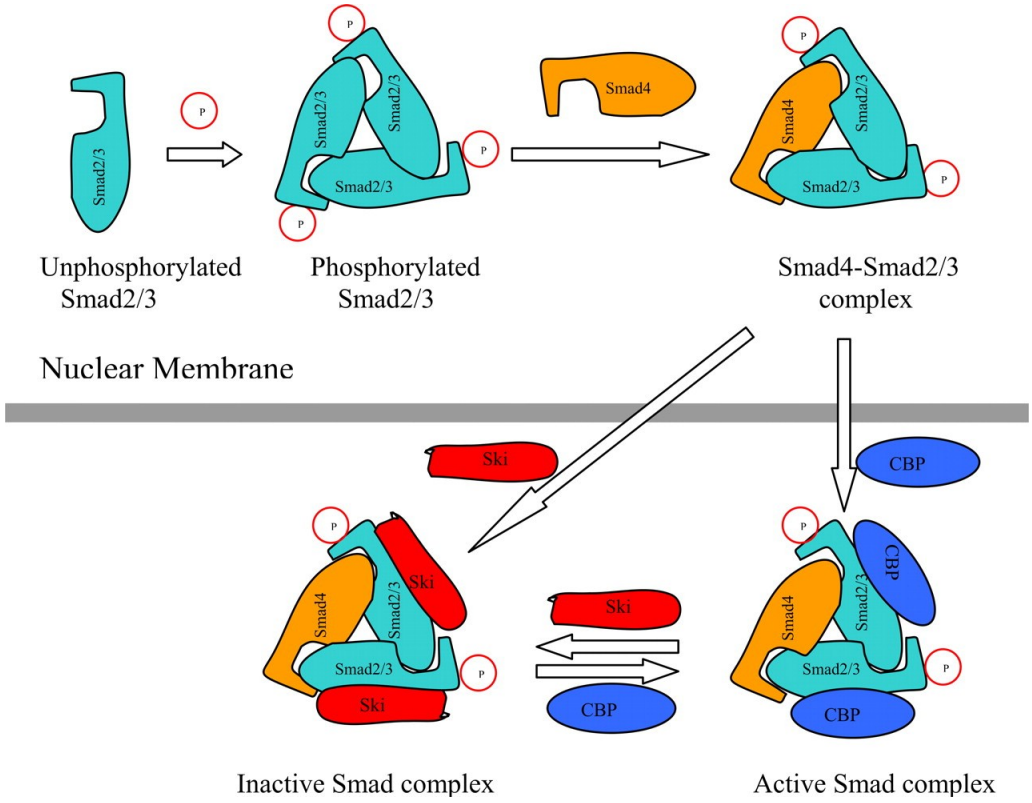


Figure 1.10: The Smad-Ski/ CBP dynamics. The R-Smads form a homotrimer upon activation. Interaction with the Co-Smad (Smad4) results in formation of heterotrimer that translocates to the nucleus, further interacting with Ski or CBP with equal affinity. Adapted from (Chen et al., 2007).

The levels of Ski or CBP would then act as a sensor mechanism for the Smads, and the same set of Smads will dictate, upon TGF-β receptor type I activation, either the activation or repression of target genes (Chen et al., 2007). Smads are an important set of proteins that are virtually essential for TGF-β signalling (Massague, 2008). Smad genes are found to be frequently altered in cancers and those mutations are found to render the TGF-β pathway defective (Attisano and Lee-Hoeflich, 2001), which links TGF-β pathway function and the integrity of Smads.

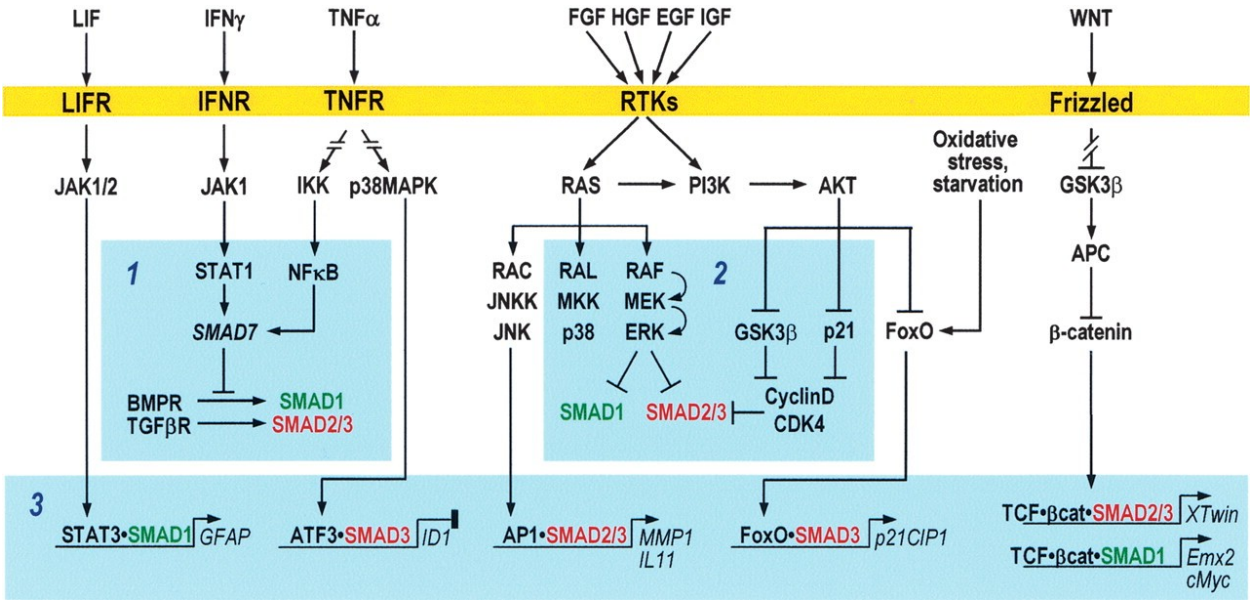


Figure 1.11: Cross-talk between TGF-β pathway and other major signal transduction pathways. Some major signal transduction pathways influence R-Smads, and ultimately affect the TGF-β pathway. IFNγ up regulates Smad7, an inhibitory Smad, growth factors affect transcription of Smad proteins, and activation of the MAPK pathway can inactivate R-Smads. Adapted from (Massague et al., 2005).

In addition to transducing TGF-β signal, Smads also provide cross-talk mechanisms among other important pathways; for example, MAPK (Mitogen-Activated Protein Kinases) can

phosphorylate R-Smad to prevent its activation (Attisano and Lee-Hoeflich, 2001). Some cross-talk mechanisms can even further increase the magnitude of effects, Ras signalling being one example that often cooperates with TGF-β pathway to affect cellular functions (Erdogan et al., 2008).

1.2.3.3 Non-canonical Pathways of TGF-β signalling

Pathways of TGF-β signalling that do not follow the canonical pathway are also important for function. The Smad proteins are not the only ones relaying TGF-β signal to the nucleus according to numerous number of studies (Massague, 2008). TGF-β receptors can interact with the Interleukin-1 receptor effector module to activate MAPK signalling cascades (Lu et al., 2007), rather than phosphorylating R-smads. TGFBR-II does not always have to interact with TGFBR-I, as another research suggests; TGFBR-I can activate Par6, independent of the type I receptor, to cause EMT in epithelial cells (Ozdamar et al., 2005). These variant pathways are termed 'non-canonical pathways,' as opposed to the canonical pathway involving Smad participation.

The fact that not only Smads can relay TGF-β signal, point to possibilities of yet unknown compensatory mechanisms or back-up systems that might complicate research. An example can be the Smad4 knock-out mice that did not show any abnormal development in TGF-β target organs, even though TGF-β family receptor ablations did result in abnormalities (Bardeesy et al., 2006). Smad4 is necessary for signalling through the canonical pathway of all TGF-β family members, since it is a common Co-Smad partner for R-smads of both TGF-β family branches (Kang, 2006); however, normal organ development without Smad4 (Bardeesy et

al., 2006) would mean there might be another back-up mechanism that allows signal transduction at a sufficient level.

1.2.4 TGF-β and Cancer

Cancers develop by acquiring inheritable traits (see figure 1.9), such as: 1) self sufficiency in growth signals; 2) insensitivity to anti-growth signals; 3) evasion of apoptosis; 4) angiogenesis; 5) limitless replicative potential; 6) the ability to invade and metastasize (Hanahan and Weinberg, 2000); 7) evasion of immune surveillance. TGF-β can inhibit tumour progression and malignancy, as it keeps cell growth in check by promoting cell cycle arrest/apoptosis in cells that are proliferating abnormally, but also it was documented to aid tumour progression and metastasis in numerous cases (Pinkas and Teicher, 2006). All the seven hallmarks of cancer can potentially be acquired by mutating TGF-β pathway components, and to have control over TGF-β signalling is to have control over cancer.

Depending on functions, the TGF-β pathway can be largely divided into two arms; one arm fighting against cancer and the other helping cancer to progress into late-stage cancer (Massague, 2008). TGF-β can be an advantage to cancer cells because the cancer cells can alter TGF-β signalling by disabling the tumor suppressive arm selectively, which requires keeping the core components intact. The tumour suppressive effect of TGF-β can be abrogated in two ways; mutation of components in the pathway so that the pathway no longer functions, or components can be modified through mutation so that they still function, but selectively. Smad proteins can be mutated to abrogate TGF-β signal, and indeed, Smad4 and 2 mutations are often found in cancers (Attisano and Lee-Hoeflich, 2001). Mutations in the receptor or other Smads are often

present in cancers (Massague, 2008). Abrogating the pathway completely would not give the cancer cells their benefits, and in general, those with modified TGF-β pathways are more problematic than the ones that have lost TGF-β signalling for good (Massague, 2008).

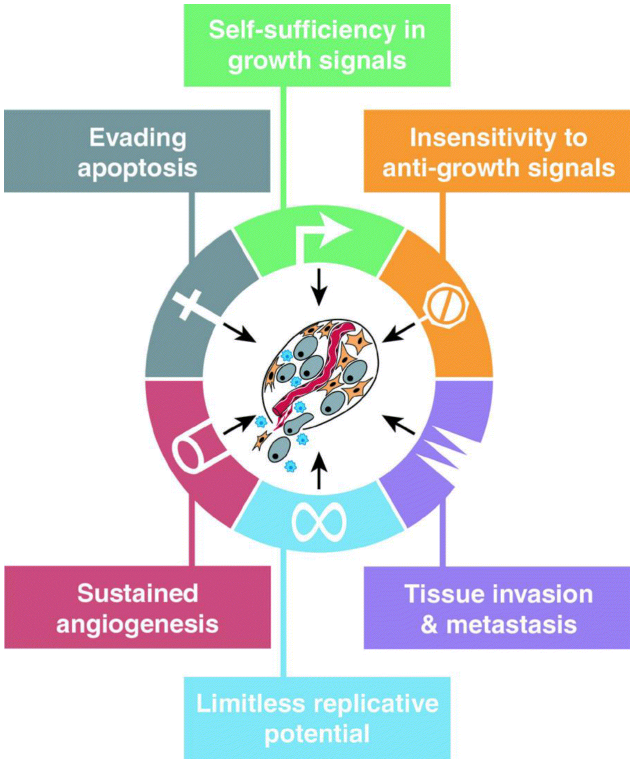


Figure 1.12: The Hallmarks of Cancer. Most cancers acquire all six traits during their development, through various mutations. Modifying the TGF-β pathway can give the cells most of the traits summarized above. Adapted from (Hanahan and Weinberg, 2000).

TGF-β can exert its effects on tumour cells by promoting differentiation, inhibiting cell cycle progression from G1 phase (Massague, 2008). In addition to the direct effects, TGF-β can also have indirect effects on cancer: In sites of injury, TGF-β functions to prevent cell proliferation and inflammation from going out of control and potentially becoming cancerous (Massague, 2008). Also, TGF-β can affect tumours by modulating the immune system, as it also

functions as an important anti-inflammatory cytokine that is essential for proper regulation of any immune response (Massague, 2008). It suppresses stroma-derived mitogens (Bhowmick et al., 2004; Massague, 2008) that may stimulate tumour growth.

1.2.4.1 TGF-β Inhibits Tumour Growth by Cell Cycle Arrest and Apoptosis

TGF-β is often present in the tumour environment, probably in an attempt to inhibit tumour growth (Massague, 2008), as it has been shown to have cytostatic effects or apoptotic effects, depending on the state of the target cell (Siegel and Massague, 2003). In the normal state, TGF-β exerts cytostatic effects to counter stimulation and when the stimulatory signals are overwhelming, TGF-β counters the effect by causing apoptosis (Guasch et al., 2007; Massague, 2008). There is a need for more investigations to determine how much of a stimulatory signal triggers apoptotic effect of TGF-β and what is involved to regulate this process. Another more indirect effect comes from fibroblasts; fibroblasts are known to be an important source of mitogens, and when TGF-β receptor type II was deleted in these cells, the epithelial cells in the vicinity started proliferating, resulting in hyperplasia (Bhowmick et al., 2004).

TGF-β receptor type II is a popular target for alterations among different cancers such as cancer of the colon and the ovaries (Engle et al., 2002; Massague, 2008). Altered methylations and other epigenetic patterns of receptor types I and II can also result in defective signal transduction, and is a common occurrence in lung cancers and bladder cancers, just to name a few (Massague, 2008). The fact that mutations that alter TGF-β pathway are frequent in cancers suggests a tumour suppressive role for the cytokine. Smad3 knockout mice exhibited accelerated healing when wounded, an effect due to rapid proliferation of keratinocytes (Ashcroft et al.,

1999). Similar results were obtained in TGF-β receptor type II knockout experiments in keratinocytes (Guasch et al., 2007). Mutations that cause ablation in the pathway have been shown to facilitate progression of polyps into carcinoma; TGF-β receptor type II ablation or Smad4 mutations alone seemed to be sufficient for the effect (Biswas et al., 2004; Takaku et al., 1998). These findings suggest that TGF-β may be the factor for inhibition of cell proliferation and inhibition of cancer progression.

In addition to loss of function mutation leading to interruption of tumour suppression, constitutively active TGF-β receptor type I inhibits tumour growth (Siegel and Massague, 2003), which demonstrates a more definite tumour inhibitory effect of TGF-β. It is important to note that disengagement of this pathway alone did not seem to be sufficient for tumorigenesis (Zhang et al., 2005). Differentiation is also one of the main effects of TGF-β on cells (Derynck and Akhurst, 2007). This is seen to be the effect of TGF-β on Id proteins (Inhibitor of Differentiation); TGF-β causes Id1 expression down-regulation in epithelial cells (Kang et al., 2003), therefore likely promoting differentiation.

1.2.4.2 TGF-β Aids in Tumour Progression

In the late stages of cancer, cancer cells usually acquire the ability to block negative factors that stunt tumour growth and use host system to their advantage. One of the most important tools is the TGF-β pathway. TGF-β can stimulate malignant progression by affecting cell-cell interactions, namely cell/tissue plasticity and survival, by enhancing angiogenesis, and suppressing the immune system to make it difficult for the host to fight cancer (Cui et al., 1996). As a reinforcing mechanism of homeostasis, TGF-β acts as a growth inhibitor in normal tissues

and in early tumours. However, cancer cells can gain resistance towards inhibitory effects or mutate signalling components of the pathway to disable the system or even use the system to their advantage (Pinkas and Teicher, 2006). It is not surprising that TGF-β core components are found intact in many types of cancers such as breast cancers and melanomas; examinations of breast cancers and melanomas revealed the selective disabling of the cytostatic arm of the TGF-β pathway. The exact components of TGF-β signalling still needs clarification (Massague, 2008),

Cancer cells can tinker the pathway to suit their needs, to eventually render all tumour suppressing properties of TGF-β defective (Massague, 2008; Yan et al., 2002). Evasion of suppression occurs through mutation of components in the pathway, such as receptor mutations and mutations in Smad proteins. Evidence show cancer cells possessing intact TGF-β receptors and Smads, but without any cytostatic response that would normally be present in normal cells (Gomis et al., 2006). Experiments have demonstrated the effects of defective components in the pathway in cell cultures and mice; Smad4 deficient mice developed spontaneous squamous cell carcinomas (Li et al., 2003) and TGFBR-II deficiency was seen to facilitate carcinoma conversion of intestinal polyps (Biswas et al., 2004). Interestingly, some genes that would have been repressed in response to TGF-β have unexpectedly shown an increase in breast cancer cells (Padua et al., 2008), which shows that the cells may be responding to TGF-β, but to respond in an opposite fashion. Metastatic breast cancer cells are shown to express intact TGF-β receptors and Smads, suggesting that they selectively lost the tumour suppressive cytostatic and apoptotic response to TGF-β (Gomis et al., 2006).

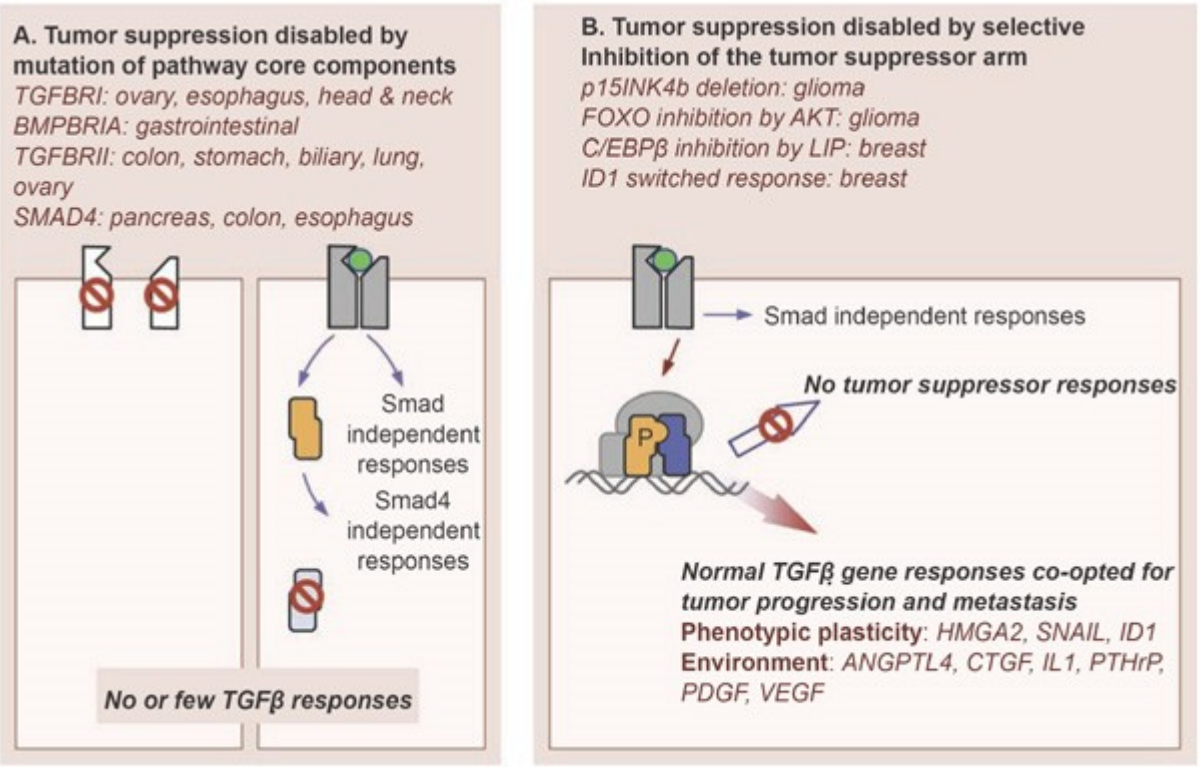


Figure 1.13: Outline of TGF-β mutations affecting normal TGF-β functions. Loss of core pathway results in abrogation of TGF-β effects, whereas selective loss of suppressor arm allows cancer to gain advantages that favour its growth and metastasis. Adapted from (Padua and Massague, 2009).

Angiogenesis

In order for the tumours to grow more than 1mm in diameter, they need to obtain sufficient blood supply, to provide them the necessary nutrients and oxygen, and to eliminate their waste products. Angiogenesis, the process of branching out new blood vessels from an existing one, is a critical step that allows tumour cells to grow beyond 1mm. TGF-β seems to play an important role in angiogenesis as TGF-β pathway component mutants that render the pathway defective were shown to be defective in angiogenesis (Dickson et al., 1995; Larsson et

al., 2001; Oshima et al., 1996). TGF-β was also shown to play an important role in modulating embryonic vascular assembly and in the establishment and maintenance of vessel wall integrity (Pepper, 1997).

EMT and Motility

TGF-β is an important regulator of development; it coordinates EMT (Derynck and Akhurst, 2007), an essential and normal process that takes place during embryonic development resulting in organogenesis and tissue formation (Derynck and Akhurst, 2007). EMT is one of the most important effects of TGF-β, a process that allows organized, non-motile epithelial cells to become disorganized, motile mesenchymal cells, which will give tumors of epithelial origin the capacity to invade and metastasize to distal sites and result in a great decrease in survival of cancer patients (Derynck and Akhurst, 2007; Xu et al., 2009). The changes that occur during the EMT process include increased migration, increased invasion, increased scattering abilities and elongation of cell shape. Mesenchymal markers such as Vimentin, Fibronectin, Snail/Slug, and N-Cadherin increase while epithelial markers such as Cytokeratin and E-Cadherin show a marked decrease.

When cancer cells obliterate the tumour suppressive arm of TGF-β, they gain an enormous benefit; it can use TGF-β to down-regulate the expression of proteins like E-Cadherin that confines them to a fixed location (Derynck and Akhurst, 2007; Thiery, 2003). EMT induced by TGF-β is most likely the key to metastasis of cancer to remote, undisturbed regions of an organism. Over-expression of TGF-β was observed to foster the growth of larger, more metastatic tumours (Stearns et al., 1999), and blocking the signalling caused inhibition of tumour progression and spread (Zhang et al., 2005). Elevated expression of TGF-β 1 was shown to be

linked to lung metastasis of breast cancer in mice which was reversed by blocking TGF-β 1 (Biswas et al., 2007), suggesting a veritable effect of TGF-β 1 in metastasis.

In addition to direct effects on tumour cells, TGF-β was also shown to direct complex processes such as myofibroblasts guiding cancer cells through collagen matrix (Allinen et al., 2004). Altered TGF-β signalling also had indirect effects on tumour cell migration by recruiting helper cells, Gr-1+CD11b+ myeloid cells, which help invasion and metastasis through its enzyme production, most notably MMPs (Matrix-Metalloproteinases) (Yang et al., 2008).

1.2.4.3. TGF-β and the Immune system

The immune system is an elaborate network composed of cells where each component is assigned distinct roles in order to eliminate foreign intruders from the body, which also includes malignant cells (Massague, 2008), as tumour cells are no longer recognized as "self" by the immune system. However, cancer cells can evade that system by employing TGF-β, which is an essential regulator of the immune system that acts mainly by suppression of the on-going immune response and blunting the magnitude of the response (Wrzesinski et al., 2007). TGF-β and its family members were shown to regulate apoptosis in hematopoietic cells by controlling the expression of a phosphatase named SHIP (Src homology 2 domain-containing 5'inositol phosphatase) in a Smad dependent manner (Valderrama-Carvajal et al., 2002).

Although inflammation is beneficial in many instances, chronic and unregulated inflammation is a factor for tumour development (Engle et al., 2002) and other disease. TGF-β defective mice are shown to suffer from uncontrollable inflammation, which often progresses to tumour development (Maggio-Price et al., 2006). Another study conducted with mice that are not

only TGF-β deficient but also lymphocyte deficient, did not develop autoimmunity but still displayed inflammatory hyperplasia in their colon, that eventually progressed to adenoma or adenocarcinoma (Engle et al., 2002), suggesting the importance of an intact lymphocyte population and TGF-β. More studies should follow in order to determine whether severe chronic inflammation alone is sufficient to provoke tumour development, or defective TGF-β had other unknown effects. So far, it seems that the immune system needs to be intact, but the narrow window of activation that is considered 'optimal' must be maintained in order to remain tumour free.

TGF-β prevents excessive inflammation by modulating CD4+ T cells, CD8+ T cells, dendritic cells, NK (Natural Killer) cells and macrophage function (see figure 1.14), playing a protective role (Massague, 2008). One mechanism of TGF-β related suppression of T cell activation seems to include IL-2 (Interleukin-2) inhibition at the transcriptional level, which can protect from chronic inflammation and autoimmune diseases (Wrzesinski et al., 2007).

However, an abundance of TGF-β can be worrisome in the context of malignancy because of its potent inhibitory effects on the immune system; TGF-β is the main factor that allows cancer cells to evade the immune system (Massague, 2008). TGF-β demonstrated negative effects on T cell activation and antigen presentation by the APCs (Antigen Presenting Cells) (Gorelik and Flavell, 2000). TGF-β also down-regulated essential weapons that cytotoxic T cells possess; Granzymes, perforins, and other molecules involved in the cytotoxic program are repressed at the transcriptional level (Thomas and Massague, 2005).

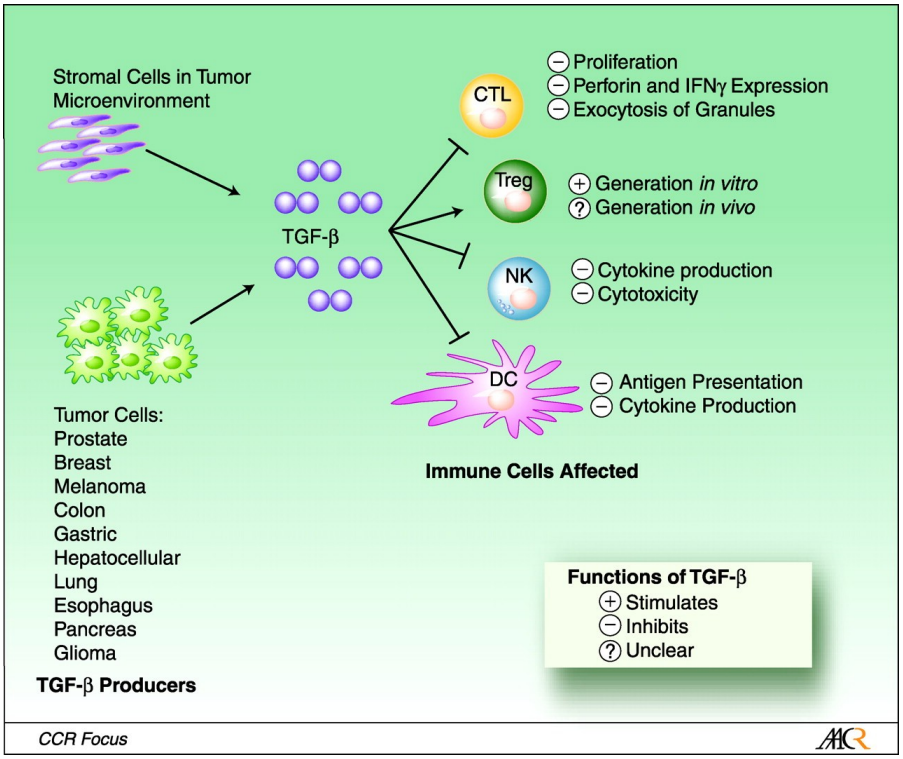


Figure 1.14: Outline of TGF-β effects on the main members of the immune system at the cellular level. The overall effect is inhibitory, which can serve beneficial functions for tumour cells. TGF-β stimulates generation of T regulatory lymphocytes that are immune suppressive, tolerance-inducing cells. TGF-β inhibits generation and proliferation of CTLs (Cytotoxic T Lymphocytes) that are mainly involved in warding off non-self intruders. NK (Natural Killer) cells, which are known to play an important role against cancer is rendered ineffective by TGF-β, and TGF-β also blunts APC (Antigen Presenting Cell) activity. Adapted from (Wrzesinski et al., 2007)

In addition, TGF-β is a factor for development of Regulatory T cells, the cells that can induce tolerance to an antigen, also adding to the anti-inflammatory, pro-tumour effect; this undesirable effect in the context of cancer is observed to reach maximal levels in advanced tumours producing significant amounts of TGF-β (Kryczek et al., 2007). Also, mice engineered to be defective in TGF-β signalling, while developing autoimmune disease was able to clear

tumours that were grafted (Gorelik and Flavell, 2000) although it is not clear whether autoimmune disease and the resulting overactive immune system cleared the tumour graft or if the process was actually specifically tumour-oriented. The mechanisms involved in all these processes are not established yet, and needs further investigation (Thomas and Massague, 2005).

1.2.5 Blocking the TGF-β Pathway

Despite the complexity of dual effects on cancer, there are research groups attempting to control the pathway by the use of inhibitors, although these attempts seem premature since some major mechanisms are not yet elucidated. With more efforts in trying to elucidate the unknown mechanisms, combined with the efforts in drug development, TGF-β signalling pathway holds immense therapeutic potential.

Because high TGF-β levels are found in many tumours and are often associated with a dismal prognosis, research in order to find TGF-β blocking drugs have begun (Wrzesinski et al., 2007), although there is still much lack of information regarding this pathway. Patients that express high levels if TGF-β in the vicinity of their tumour were estimated to be 18 times more likely to suffer from relapse of the disease (Friedman et al., 1995). Autocrine activation of TGF-β pathway in cancer is linked to increased metastasis, invasions, and proliferation (Yan et al., 2002). Neutralization of TGF-β is now proposed as a solution for tumour-derived TGF-β-induced down-regulation of T cells, and ultimately the down-regulation of the immune system (Thomas and Massague, 2005). Blocking TGF-β is predicted to be beneficial in prevention of metastasis, with added benefits of enhancing the uptake of other cancer drugs (Derynck and Akhurst, 2007). Indeed, laboratory observations show while TGF-β increase caused by radiation

resulted in more metastases, this effect was counteracted by administration of TGF-β neutralizing antibody (Biswas et al., 2007).

However, based on discoveries covered in previous sections, the thought of blocking TGF-β altogether with the use of blocking agents is difficult to fathom. An organism consists of various cell types and tissues in different environments that can be variously affected by TGF-β. TGF-β signalling system, a highly contextual program regulating a plethora of genes, cannot be expected to be turned on/off at will and not have any serious side-effects. Although clinical trials so far have shown promising results and no side effects serious enough to stop the program (Yingling et al., 2004), the long term effects are not known. As years pass with clinical trials it would not be surprising when TGF-β blocking drugs present some serious side effects such as autoimmune diseases and improper organ function, knowing that there are diseases associated with insufficient TGF-β signalling such as the Marfan syndrome and Loeys-Dietz syndrome that result in vascular abnormalities (Loeys et al., 2005).

To block the whole pathway altogether without discrimination, there are still too much unknowns that can lead to unexpected, undesirable effects. The difference between the isoforms of TGF-β needs recognition, and unknown mechanisms such as that of the T cell activation suppression by TGF-β (Thomas and Massague, 2005) should be elucidated to obtain a more efficacious drug with fewer side effects. Every single component of the TGF-β signalling pathway is possibly a subject of mutation, and alterations can also occur at the epigenetic level (Massague, 2008). Mutations that alter component characteristics may be the most important cause of differential effects of TGF-β on tumour tissues. Overall, effects seem to vary depending on cell types and lines, which would mean effects of TGF-β on certain cells, cannot be assumed before actually testing on the cells. Also, judging from the complexity of the system, species

variations are also likely to exist, which is a point to consider since most *in-vivo* experiments are done in mice. Other animal systems must be tested to select the ones bearing most similarities to humans to conduct more meaningful experiments.

1.3 Overview of Menin

Menin is the product of MEN1 (multiple endocrine neoplasia type 1), and is a novel protein with no obvious function (Yang and Hua, 2007). Several partners are proposed and therefore several roles, but the exact functions of the protein is yet to be unveiled (White and Doherty, 2008). Up to date, the most prominent role it might play seems to be cell-growth regulation, as researchers have seen a noticeable effect of Menin knock-out/reconstitution on cell cycle transition, apoptosis, and levels of expression of cell cycle regulators like p18 INK4C, p27Kip1, and cyclin D1 (Hussein et al., 2007). It has also been shown that mutational inactivation of Menin can be linked with the loss of growth control in many types of tissues, suggesting that the gene may play an important role in tumorigenesis in general, not limited to MEN1 affected families with endocrine tumors (Marx et al., 1999a).

The term Multiple Endocrine Neoplasia 1 was coined in 1968 (Steiner et al., 1968), and is used to describe a disorder that involves developing relatively uncommon endocrine tumours in two or more hormone producing tissues (Piecha et al., 2008; White and Doherty, 2008). The gene most commonly found to be mutated in the afflicted patients was identified on chromosome 11q13 and cloned in 1997, which was named MEN1 after the disease (Chandrasekharappa et al., 1997; Lemmens et al., 1997).

1. 3. 1 The MEN1 Gene and its Product Menin

1.3.1.1 Multiple Endocrine Neoplasia

MEN1 is a disease that results from mutations in the MEN1 gene, which results in deficiencies in the functional Menin protein. The prevalence of this disease is 3 per 100,000 with the same rates for both males and females (Dreijerink et al., 2006). Multiple tumors develop either in one tissue or several tissues, and they tend to reoccur in MEN1 (Piecha et al., 2008), but the most prominent feature of this rare condition is the development of "the three P's:" Parathyroid hyperplasia, pancreatic endocrine tumors of islet cells, and pituitary endocrine tumors (Brandi et al., 2001; White and Doherty, 2008). To be classified as a MEN1 case, at least two of the three P's have to be present (Brandi et al., 2001; White and Doherty, 2008). The three P's are the most common conditions affecting MEN1 patients, although they are not the only afflictions. Non-endocrine tumors are relatively rare, but they do occur. Most MEN1 tumors are well-differentiated, histologically benign, and are rarely metastatic. The symptoms include hyper-secretion of hormones and mass effect (Piecha et al., 2008). This is a devastating disorder as half of all MEN1 patients are expected to die before they reach the age of 50 (Doherty, 2005).

The disease can be either hereditary or sporadic (Dreijerink et al., 2006), where hereditary MEN1 shows an autosomal dominant pattern of inheritance (Piecha et al., 2008). Sporadic cases are extremely rare, as this implies "two-hits" made on separate organs independently (Dreijerink et al., 2006; White and Doherty, 2008). This type of pattern can be seen in tumors that are associated with mutations in a tumor suppressor gene and can be explained by the two-hit hypothesis, the loss of both alleles leading to tumor development if a gene is a tumor suppressor (Knudson, 1971), the classic example being the Retinoblastoma gene.

The mutations that cause inactivation of Menin, of which 400 different ones were found (Dreijerink et al., 2006), are spread throughout the gene without any hot-spots and there is no genotype to phenotype correlation, which complicates research (Guo and Sawicki, 2001; Marx et al., 1999b; Tsukada et al., 2001). However, as several groups have developed and are working on knock-out models (Hussein et al., 2007; Ji et al., 2007) there will be increasingly more information about Menin available.

Carriers of MEN1 mutations must be examined periodically for any tumor development as it is only a matter of time for the carriers to develop MEN1 related tumors (Piecha et al., 2008). The penetrance of MEN1 at the age of 50 is near complete (Dreijerink et al., 2006; White and Doherty, 2008) and although most MEN1 related tumors are benign, the lack of regular checkups can lead to serious complications and even earlier death (Piecha et al., 2008). The screening procedure consists of a series of biochemical tests to detect anomalies in serum calcium levels, serum Parathyroid hormone levels (PTH), fasting gastrin levels, fasting glucose levels combined with computed tomography, endoscopy, MRI imaging (Piecha et al., 2008).

1.3.1.2 Menin

Menin is located on chromosome 11q13 in humans and it is highly conserved among humans, mice, rats, Zebrafish and even fruit flies (Dreijerink et al., 2006). The gene is composed of 10 exons, which encodes for the 610 amino-acid protein Menin (Chandrasekharappa et al., 1997; Piecha et al., 2008). It is a 67kDa nuclear protein that is expressed ubiquitously and abundantly throughout the body (Guru et al., 1998). Higher levels of expression are observed in actively proliferating cells, and there is a difference in localization in meiotic cells compared to

somatic cells (Suphapeetiporn et al., 2002) which suggests different regulation systems are at work in different cell types and Menin might even play a different role depending on the site.

Menin, has been shown to interact or bind numerous partners (White and Doherty, 2008), most of them being nuclear factors. It was shown to bind transcription factors, DNA processing machinery, cytoskeleton-associated proteins and components of DNA repair mechanisms (Agarwal et al., 1999; Piecha et al., 2008; Poisson et al., 2003; Sukhodolets et al., 2003). It was also shown to directly bind DNA in a non-specific manner (Attisano and Lee-Hoeflich, 2001; La et al., 2004). Mutations in MEN1, especially the ones near the C' terminus prevented DNA binding and caused the cell to fail at repressing cell proliferation, cell cycle progression at the G2/M phase (La et al., 2004). The positive charges in the two NLS regions were shown to direct DNA binding as well as repressing cell proliferation (La et al., 2004).

Menin is widely recognized as a tumour suppressor protein based on clinical and experimental observations (Piecha et al., 2008). Inactivation of Menin results in increased proliferation, cell cycle progression, and inhibition of apoptosis (Schnepp et al., 2006), and all these effects were reversed when Menin was reconstituted (Hussein et al., 2007), which supports its putative identity as a tumour suppressor. Menin seems to act as either a factor in the DNA repair process or as a protective factor that prevents DNA damage (Sakurai et al., 1999). It is a critical factor in the TGF-β pathway for inducing cell cycle arrest in pituitary tumour cells (Kaji et al., 2001). Interestingly, a Menin-null cell line developed and observed in a study displayed altered ECM component expression; those ECM components that are important for the developing heart and also known to be regulated by TGF-β, were decreased (Ji et al., 2007). The same cell line was shown to respond poorly to TGF-β (Ji et al., 2007).

1.3.2 Menin Structure and Sites of Importance

Menin is a 67kDa nuclear protein that does not share any homologies with any other known protein. There are no clear functional domains, although there is some evidence that suggests it is a GTPase protein. It contains 2 Nuclear Localization Signals, and has numerous potential phosphorylation sites. The regulators that control Menin phosphorylation are not known at this point, but the major phosphorylation site of Menin seems to be S543 and some phosphorylations occur on S583, both being serine residues (MacConaill et al., 2006).

Mutations in Menin tend to cluster around JunD-interacting areas of Menin (Agarwal et al., 1999). JunD binding region was reported to have no effect on Smad3-Menin interaction (Kaji et al., 2001). Nonsense mutations occurs more frequently in exons 2, 4, 7, 9, and 10 (Wautot et al., 2002). In the two NLS regions, no missense mutations were reported, despite the amount of mutations described (Wautot et al., 2002).

1. 3. 3 Proposed functions of Menin

Menin interacts with numerous other partners including Jun D (Agarwal et al., 1999; Gobl et al., 1999), NF-κB (Heppner et al., 2001), and MH2 domain of Smad3 (Kaji et al., 2001; Sowa et al., 2003) of the TGF-β pathway and others such as Smads responsible for BMP signaling in bone cells (Hussein et al., 2007; Sowa et al., 2003). Not surprisingly, Menin was reported to exert control over gene expression of several genes such as telomerase (Sukhodolets et al., 2003), several hormones (Namihira et al., 2002; Sayo et al., 2002), and cyclin dependent

inhibitors like p18 INK4C and p27 Kip1 (Fontaniere et al., 2006; Karnik et al., 2005; Milne et al., 2005). In most cases, Menin was shown to control genes through affecting transcription, involving epigenetic regulators (Dreijerink et al., 2006), linking signalling pathways to chromatin modification.

It has been shown many times that the "Loss of Heterozygosity" of Menin stimulates tumor formation in endocrine tissues, but the effect is not clear in non-endocrine tissues. Menin was shown to be involved in multiple processes like chromatin remodeling and gene transcription, but unfortunately the exact function of Menin still remains elusive and the effect of mutations in MEN1 gene is not clear, especially in non-endocrine tissues. Factors like JunD exert different effects on the cell depending on the presence of Menin; In the presence of Menin, JunD acts as a growth suppressor (Marx, 2005), wherease if Menin is absent from the environment, JunD acts as a growth promoter (Agarwal et al., 2003; Knapp et al., 2000). Some researchers have labelled Menin as a general regulator of transcription (Scacheri et al., 2006).

Menin as a GTPase

Menin is considered as a tumor suppressor protein with a nuclear localization. It also exhibits efficient GTPase activity in the presence of a GAP named nm23, a cytoplasmic GTPase activating protein for the Ras related GTPase Rad (Ohkura et al., 2001; Yaguchi et al., 2002). Nm23 is a putative metastasis suppressor (Steeg et al., 1988) that belong to a family of nucleoside diphosphate kinases that are major suppliers of nucleoside triphosphates in the cell using phosphates from ATPs (Yaguchi et al., 2002). Nm23 and Menin, when they are separate, were shown not to have any GTPase activity at all (Yaguchi et al., 2002). Further adding to the evidence, Menin was shown to have a low-affinity but specific binding to GTP in a magnesium-

dependent manner as with other GTPases (Feuerstein et al., 1987), and upon examining its sequence GTP-binding sequence motifs present in all known GTPases (Yaguchi et al., 2002). However, the biological significance of Menin's GTPase activity remains unknown.

Menin as a pro-apoptotic factor

Among many of its proposed roles, Menin was observed to be important in apoptosis, one of the most important protective mechanisms against tumour formation. Over-expressing Menin was shown to increase apoptosis in an insulinoma cell line (Sayo et al., 2002). A similar experiment using a murine cell line demonstrated over expression of Menin can actually trigger apoptosis (Schnepp et al., 2004). Loss of Menin in cells hindered initiation of apoptosis triggered by tumour necrosis factor α (TNF- α) and UV irradiation, and replenishing Menin in those cells restored their ability to go through apoptosis (White and Doherty, 2008).

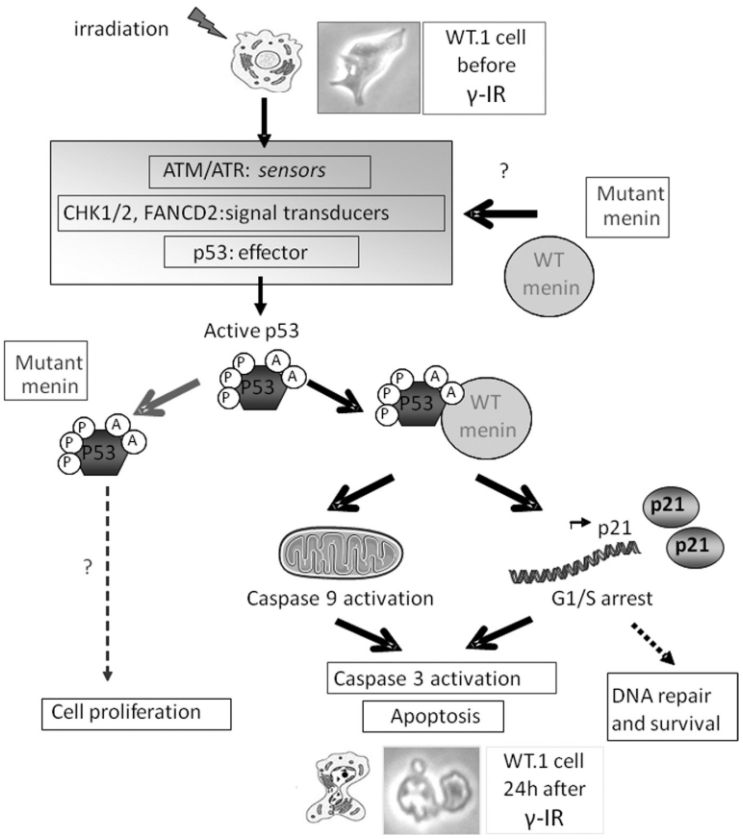


Figure 1.15: Menin contributes to apoptosis. Menin was shown to play a role in apoptosis by activating transcription of Caspase 8, an initiator caspase. It is also involved in activation of Caspase 9, another initiator Caspase, and p21 that plays a role in DNA repair or apoptosis. Adapted from (Bazzi et al., 2008).

Evidence suggests that Menin might act as a pro-apoptotic factor by inducing the expression of caspase 8 (Varfolomeev et al., 1998), which is an essential initiator caspase. Menin binds to the 5' untranslated region (5' UTR) of the Caspase 8 gene and activates transcription, while the absence of Menin reduced acetylation of the Caspase 8 gene (La et al., 2007). Menin mutants were found to lose their ability to bind to the 5'UTR of the Caspase 8 gene, failing to activate Caspase 8 gene transcription and TNF-α mediated apoptosis (La et al., 2007).

Menin causes cell cycle arrest and growth inhibition

Menin is shown to act as an important factor in cell cycle, by modulating the expression of cyclin D1 gene, cyclin D3 and CDK4 (cyclin dependent kinase 4) (Ratineau et al., 2004), all of which forms a kinase complex that promotes G1 to S transition (Sherr, 1996). Also, evidence suggests that Menin plays a role in TGF-β mediated growth inhibition by participating in transcriptional regulation (Guru et al., 1998) and cell cycle control (Kaji et al., 1999) brought on by TGF-β. Menin re-expression in Menin deficient cell lines demonstrated that Menin may be an important factor in cell cycle arrest at G1 phase (Schnepp et al., 2006).

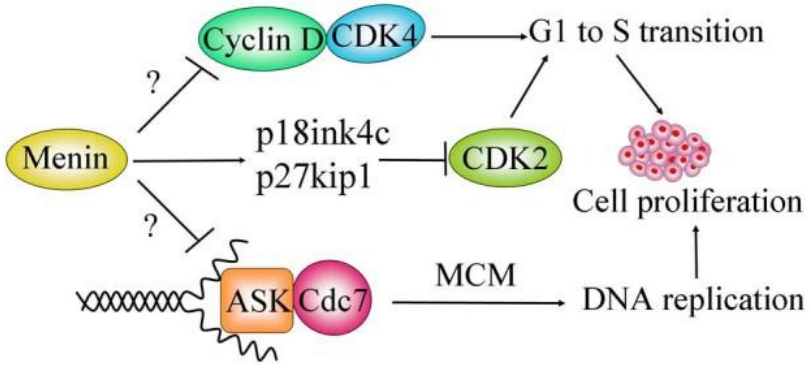


Figure 1.16: Menin May Regulate Cell Cycle Transition. Menin was shown to down regulate genes that play important roles in cell cycle transition from G1 to S phase, such as Cyclin D, CDK4 and ASK (initiator of DNA replication). Menin up regulates CDK inhibitors p18 and p27, further preventing cell cycle transition. Adapted from (Yang and Hua, 2007).

Menin as a protector of chromosomes

Genomic stability seems to involve Menin, as Menin was shown to be linked in many cases with increase stability. MEN1 patients carry chromosomal breakage even in their "normal" cells (Gustavson et al., 1983; Scappaticci et al., 1991). Menin also interacts with DNA repair

proteins such as RPA2, FanCD2 (a protein involved in a BRCA1 – mediated DNA repair pathway), and CHES1 (Busygina et al., 2006; Jin et al., 2003; Sukhodolets et al., 2003). Even *Drosophila* was shown to require its Menin homolog for a proper stress response (Papaconstantinou et al., 2005) and the absence of its Menin homolog made it sensitive to DNA damage (Busygina et al., 2004).

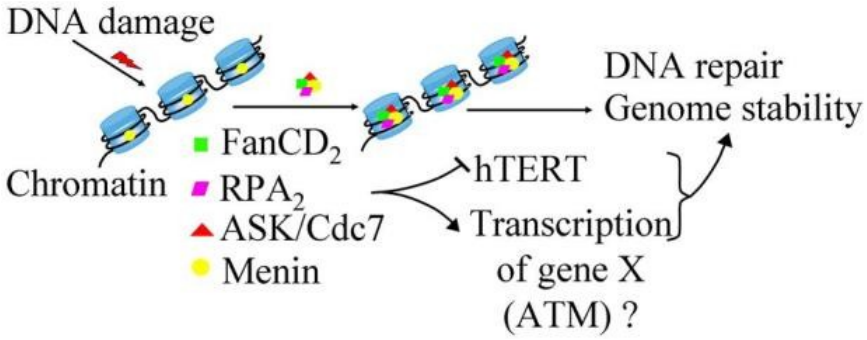


Figure 1.17: Menin may be a required factor for Genome stability. Menin was shown to interact with various nuclear factors that are known to be crucial for DNA repair processes and in sensing DNA damage. Menin was shown to increase its affinity for FanCD2 upon DNA damage. The interaction results in inhibition of hTERT transcription and possibly an enhanced transcription of a factor responsible for increasing genomic stability. Adapted from (Yang and Hua, 2007).

Menin in hormonal regulation

Menin is likely to be an important factor for hormonal regulation, as the lack of the protein is often linked to endocrine disorders, especially the ones concerning the pituitary gland (Namihira et al., 2002; Yang and Hua, 2007). One way that Menin achieves this is by acting as a key regulator in signalling events of Activin, a TGF-β family member (Lacerte et al., 2004).

Menin was shown to regulate development and maintenance of lactotrope cells of the pituitary gland, and suppress secretion of the hormone prolactin in response to Activin, together with the Smad proteins that are responsible for relaying TGF-β signal (Lacerte et al., 2004; Lebrun, 2009; Namihira et al., 2002). As it does downstream of TGF-β signalling, Menin cooperates with Smad proteins to control expression of key regulatory factors to ultimately control pituitary prolactin levels (Lacerte et al., 2004).

1. 3. 4 Menin in the TGF-β pathway

Menin has been shown to cooperate with TGF-β to supress cell proliferation (Kaji et al., 2001), suggesting a role for Menin in the TGF-β mediated effects. The most important evidence is that Menin binds physically and specifically to Smad3, the major component of the TGF-β pathway (Kaji et al., 2001). The effect of Menin is known to occur downstream of Smad3 and Smad4 interaction, and it is believed to aid binding of the Smad complex to the DNA (Kaji et al., 2001). Other effects of TGF-β might require Menin as a study has shown that in MEFs (Menin-null Mouse Embryo Fibroblasts), expression of ECM proteins that are known to be induced by TGF-β were decreased (Ji et al., 2007). Inactivation of Menin was shown to specifically promote endocrine tumour formation (Hu et al., 1998; Kaji et al., 2001), which suggests that the TGF-β pathway is not the only one Menin is participating in, and there is likely to be a tissue specific component in regulating Menin.

1. 3. 5 A possible regulatory mechanism for Menin

Menin is largely known as a nuclear protein, but it was shown to have a different cellular localization depending on the cell cycle. It is expressed ubiquitously, and most of the roles it was associated with are not considered tissue-specific (Dreijerink et al., 2006), which is curious since mutation of MEN1 results in endocrine diseases more than non-endocrine diseases. Expression levels of Menin was shown to fluctuate according to cell cycle, and the expression of Menin increased as cells entered S phase (Kaji et al., 1999). TGF-β can stimulate Menin expression with as short as 30minutes of stimulation, and was observed to reach the maximum from one to three hours in rat pituitary cell line GH4 (Kaji et al., 2001). Recently, type III intermediate filaments Vimentin and Glial fibrillary acidic protein(GFAP), have been shown to interact with Menin, suggesting they might play a role in regulating the cellular localization of the protein, which seems to differ according to the cell cyles (Lopez-Egido et al., 2002).

1.3.6 Menin in other pathways

Other than the TGF-β pathway, Menin also seems to be essential in the canonical Wnt/ β-catenin signalling in tumour cells. Menin physically binds to the proteins involved in the pathway such as β-Catenin and TCF3 (Chen et al., 2008). It modulates downstream target genes via trimethylation (Chen et al., 2008). Menin also plays a role in bone development, and it is required for the commitment of multipotential mesenchymal stem cells, suggesting it plays a role in the BMP pathway by interacting with BMP regulated Smads such as Smad1 and Smad5 (Hendy et al., 2005).

1.4 Objectives and Hypothesis

TGF-β has anti-tumor effects during premalignant stages, as it can promote cell cycle arrest, apoptosis in rapidly proliferating tumor cells. However, it can facilitate cancer progression in the late stages due to its ability to elicit trans-differentiation (EMT), by permitting the tumor cells to gain motility and invasive properties characteristic of a mesenchymal cell type. Menin was repeatedly shown to act downstream of many TGF-β family members (TGF-β, Activin, BMPs) to regulate some of these growth factor biological effects, particularly at the level of tumor suppression (Hendy et al., 2005; Kaji et al., 2001; Sowa et al., 2004). However, a potential role for menin in mediating the TGF-β pro-metastatic responses has not yet been investigated. My hypothesis is that menin, a known tumor suppressor, may also function as a factor for progression of cancer by participating in the TGF-β signaling pathway and its tumor progressive arm. My research project specifically aims at elucidating the role and contribution of menin downstream of TGF-β -induced cell migration, invasion and EMT in aggressive breast cancer cells.

1.5 Thesis Organization

The introduction of this thesis covered necessary facts and recent findings that need to be discussed in order to have a deeper understanding of the topics presented. Chapter II explains all the reagents and procedures that were used. The results are presented in Chapter III and subsequently discussed in Chapter IV. Finally, in chapter V, the thesis is summarized and some suggestions for the future are given.

Chapter II: Materials and methods

2.1 Cell culture

All cell lines used are TGF-β responsive cells that have intact pathway components. Human mammary adenocarcinoma cell lines MCF7 (low invasiveness, many breast tissue-specific markers are intact), MDA-MB-231 (invasive phenotype, and many breast cell markers are lost), SCP2 (a metastatic subclone of MDA-MB-231, metastasizes to the bone), and SCP25 (a metastatic subclone of MDA-MB-231) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO) supplemented with 10 % Fetal Bovine Serum (FBS, GIBCO), 2mM L-Glutamine (GIBCO), 1000 units/ml penicillin and 100μg/ml streptomycin (GIBCO). NMuMG (Normal mouse mammary gland) epithelial cells, which are good models for studies involving cell adhesion and growth factors, were cultured in DMEM supplemented with 10% FBS, 2mM L-Glutamine, 1000 units/ml penicillin and 100μg/ml streptomycin, and insulin. Cells were grown at 37 ºC in a humidified atmosphere containing 5 % CO_2. Cells were passaged every third day (three times a week). Cells were detached from cell culture plates using trypsin (0.25%, Wisent), followed by washing with DMEM containing 10% FBS to neutralize the trypsin.

2.2 Plasmid constructs

The 3TP-lux reporter construct was a gift from Dr. Joan Massague (Memorial Sloan-Kettering Cancer Center, New York). This promoter construct is a tool developed specifically for measuring TGF-β activated gene transcription activity. The construct contains TGF-β responsive

element (Smad3 binding sites) of the Plasminogen Activator Inhibitor 1 (PAI-1) promoter region and is used to assess TGF-β/activin response.

2.3 Reagents

Human TGF-β1 #100-21R was purchased from Pepro Tech Inc. Rabbit monoclonal antibody against Menin was purchased from Bethyl Inc. Goat anti-rabbit Horse Radish Peroxidase (HRP) were purchased from Santa Cruz Biotechnology Inc., CA. A-sepharose, G-sepharose beads were purchased from Amersham Biosciences/GE Healthcare (Quebec, Canada). Affinity purified rabbit anti-Menin antibody (A300-105A) was purchased from Bethyl Laboratories Inc. Anti Zo-1, N-cadherin, E-cadherin, Vimentin were bought from BD Biosciences. Anti β-tubulin (3F3-G2), Smad 2/3 (N19), and Smad 3 (FL425) antibodies were purchased from Santa Cruz. Anti αParp antibodies (33-3100) were purchased from Zymed, and anti-phosphosmad 2 (Ser 465/467) antibodies were bought from Cell Signalling.

2.4 Cell transfections

Menin expression was knocked down using small interfering RNA (siRNA) from Santa Cruz Biotechnology Inc., CA (h: sc-35922, m: sc-35923), using Lipofectamine 2000 (invitrogen). Cells were seeded onto a 6-well plate in DMEM supplemented with 10% serum to let them attach and grow overnight. The following day, the cells were put in DMEM without serum and the cocktail containing the siRNA, Lipofectamine 2000, and Optimem (Invitrogen) was added after. The cocktail was applied overnight, around 16 to 20 hours. The cells were either collected or stimulated in starvation medium at this point. Starving the cells arrests their growth, which is

desireable for stimulation experiments since the reaction to a stimulant can be evaluated in a uniform population.

2.5 Cell viability assay (MTT colorimetric assay)

Cells were trypsinized and plated in 6-well dishes in complete medium (DMEM supplemented with 10%FBS and antibiotics as mentioned under section 2.1). The following day, cells were transfected or not with Menin siRNA (described under section 2.3). The next day, cells were washed with phosphate-buffered saline (PBS 1X) at pH 7.4 (8g NaCl; 2g KCl; 11.5g Na_2HPO_4 H_2O; 2g KH_2PO_4 per liter for PBS 10X), trypsin-digested to detach, and plated in triplicates or quadruplicates in 96-well plates. After allowing the cells to fully attach to the plate surface overnight, human cells were starved in DMEM without FBS supplementation, with or without 100pM TGF-β1. Mouse cells (NMuMG) were starved in DMEM supplemented with 2% FBS.

After a given amount of stimulation/starvation, the yellow tetrazolium salt (MTT) is dissolved in 1XPBS (5mg/ml) and then added to the cells. The dye is successfully reduced only in metabolically active cells to form insoluble purple formazan crystals in the cells. Following 2 hours of incubation at 37ºC, the crystals were then dissolved by the addition of DMSO. Sorenson's Glycine buffer (0.1M glycine, 0.1M NaCl, pH to 10.5) was added to the DMSO-dye mixture to adjust the pH. The amount of color was then quantified by measuring the absorbance at 570nm using a Microplate reader (BIOTEK Instruments Inc.).

2.6 qRT-PCR

2.6.1 RNA extraction

Following stimulation of the cells with TGF-β for an appropriate amount of time, total cell RNA was extracted using Trizol (Invitrogen) according to the manufacturer's protocol. The cells were lysed directly in the culture dish by addition of Trizol and the resulting mix was completely homogenized using a pipette. RNA was extracted by adding Cloroform to the Trizol mix followed by centrifugation. Then the RNA was precipitated adding isopropanol followed by washing with 70% ethanol. The RNA pellets were air-dried, dissolved in RNase free water (GIBCO), and incubated at 65ºC for complete dissolving of the pellet. The total RNA extracts were quantified by measuring the absorbance at 260nm (Optimal density OD 260 nm of 1= 40 µg/ml of single stranded RNA) using a Microplate reader.

2.6.2 Reverse Transcription

cDNA synthesis was carried out using oligo-dT primers, using 2µg of total RNA and M-MLV reverse transcriptase (Invitrogen). Thermal cycling conditions were set as: 60min at 37 ºC, 10min at 99 ºC, and then cool to 4 ºC. The resulting cDNA was diluted with RNase free water (GIBCO) and stored in -20 ºC freezer until use.

2.6.3 Primers

The primers were designed using Primer3 program linked to the National Center for Biotechnology Information (NCBI). Both human and mouse N-cadherin, Twist1 primers were designed using this program, followed by a BLAST(Basic Local Alignment Search Tool) search to confirm specificity. All of them were obtained from Invitrogen.

Human N-cadherin

Foreward primer: TGAAACGCCGGGATAAAGAACGCC

Reverse primer: GGTCTTTCATCCATTCGTCGGATTCCC

Human Twist1

Foreward primer: ACGCCTTCTCGGTCTGGAGGAT

Reverse primer: GCCCCACGCCCTGTTTCTTTGAA

Mouse N-cadherin

Foreward primer: CAAATGCCCTGAATGGAATGCTGCG

Reverse primer: GGCTGTGTTTGAAAGGCCATAAGTGGG

Mouse Twist1

Foreward primer: TACGCCTTCTCCGTCTGGAGGAT

Reverse primer: CCCCACGCCCTGATTCTTGTGAA

GAPDH

Foreward primer: GCCTCAAGATCATCAGCAATGCCT

Reverse primer: TGTGGTCATGAGTCCTTCCACGAT

2.6.4 qRT-PCR

20µl of cDNA was used as template for quantitvative Polymerase Chain Reaction (q-PCR) using home-made *Taq* polymerase enzyme. After a complete denaturing step of 10minutes at 94 ºC, 40 cycles of denaturation, annealing, and elongation were repeated. Cycling conditions used were: 10 seconds at 94 ºC, 10 secondes at 58 ºC, and 15 seconds at 72 ºC. Final melting curve was obtained by heating the samples up to 99 ºC.

2.7 Luciferase Reporter assay

2.7.1 Transfection

For reporter assays, β-galactosidase encoding plasmids were co-transfected with the luciferase reporter construct to serve as an internal control. All the values obtained from the luciferase assay were normalized using values obtained from β-galactosidase activities of each sample. 1µg of each plasmid were mixed into the transfection cocktail as discussed under section 2.4 cell transfections.

2.7.2 Luciferase assay

Cells were washed with cold 1X PBS pH7.4, then lysed and harvested on ice using luciferase assay extraction buffer (1% Triton X-100, 15mM $MgSO_4$, $mM EGTA, 1mM DTT, 25mM glycylglycine, H_2O). After centrifugation and removal of pellet, 5µl of assay cocktail (H_2O, 0.1M ATP, 0.5M KH_2PO_4 pH7.8, 1M $MgCl_2$) is mixed with 45µl cell lysate in a well of white, opaque 96-well plate. Reading is done using luminometer (EG&G BERTHOLD microplate luminometer LB96V) that injects luciferin solution (25mM Luciferin stock, 0.1M KH_2PO_4).

2.7.3 β-gal assay

With the exact same lysates obtained for luciferase assay, β-gal assay was performed. 10µl of cell lysate was mixed with β-gal buffer (60mM Na_2HPO_4, 40mM NaH_2PO_4, 10mM KCl, 1mM $MgCl_2$, 50mM β-mercaptoethanol, H_2O) containing 1.5mg of ONPG per 1ml of β-gal buffer,

in a clear 96-well plate followed by incubation at 37 ºC until the samples develop faint yellow color. The color was measured at 420nm using a Microplate reader.

2.8 Immunoprecipitation

Cell lysates were prepared in the same way as described under 2.8.1 sample preparation up to the centrifugation step, without DTT in the lysis buffer. After removal of pellet, cell lysate samples were mixed with 1µg of antibody [Smad3 FL425 (Santa Cruz), Menin (Bethyl)] and the total volume was adjusted to reach 1ml. The mix was incubated overnight at 4 ºC, while continually shaking. The next day, 40µ of either a 50% protein-A or protein-G sepharose beads (Santa Cruz) mix was added and further incubated in the same condition for 2 hours. After incubation, the beads were then collected and washed three times to remove non-bound proteins. The beads were then mixed with 2XSDS loading dye (Tris-HCl pH 6.8, SDS, glycerol, bromophenol blue) and heated to 100 ºC for 5 minutes to elute protein from the bead. The beads were separated from liquid and were discarded and the remaining liquid containing the separated proteins was kept at -20 ºC until an SDS-PAGE (see under western blot analysis) was ready to perform.

2.9 Western blot analysis

2.9.1 Sample preparation

Cells were washed with cold 1X PBS pH7.4, then lysed and harvested on ice using lysis buffer containing 1% Triton X-100 (50 mM Tris-Cl ph7.4, 150mM NaCl, 1% Triton X-100,

1mM EDTA, 1mM EGTA, 1mM DTT, 100µM $NaVO_4$, 50mM β-glycerophosphate) supplemented with 100µM phenylmethylsulphonyl fluoride (PMSF), 10µg/ml aprotinin, 10µg/ml leupeptin, and 10µg/ml pepstatin. After centrifugation and removal of pellet, the supernatant was mixed with 6XSDS loading dye (125mM Tris-HCl pH 6.8, 2% SDS, 20% glycerol, 0.2% bromophenol blue), 20ul of buffer for 100ul of lysate and were kept at -20 ºC until ready to use. Samples were heated to 100 ºC for 10minutes to completely denature the protein strands.

2.9.2 Protein quantification

Total protein concentration of each sample lysate was measured using Micro BCA Protein Assay Kit (Pierce), according to the manufacturer's instructions. Protein lysates were diluted with water (1 in 50), followed by the addition of BCA reagent and incubation at 37 ºC for approximately 30minutes. A standard is also prepared in triplicates with bovine albumin on the same plate, with the addition of the same reagents. The optical density was measured using a Microplate reader, at 562nm. Readings of samples will be compared to the standard curve obtained to calculate protein concentration.

2.9.3 SDS-PAGE and transfer

Sample lysates containing 80µg of protein were separated by SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis), using electrophoresis apparatus from BIO-RAD. After separation in the gel, proteins were transferred to 0.2 µm nitrocellulose

membranes (BIO RAD) to proceed with detection of specific proteins through immunoblotting. A semi-dry transfer apparatus (The W.E.P. Company) was used, and the proteins were transferred in a puddle of transfer buffer (Tris-Cl, Glycine, methanol) at 15 volts for approximately 75 minutes where the volts were kept constant.

2.9.4 Immunoblotting

The nitrocellulose membrane was blocked by soaking in blocking buffer (10mM Tris-Cl pH7.5, 150mM NaCl, 0.05% Tween 20, 5% non-fat dry milk) for 1 hour. Following a wash, a gelatin solution (10mM Tris pH 7.5, 150mM NaCl, 0.05% Tween20, 0.25% Gelatin, 0.05% NaAzide) containing the appropriate antibody was applied to the membrane and was left gently rocking for 16 hours at 4 ºC. The memebrane was washed with washing buffer (50mM Tris pH 7.5, 200mM NaCl, 0.05% Tween 20) and was incubated in blocking buffer containing secondary antibody linked with horseradish peroxidise for one hour at room temperature. After three 10 minute washes with washing buffer, the membrane is treated with home-made ECL chemiluminescent reagents (luminol, p-coumaric acid, H_2O_2) to visualize the bands detectd by antibodies using an Alpha Innotech imager (Cell Biosciences).

2.10 Microscopy

Cells were grown on 13mm non-coated glass coverslips by fitting the sterilized glass slides on to the bottom of the wells of 6-well plates, filling the wells with DMEM supplemented with FBS and then seeding the cells. When the cells reached 90% confluency, they were transfected as described under 2.4 transfections section. After transfection and appropriate amount of stimulation, cells were fixed with 4% paraformaldehyde in 1XPBS at room

temperature for 15minutes. Fixed cells were permeablized by treating with 0.5% Triton-X-100 in 1XPBS for 30 minutes, washed three times with 1XPBS, and blocked with 4% BSA in 1XPBS for 30 miutes. Then the cells were incubated for 1 hour with the primary antibody diluted in 1XPBS (1:500 dilution of rabbit anti-Menin antibody). After three washings with 1XPBS, the cells were incubated for 1 hour with antibodies linked to a fluorophore diluted in 1XPBS (Alexa Fluor 488 goat anti-mouse IgG H+L, Alexa Fluor 568 goat anti-rabbit IgG H+L, both from Invitrogen). Cover slips were mounted and observed using a LSM-510 Laser scanning microscope (Zeiss).

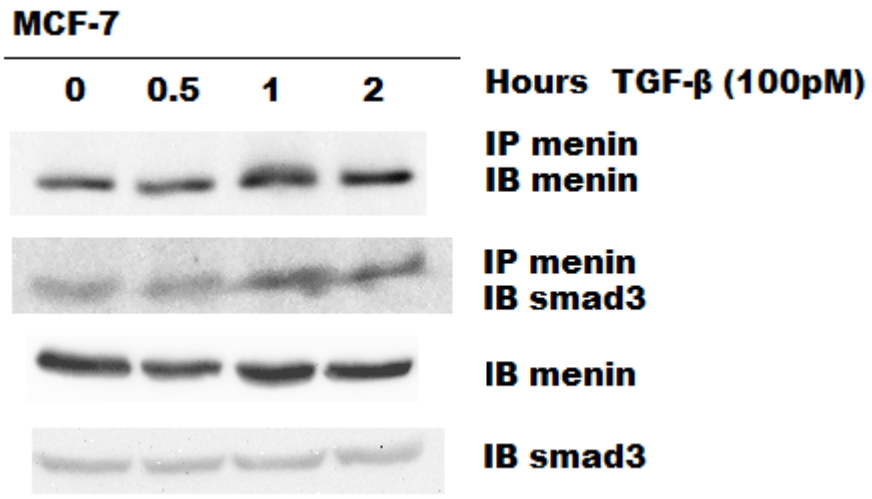
Chapter III: Results

3.1 Menin interacts with Smad3 in Breast Cancer Cells

Menin is a ubiquitously expressed protein that is conserved among a wide variety of species. It seems to act differently in endocrine cells compared to non-endocrine cells, since its mutation is linked frequently with endocrine tumors while non-endocrine tumors are relatively rare. This suggests that there is a tissue-specific regulation of Menin, as it is always present in all types of cells, ruling out the possibility of differential expression of Menin being responsible for the different rates of tumor development in different tissues. Co-immunoprecipitation experiments proved that Menin interacts physically and specifically with Smad3 of the TGF-β pathway in Rat pituitary GH4C1 cell lines (Kaji et al., 2001). To verify whether Menin interacts with Smad 3 in cell lines of breast tissue origin, different breast cancer cell lines MCF-7 and SCP2 (a subclone of MDA-MB-231) that are highly responsive to TGF-β stimulation were used. These breast cancer cell lines exhibit different level of malignancy, SCP2 being highly tumorigenic and metastatic, and MCF-7 being poorly tumorigenic and non-metastatic.

Cells were treated with TGF-β for different lengths of time, and the cell lysates were collected to immunoprecipitate either Menin or Smad3 to immunoblot for the other. The results revealed the interaction of Menin and Smad3 in short time frames but not in a relatively longer, 24 hour time frame (data not shown) suggesting that Smad 3-Menin interaction is quickly induced and relatively short-lived.

A. Non-metastatic Breast Cancer Cells



B. Metastatic Breast Cancer Cells

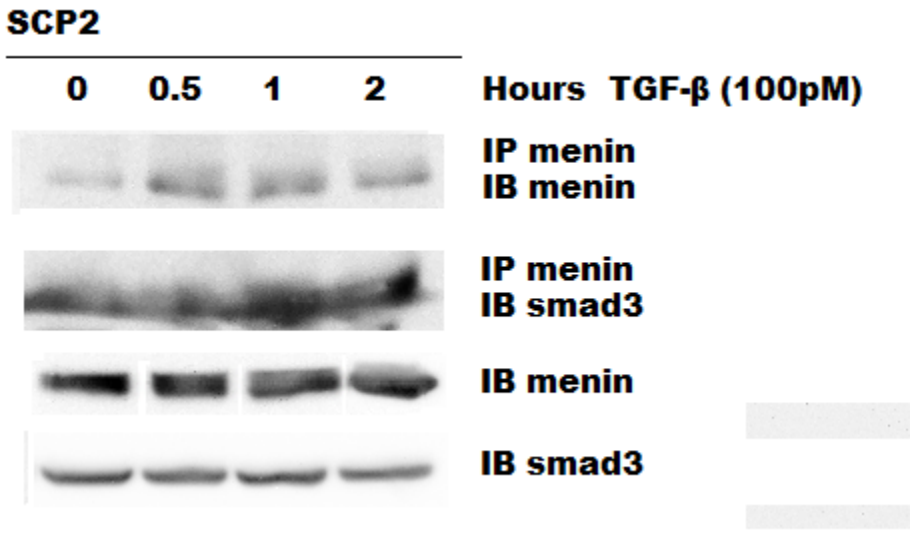
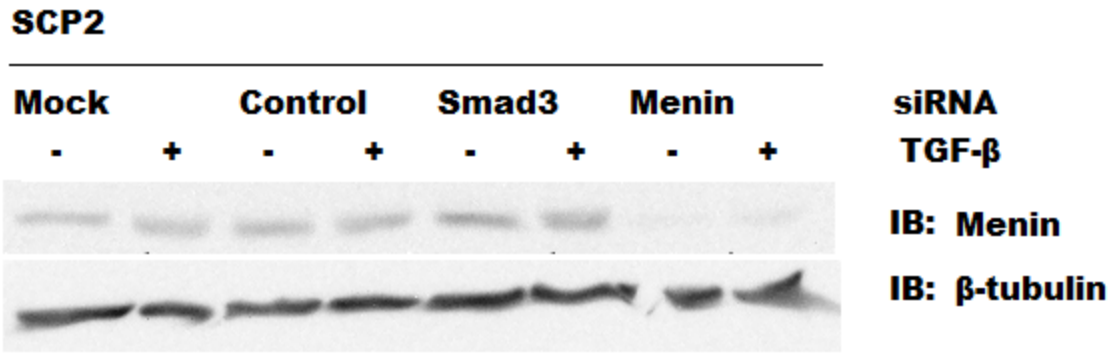


Figure 3.1: Menin interacts with Smad3 in cell lines of breast tissue origin. MCF-7 and SCP2 cells (subclones of MDA-MB-231) were stimulated or not with TGF-β for different lengths of time as indicated. Total cell lysates collected from treacted cells were incubated with either Smad3 or Menin antibodies to immunoprecipitate one and detect the other for confirmation.

A. SCP2



B. NMuMG (Normal mouse mammary gland cells)

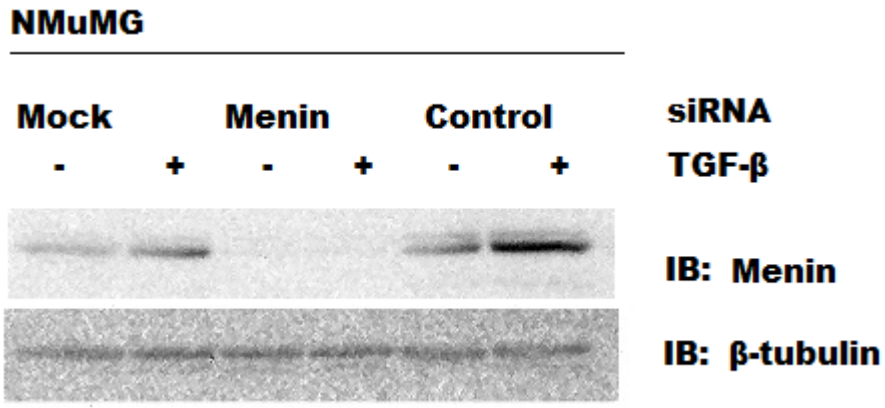


Figure 3.2: Comparison of knock-down efficiencies of Menin siRNA. SCP2 and NMuMG cells were transfected with specific siRNA targeted towards Menin using Lipofectamine 2000. The lysates collected from treated cells were analyzed through western blotting. The membrane was probed with rabbit anti-Menin antibody and mouse anti-β-tubulin antibody. β-tubulin served as loading controls. The lysates analyzed in figure 3.2 B were collected using the leftover extracts from a luciferase assay, shown in figure 3.3.

3.2 Efficiency of siRNA

The choice of siRNA (small interfering RNA) was essential in this study, as it was important to compare and contrast the effects of TGF-β in the presence of Menin and in the absence of Menin. Two types of siRNA were used in this project, one against human Menin and the other against mouse Menin, both purchased from Santa Cruz. Cells were transfected with increasing amounts of Menin siRNA (data not shown) to determine the optimal concentration of siRNA, which was then applied to all subsequent experiments. The optimum level of knock-down was achieved with 60nM concentration of siRNA (data not shown).

As Menin was successfully knocked down by siRNA specific for Menin, siRNA from Santa Cruz was used in the following sections to assess the function of Menin in the TGF-β pathway. The concentration of siRNA in each experiment was set at 60nM. Knocking down Menin did not alter expression of other proteins. Another point to note from this figure is that the amount of Menin protein seems to moderately increase with TGF-β treatment, as it can be shown from both figures. This is in fact consistent with previously published data in the literature (Kaji et al., 2001; Lacerte et al., 2004; Lebrun, 2009).

3.3 Tumor suppressive effects of TGF-β

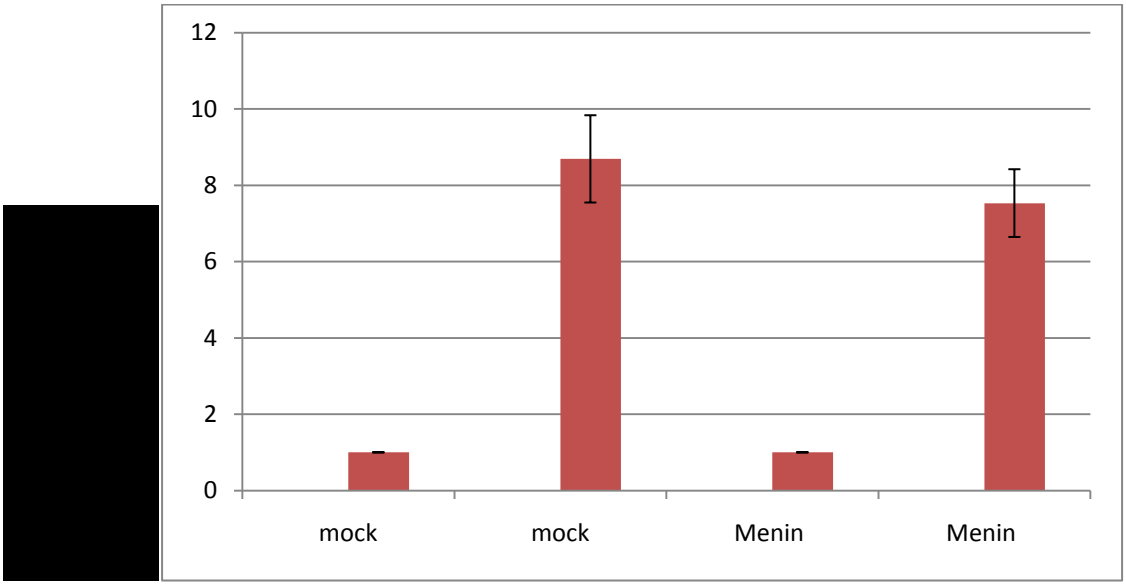
3.3.1 Menin Knock-down effects on TGF-β transcriptional activation

To determine the role of Menin in TGF-β-mediated activation of gene transcription, a TGF-β-responsive gene promoter construct was employed. 3TP-lux contains three consecutive TREs (TPA responsive elements) and a portion of the PAI-1(Plasminogen Activator Inhibitor 1) promoter, which contains Smad 3 binding elements, and it gives sensitive readings which makes it useful for this purpose. Cells were co-transfected all at the same time with promoter constructs

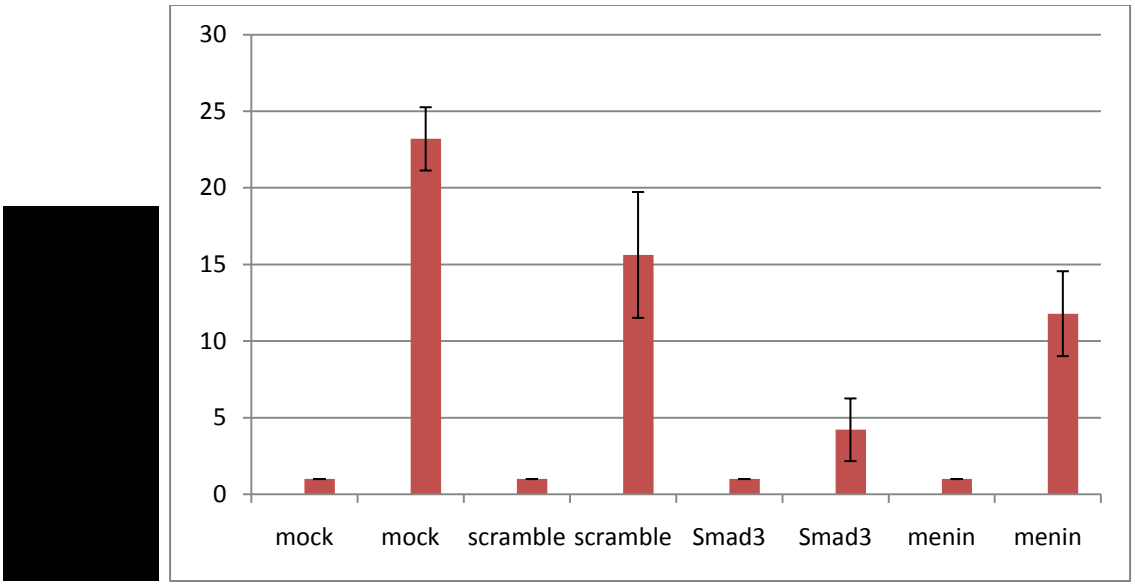
with or without siRNA, without doubling the Lipofectamine reagent compared to siRNA transfections. After 16 hours of treatment, the cells were stimulated or not with TGF-β in serum-free medium without addition of any other growth hormones (see 2.4 cell transfections for more information). Lysates were collected after 24 hours of treatment with TGF-β, and the luciferase activity was assessed.

As shown in the following figure, none of the breast tissue cell lines tested displayed any difference in the level of activity in the presence or absence of Menin. This suggests that Menin might not be essential when it comes to transcriptional regulation by TGF-β in breast cancer cell lines although it was shown to be important in other cell types (Hendy et al., 2005; Sowa et al., 2004), suggesting a possible tissue specific role of Menin.

A. NMuMG

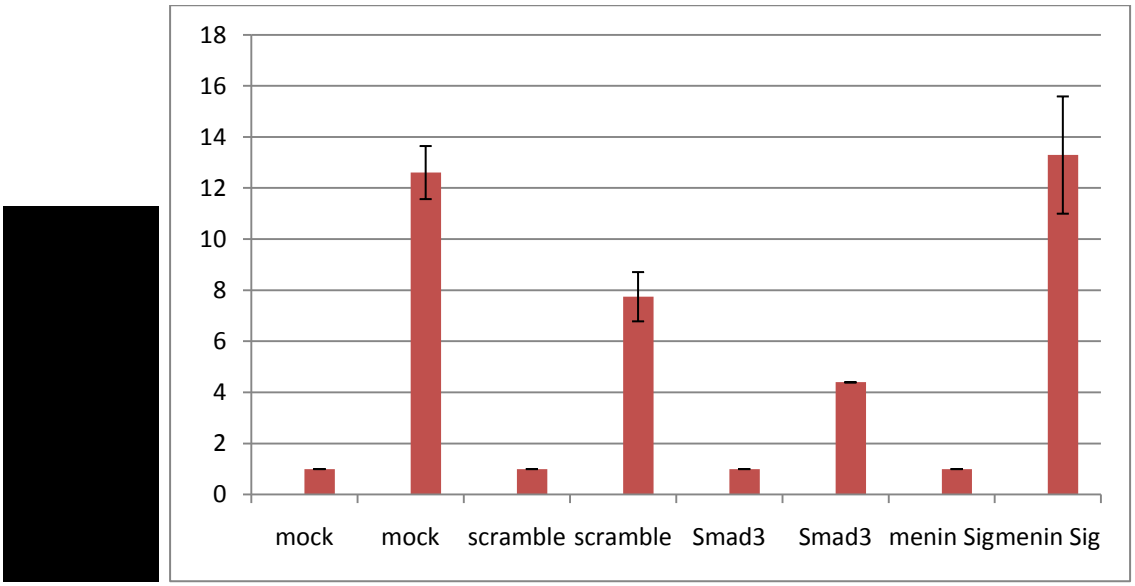


B. MDA-MB-231



TGF-β: - + - + - + - +

C. SCP2



TGF-β: - + - + - + - +

Figure 3.3: Knocking Down Menin in breast cancer cell lines does not seem to affect transcriptional activation brought on by TGF-β. NMuMG, MDA-MB-231, and SCP2 cells were co-transfected with 1μg of 3TP-lux reporter plasmid, 1μg of β-galactosidase expression plasmid as a control, and with or without 60nM Menin siRNA. After 16 hours of transfection, the cells were treated or not with 100pM TGF-β for 24 hours. The TGF-β response was measured by relative luciferase activities and were normalized to the relative β-galactosidase activities. Results represent the average and standard deviations of three independent experiments.

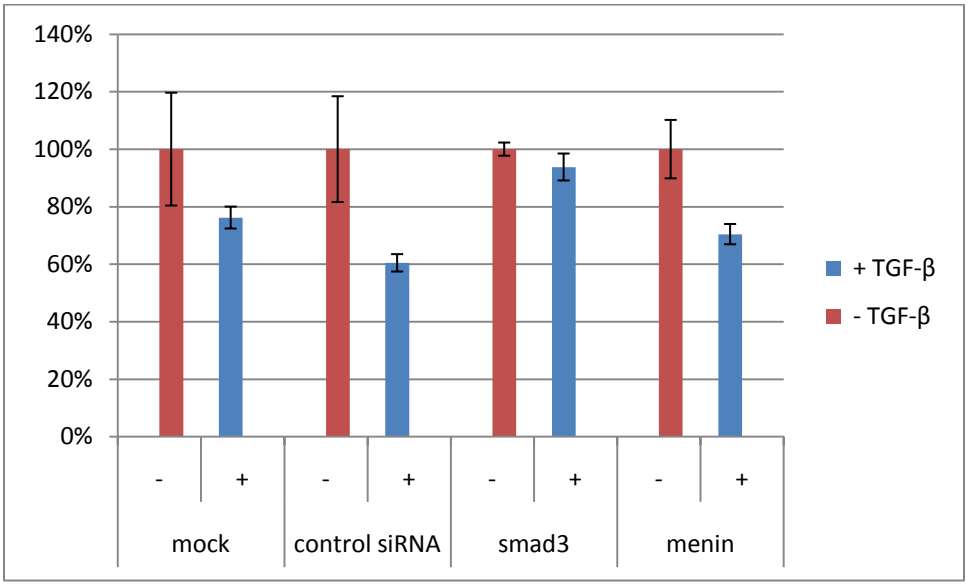
3.3.2 Menin knock-down and TGF-β mediated inhibition of cell proliferation

TGF-β is known to cause cell cycle arrest in various cell lines. To assess the role of Menin in TGF-β-induced cell cycle arrest, cells were transfected or not with Menin siRNA to knock down the levels of the protein. After transfection and stimulation with TGF-β for varying lengths of time (minimum 72 hours), cell viability was measured by performing MTT assay. MTT assay is an indirect method of measuring cell viability, as it measures the amount of water-insoluble purple formazan crystals formed by active enzymatic processing of water soluble tetrazolium dye (MTT).

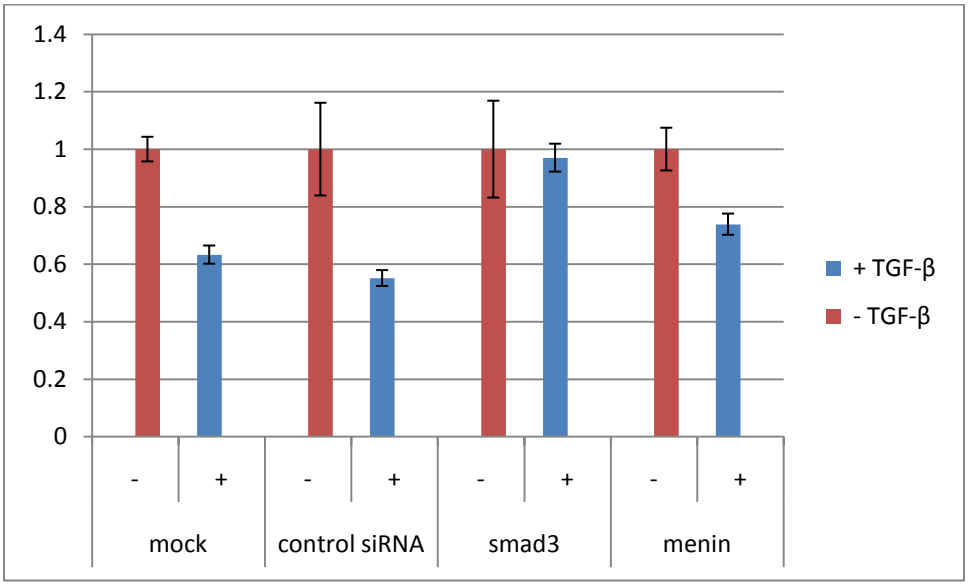
TGF-β stimulation in non-treated cells resulted in 20 ~ 60% decrease in cell viability (growth inhibition). Menin does not seem to affect this aspect of TGF-β effects as it can be seen to make very little difference on cell viability. This suggests that Menin might not play a significant role in TGF-β mediated cell growth control. MDA and SCP2 cells were omitted as they have already selectively lost their response to TGF-β in terms of cell growth control (data not shown).

A) MCF7 breast cancer cells

MTT assay – 72 hours



MTT assay – 96 hours



B) NMuMG

MTT assay – 96 hours

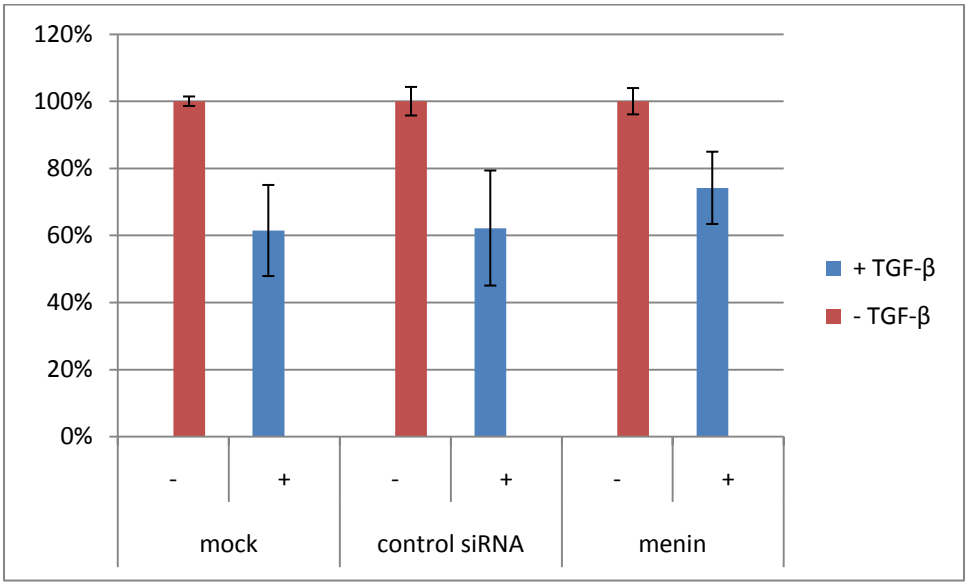


Figure 3.4: Cell viability does not seem to be greatly affected by the levels of Menin. MTT assays were performed on MCF-7 breast cancer cells and normal breast cell line NMuMG. MCF-7 and NMuMG cells were mock transfected or transfected with siRNA specific towards Menin. Then the cells were treated or not with TGF-β for the amounts of time indicated in the figure before assessing cell viability by measuring the amount of resulting formazan crystals. Values are representative of experiments performed in quadruplicates. Results from panel B represent the average and standard deviations of three independent experiments. The units used to depict cell viability are arbitrary units, setting the reading obtained from untreated cells as 100%.

3.4 Tumor promoting effects of TGF-β

3.4.1 TGF-β affects cell migration, cell shape, and EMT

TGF-β is a known factor that causes EMT. EMT is an important process in development, as it can let cells switch phenotypes and form appropriate structures depending on the need.

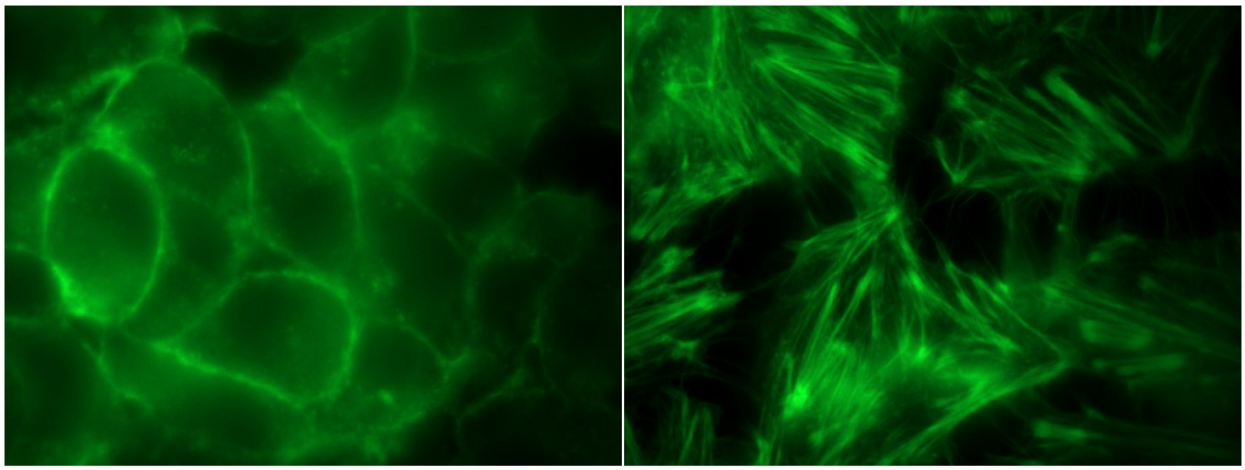
When EMT occurs in tumors, it is dangerous as it gives tumor cells the capacity to invade and metastasize. EMT involves the loss of epithelial cell polarity, separation of cells and acquisition of motility (Vincent-Salomon and Thiery, 2003). EMT can also be noted by the change of Cadherin expressions. The level of Cadherin expression dictates the strength of cell-cell adhesion (Steinberg and Takeichi, 1994), and Cadherin type is known to determine the specificity of the interactions(Nose et al., 1988).

To evaluate the role of Menin in TGF-β, NMuMG and SCP2 cells were used. NMuMG cells were transfected or not with siRNA duplexes specific to Menin to knock-down Menin, followed by stimulation with TGF-β. After 24 hours of stimulation, the cells were stained with EMT markers, F-actin or N-Cadherin, to observe changes under confocal microscope. SCP2 cells were used to evaluate migratory capacities of cells after transfection with siRNA to knock-down Menin.

As shown in Figure 3.5 (A), TGF-β stimulation results in formation of strong stress fibers in relatively thick, organized bundles. This is an important sign of EMT, as stress fibers are traits of fibroblasts, and these fibers are closely associated with cell adhesion and motility (Hotulainen and Lappalainen, 2006). Knock-down of Menin seems to cause weakening of stress fiber bundles, thinning them and shortening them, and weakening of stress fiber directionality can also be observed.These results suggest Menin might play a role in TGF-β mediated EMT and cell motility, through an unknown mechanism that involves actin assembly.

A) Mock transfected cells

TGF-β: - +



B) Menin knocked down with specific siRNA

TGF-β: - +

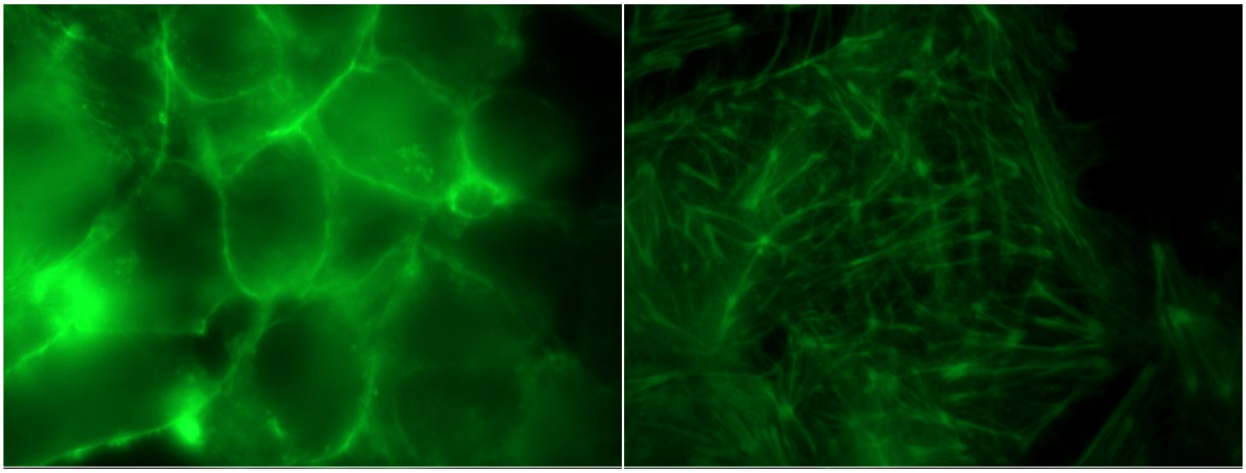


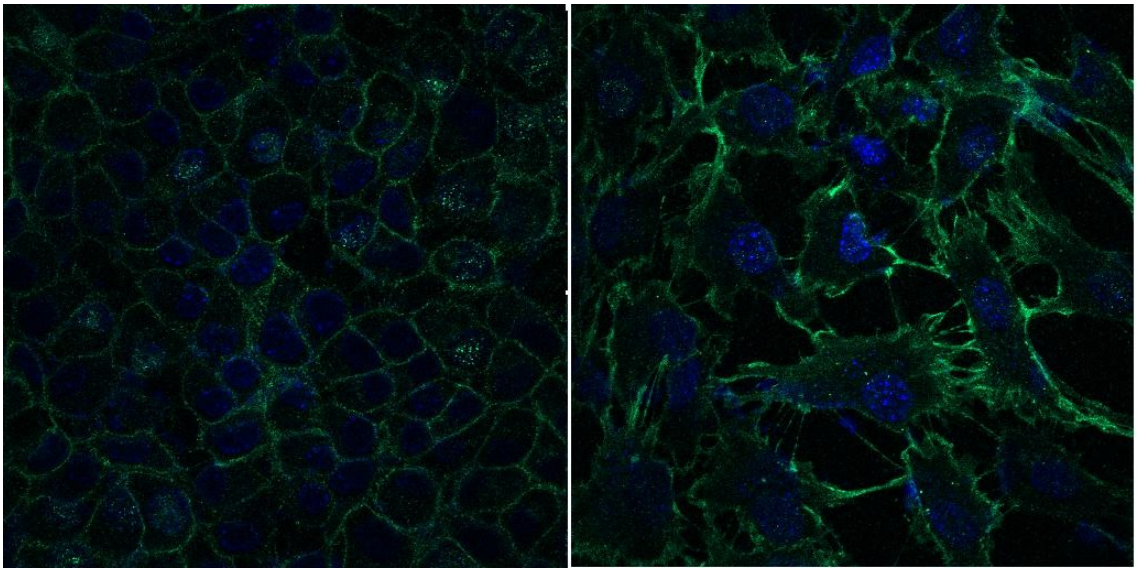
Figure 3.5: Stress fiber bundle (F-actin) formation before and after 24 hours TGF-β stimulation (100pM) in NMuMG cell line. NMuMG cells grown on non-coated glass coverslips (13mm) were mock transfected (A) or transfected with siRNA specific towards Menin (B). Cells were stimulated or not with TGF-β for 24 hours before they were stained using anti-F-actin antibody followed by appropriate secondary antibody linked to green fluorophore. The images shown are representative images selected from at least six pictures taken from different visual fields.

Changes in N-Cadherin levels are one of the classic signs of EMT. TGF-β is known to up-regulate N-Cadherin, a cell-cell adhesion molecule that is associated with cell motility and increased invasiveness of tumor cells: N-Cadherins are known to localize at the ends of F-actin microfilament bundles, and blocking antibody specific towards N-Cadherin are known to suppress spreading and migration of cells, inhibiting wound repair (Jones et al., 2002).

As shown in figure 3.6 (A), TGF-β normally brings about up-regulations in N-Cadherin levels, especially at the tips of the cell projections between neighbouring cells. Cells treated with siRNA specific towards Menin seemed to have undergone no change or very little change with TGF-β stimulation. Menin Knock-down cells stimulated with TGF-β lack the N-Cadherin up-regulation at the cell projections, which is present in mock transfected cells stimulated with TGF-β. In addition to the difference observed in N-Cadherin, cell morphology seems to differ; mock transfected cells went through a change in cell shape, spreading out and forming finger-like projections, while Menin-specific siRNA treated cells failed to undergo much change.

A) Mock transfected cells

TGF-β: - +



B) Menin knocked down with specific siRNA

TGF-β: - +

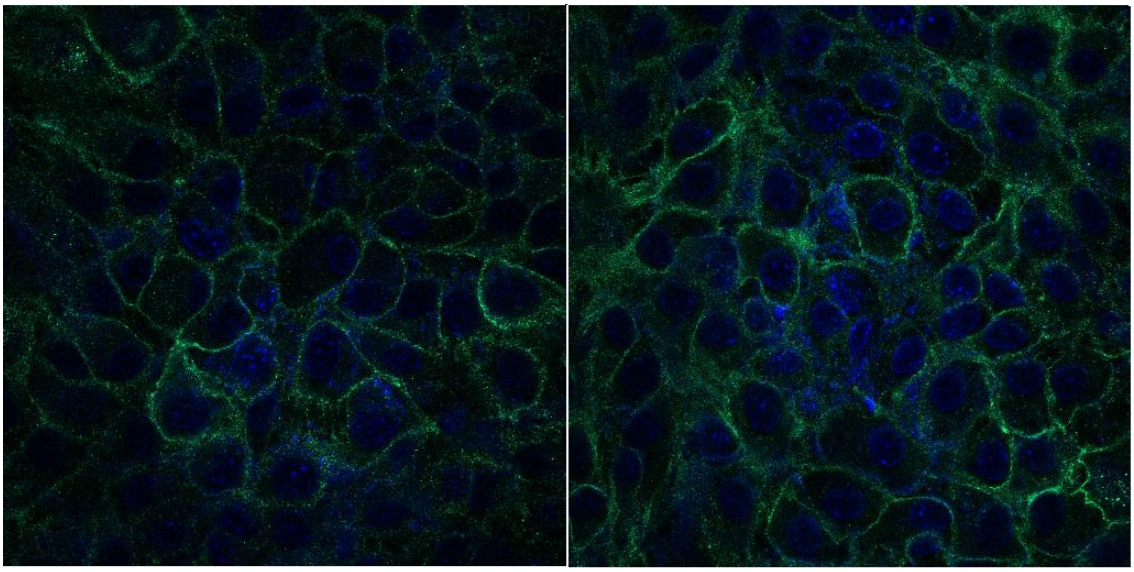
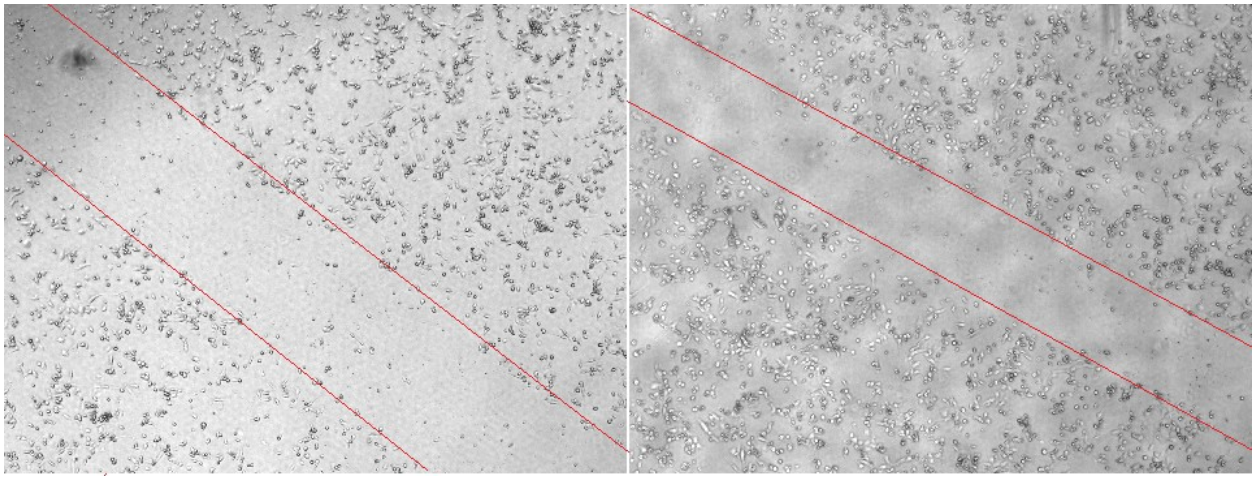


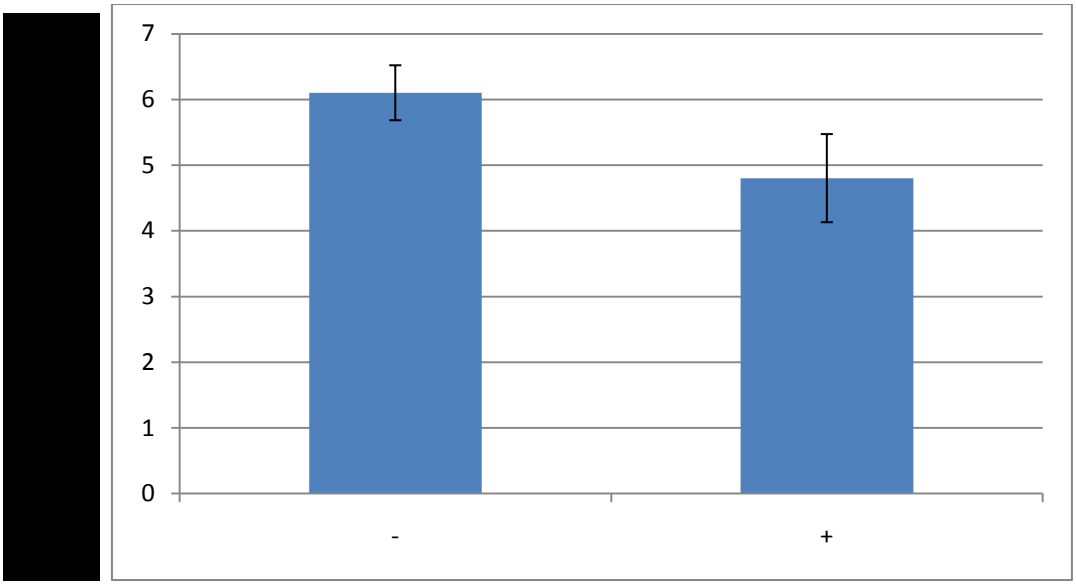
Figure 3.6: N-Cadherin localization, cell morphology before and after 24 hours TGF-β stimulation. NMuMG cells grown on non-coated glass coverslips (13mm) were mock transfected (A) or transfected with siRNA specific towards Menin (B). Cells were stimulated or not with TGF-β for 24 hours before they were stained using anti-N-Cadherin antibody followed by appropriate secondary antibody linked to green fluorophore. The images shown are representative images selected from at least six pictures taken from different visual fields.

Migration is a dynamic phenomenon that is fundamental in various physiological processes such as inflammation, wound-healing, angiogenesis, and embryonic development (Schmidt et al., 1994). It is an important process that is involved in cancer metastasis (Hanahan and Weinberg, 2000). TGF-β is known to mediate these processes, and to determine if Menin participates in bringing about this aspect of TGF-β, a set of cell migration assay known as "the wound healing assay" was performed. This assay involves letting cells grow on cell culture plates, followed by making a consistent narrow scratch after the appropriate transfections, and stimulation with TGF-β for at least 48 hours. The wound was compared under a microscope after completion of 48 hours of stimulation.

(A) Mock transfected SCP2 cells

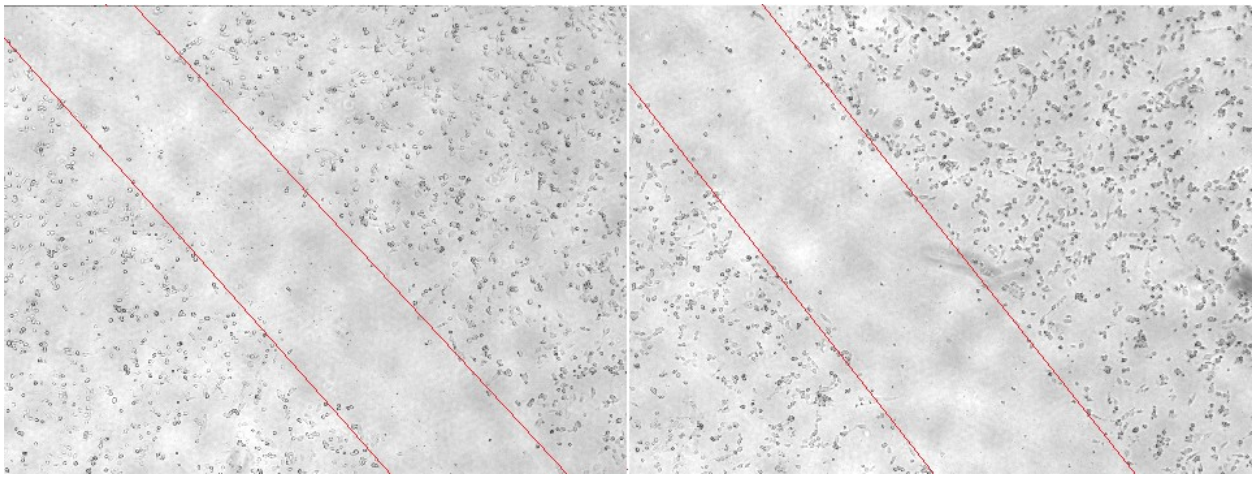
TGF-β: - +





(B) Menin siRNA transfected SCP2 cells

TGF-β: - +



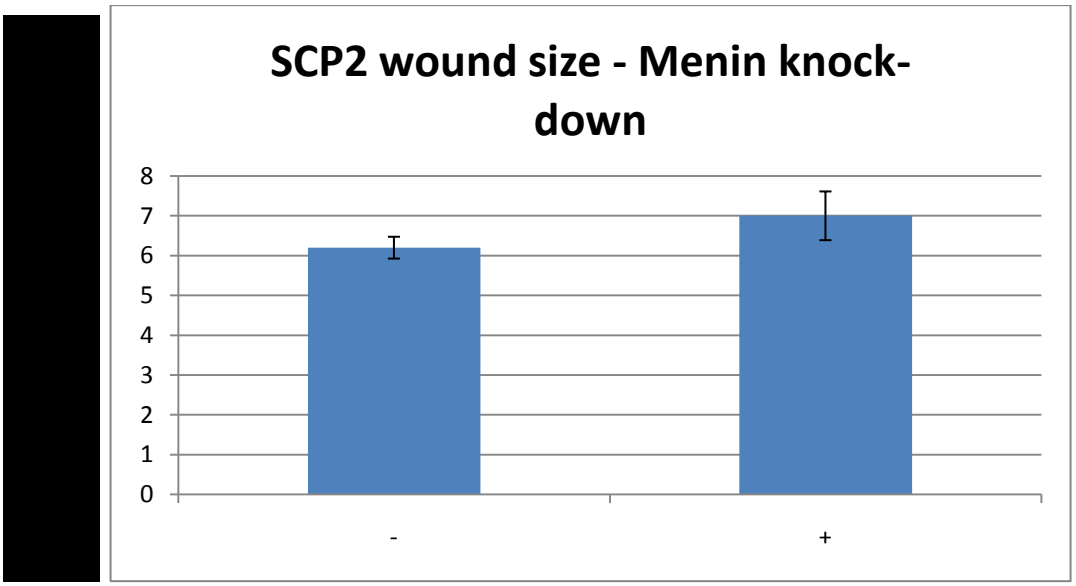


Figure 3.7: TGF-β mediated wound healing in SCP2 cells. SCP2 cells grown on 6-well plates were mock transfected (A) or transfected with siRNA specific towards Menin (B). A thin scratch was made directly on cells in a straight line to observe and compare the amount of wound closure after stimulation. Cells were stimulated or not with TGF-β for 48 hours before they were observed under the microscope. The images shown are representative images selected from at least six pictures taken from different visual fields. The graph is an average of six visual fields.

As it can be seen from figure 3.7, there is a good possibility that Menin might play an important role in TGF-β mediated cell migration. In mock transfected cells, 48 hours of stimulation with TGF-β resulted in wound closure by more than 20% compared to the unstimulated (similar to the initial scratch- data not shown). In cells transfected with siRNA duplex targeted towards Menin, the wound does not close at all.

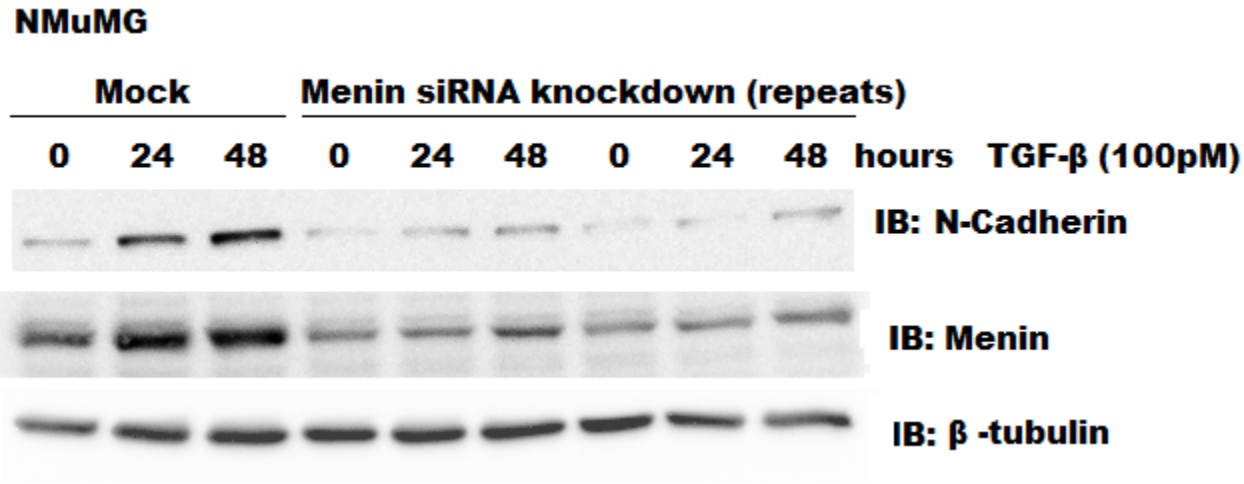


Figure 3.8: The effect of Menin knock-down on levels of N-Cadherin. NMuMG cells were transfected with specific siRNA targeted towards Menin, followed by stimulation with TGF-β for up to 48 hours. A western blot was performed on NMuMG lysates collected after transfection and stimulation. The membrane was probed with anti-N-Cadherin antibody (upper panel), anti-Menin antibody (middle panel), and anti-β-tubulin antibody (lower panel) for loading control. The lysates analyzed were collected using Triton X lysis buffer. Negative results (blots performed with E-cadherin, SIP-1, Vimentin) are not shown.

3.5 Effect of Menin on N-cadherin transcript level and protein level

N-Cadherin is an important factor in cell migration and EMT process, and is a gene that is known to be upregulated by TGF-β. To determine the effect of Menin on TGF-β mediated N-Cadherin upregulation, some qRT-PCR (quantitative real-time polymerase chain reaction) was performed using RNA from NMuMG normal mammary gland cells as these are known to be a good model for EMT (Xie et al., 2003).

NMuMG cells were transfected with siRNA targeted against Menin followed by TGF-β stimulation. As shown in figure 3.8, knock down of Menin using specific siRNA not only resulted in diminished Menin protein levels, but also a drastic reduction in N-Cadherin levels. TGF-β stimulation induces strong levels of N-Cadherin expression, but Menin knock down cells failed to express strong levels of N-Cadherin, suggesting a role for Menin in TGF-β mediated EMT process. The level of change of N-Cadherin mRNA upon knocking down Menin needs further experimentation with various time points to be meaningful (preliminary time-course data not shown).

To summarize, this thesis explores the role of Menin in the TGF-β pathway. Menin seems to be upregulated by TGF-β itself (see figure 3.8), and although it does not seem to play an important role in TGF-β mediated tumor suppressive effects (cell cycle arrest and apoptosis), it does seem to have an effect on phenomena related to TGF-β mediated tumor progression (cell migration, EMT). This thesis shows that Menin may not have an effect on TGF-β activation of gene transcription mediated by Smad 3 using 3TP-lux luciferase construct and siRNA targeted towards Menin specifically. Menin might not play an important role in TGF-β mediated suppression of cell proliferation, as shown by the MTT assay, an indirect method of measuring

cell viability (to obtain an idea of the cell population). Contrary to its role as tumor suppressor, Menin was shown to possess a potentially tumor progressive role through participating in the TGF-β pathway: Menin might affect cell migration as shown in the wound-healing assay (see figure 3.7); It might affect the EMT process by potentially controlling N-Cadherin levels or actin assembly (see figure 3.5).

Chapter IV: Discussion

TGF-β is a multifunctional cytokine, developed to control numerous processes with its main goal being the maintenance of homeostasis (Cui et al., 1996). The cytokine is known to exert tumour suppressive effects in normal cells and in early stage cancer cells but late stage cancer cells, through acquisition of mutations in the genes pertaining to the TGF-β pathway, can use the cytokine to their advantage (Massague, 2008).There are multiple evidences suggesting TGF-β contribution to cancer progression and metastasis (Cui et al., 1996; Massague, 2008), which is mainly due to TGF-β mediated EMT that contributes to cancer progression in several ways such as actin reorganization, upregulation of mesenchymal markers, and increased cell motility (Boland et al., 1996; Derynck and Akhurst, 2007; Padua and Massague, 2009). In addition, TGF-β modulation allows cancer cells to evade immune surveillance (Li and Flavell, 2008), the natural mechanism that the body uses to detect and fight off foreign matters, which includes cancer cells. It is thus no surprise elevated levels of TGF-β are found in tumors *in vivo* (Cui et al., 1996). These negative properties of TGF-β led to the development of TGF-β blockers, which consists mainly of large molecules such as antibodies, to eliminate the tool that the cancer uses to its advantage (Massague, 2008). However, the pathway is far from simple and further uncovering and dissecting components in the TGF-β pathway is essential to narrow down drug targets and improve cancer treatment.

Menin is an elusive protein with no definite function that was shown to interact with Smad 3, a major player in the TGF-β canonical pathway (Kaji et al., 2001). In this study, the role

of Menin in the TGF-β pathway was investigated through a battery of experiments testing for all known effects of TGF-β.

4.1 Possible Regulation of Menin expression by TGF-β

Menin is a widely expressed protein that assumes nuclear localization except for during cell division and immediately after cell division, where it was shown to be cytoplasmic (Huang et al., 1999). This cytoplasmic sequestration is possibly mediated by type III intermediate filaments (Lopez-Egido et al., 2002) which increases after stimulation with TGF-β (Derynck and Akhurst, 2007), and its expression level was shown to be regulated by a feedback from the protein itself (Zablewska et al., 2003). From the western blot results obtained, Menin also seems to be a target of the TGF-β pathway, as it can be seen from figure 3.8 A where stimulation of NMuMG cells with TGF-β resulted in an increase of the protein level in 24 hours. Further investigation is required to determine if this up-regulation of Menin is present in breast cancer cell lines and at what level the regulation takes place, and if the regulation is a direct rather than an indirect one. Also, the possibility of Menin phosphorylation after TGF-β stimulation should be examined.

4.2 The effect of Menin on TGF-β mediated cellular responses

4.2.1 Effect on Cytostasis and Apoptosis

TGF-β has already been shown to be a potent cell growth suppressor (Roberts et al., 1986), and Menin itself also plays a role in apoptosis. Over-expression of Menin was shown to

induce apoptosis in mouse embryonic fibroblasts (MEF cells are often used in cancer research due to their similarities shared with human fibroblasts), while loss of Menin caused loss of the apoptotic response (Schnepp et al., 2004). Furthermore, mutating the gene MEN1 was shown to acutely enhance proliferation of pancreatic islet cells (Schnepp et al., 2006), and reduction of Menin expression was shown to enhance cell proliferation in intestinal epithelial cells (Ratineau et al., 2004). It has been shown that Menin might act through the induction of CDK inhibitors, p27 and p18 (Karnik et al., 2005), which were also shown to be responsive to TGF-β.

Since Menin and TGF-β appear to have similar effects, the possible contribution of Menin in the TGF-β mediated inhibition of cell proliferation was assessed using MTT cell viability assay. Contrary to what was expected, the results showed that Menin likely to be not involved in regulating cell proliferation downstream of TGF-β, as cells transfected with siRNA duplexes specifically targeting Menin did not differ from mock transfected cells. This suggests that Menin and TGF-β might act independently on cell cycle arrest/inhibition of proliferation/apoptosis, and Menin does not play a role in TGF-β mediated cell growth control.

4.2.2 Effect on Transcriptional Activation of TGF-β regulated genes

TGF-β stimulation results in activation of the Smad proteins to relay the signal to the nucleus and make changes in gene transcription. Each Smad 4/R-Smad/cofactor complex targets a different set of genes, and the complex recruits coactivators or corepressors and chromatin remodelling factors to control each gene (Massague, 2008). Menin was found in histone methyltransferase complexes, it participates in transcriptional regulation of hormone responsive genes, and it was shown to be a cofactor for Smad dependent transcription in certain cell lines

(Sowa et al., 2003). Based on these data obtained from the literature, it appeared likely that Menin might act as an essential cofactor in the transcriptional activation of genes responsive to TGF-β in cells of breast tissue origin.

To test this aspect of TGF-β mediated effects, a reporter construct was employed along with specific siRNA to knock down Menin levels. 3TP-lux construct, which contains three TPA responsive elements and a portion of the PAI-1 promoter linked to a luciferase gene as a reporter, is highly responsive towards TGF-β stimulation and it serves as a good tool to test TGF-β activated gene transcription (Wrana et al., 1992). Cotransfection of the construct and siRNA followed by 24 hour stimulation and assessment of the luciferase activity in each sample suggested no change in reporter activity after Menin knock down (see figure 3.4). This suggests that there may be some level of difference among tissues in Menin mechanism of action. In cells of breast tissue origin, Menin might not play a great role in TGF-β mediated transcriptional activation, but rather, it might play yet an unknown role that needs to be investigated.

4.2.3 Induction of EMT

EMT is a complex process that involves the loss of polarity, reorganization of the cytoskeleton, and re-distribution of organelles (Thiery, 2003), ultimately changing the cell phenotype. TGF-β is a known modulator of cell phenotype in vivo (Cui et al., 1996; Roberts et al., 1986), and because of this property of TGF-β, it increases malignant conversion rate despite its potent effect in suppressing the induction of tumors (Cui et al., 1996). Since Menin is an interacting partner of Smad 3, there was a possibility that Menin might play a role in regulating proteins related to EMT. The role of Menin in the TGF-β mediated EMT was investigated, by

analyzing the change in levels of EMT marker proteins such as N-Cadherin, Vimentin, E-Cadherin, SIP-1.

The only noticeable effect was seen in N-Cadherin levels (see figure 3.8 A). Compared to the mock transfected cells, cells transfected with siRNA targeted specifically towards Menin, showed a noticeable decrease in N-Cadherin levels.

To check the level of N-Cadherin mRNA transcripts, specific primers were designed and were used to perform quantitative PCR on total RNA converted to cDNA after treating the cell or not with siRNA targeting Menin and TGF-β stimulation for 24 hours. The results of the PCR did not show much difference in N-Cadherin transcript levels of mock transfected cells and cells transfected with siRNA targeted against Menin.

Based on the obtained data, Menin seems to help bring about changes associated with EMT, in response to TGF-β stimulation. However, the level of action of Menin needs to be confirmed, as it does not seem to act on transcription although it does interact with Smad 3 (see figure 3.1) and it does affect N-Cadherin protein levels (see figure 3.8 A).

4.2.4 Effect on Migration

TGF-β is an important factor affecting migration, by regulating the expression of ECM proteins and cell adhesion molecules such as the Cadherins. N-Cadherin is of special interest, as it is known to actively promote cell motility and invasion (Nieman et al., 1999), and was shown to be a required factor for cell migration during TGF-β mediated EMT process. Since Menin was also shown to be important for the ECM and cell adhesion (Ji et al., 2007) and Menin seemed to

affect N-Cadherin levels (see figure 3.8A), the possibility of Menin playing a role in TGF-β induction of cell migration needed to be confirmed.

To assess the effect of Menin in TGF-β mediated cell migration, a wound-healing assay was employed. SCP2 metastatic breast cancer cell line, which has lost all cytostatic response to TGF-β, was used. The wound made onto mock transfected cells containing intact Menin was shown to regress after treatment with TGF-β, whereas the wound made on cells that were transfected with specific siRNA to knock down Menin did not display any sign of regression. This may be the result of Menin knock down resulting in downregulation of N-Cadherin protein levels, as N-Cadherins are known to be an important factor in cell migration (Nieman et al., 1999).

Examination of stress fiber formation and N-Cadherin distribution indicates a possible requirement of Menin in the TGF-β pathway to properly convert cell characteristics from non-motile to motile. N-Cadherin localization to the finger-like projections which was seen in mock transfected cells seemed to be absent in Menin knock down cells, and in fact, the cell morphology was seen to be drastically different from the mock, missing the finger-like projections altogether. Stress fibers are traits of fibroblasts, and are needed for contractile motion which aids in cell motility. Knocking down Menin seems to interfere with the organization of the fiber bundles, making the bundles thinner and more irregular which might explain the results obtained in the wound healing assay.

Chapter V: Conclusion

5.1 Conclusion

Menin, the protein product of MEN1 gene, is a protein ubiquitously expressed throughout the body in abundance (Dreijerink et al., 2009). Its effects seem to differ in endocrine tissues and non endocrine tissues, judging from the fact that its mutation is more often linked with endocrine tumors than non-endocrine (Guo and Sawicki, 2001). Although it does not have a clear role defined, results from many different groups seem to suggest a tumor suppressive role for Menin. Menin was also shown to play a significant role in the TGF-β pathway, by interacting with Smad3, which is the cytoplasmic component of the TGF-β pathway, involved in interacting with cofactors to activate/repress genes in the nucleus upon TGF-β signal activation (Kaji et al., 2001).

TGF-β effects are divided into two large arms, one being the tumor suppressive and the other being tumor progressive (Massague, 2008). Menin, by interacting with Smad 3 downstream of TGF-β, likely plays some role in response to TGF-β. Menin is widely accepted as a tumor suppressor protein, stemming from the observation that in the absence of the protein, tumors tend to develop. If it had a role downstream of TGF-β, it seemed logical that Menin would play a role in the tumor suppressive arm of TGF-β. Contrary to expected, Menin does not seem to play a significant role in controlling the cell growth and activating apoptosis in mammary epithelial cells and tumors derived from mammary epithelial cell types, based on cell proliferation assay (MTT assay) results. Although Menin does seem to interact with Smad3 in cell lines of breast tissue origin, it is possible that in the types of cells tested, Menin-Smad3

interaction to affect transcription is not the major mechanism of action of Menin in the TGF-β pathway.

It is possible that Menin does play a role in the TGF-β pathway, since its absence was observed to lower the levels of N-Cadherin protein and affect F-actin organization. This would suggest Menin functions to regulate TGF-β related tumor progressive effecs, which contradicts its name as a tumor suppressor. However, this is not the only observation that Menin might have pro-cancer roles. Menin was recently shown to be essential in leukemogenesis, by interacting with MLL (Myeloid/lymphoid or mixed-lineage leukemia), which is a large nuclear protein involved in maintenance of gene expression during development (Yokoyama and Cleary, 2008).

5.2 Future directions

The role of Menin is still unclear, but there is some evidence that suggests that it might be playing a role in the TGF-β pathway, particularly the tumor progressive arm of TGF-β. This proposes a possible new role of Menin: A protein accepted widely as a tumor suppressor protein actually participates in a pathway known to promote cancer progression to exert its effects. The following studies may be of interest: 1) Assessment of EMT characteristics after siRNA knock-down of Menin followed with a much longer time course, as in some studies EMT changes were shown to be more noticeable as the length of stimulation was extended; 2) assess the role of Menin in modulating N-cadherin gene, or Twist1 gene that was recently shown to have control over N-Cadherin transcription(Alexander et al., 2006); 3) investigate whether or not Menin is phosphorylated by TGF-β stimulation; 4) investigate the involvement of histone deacetylase/acetyltransferase complexes in the mechanism of action of Menin, by co-

immunoprecipitation experiments to confirm physical interaction and incorporating histone acetylase/deacetylase inhibiting compounds into experiments. Further investigation of the role of Menin in TGF-β mediated control over EMT related genes other than N-Cadherin also seems to be a promising project.

Also, to strengthen the conclusion that TGF-β mediated cell cycle arrest and apoptosis is indeed independent of Menin, cell cycle analysis should be performed to be paired with apoptosis measurements.

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